An Investigation Into The Effect Of Maternal Exposure To Nicotine And Copper On Neonatal Lung Development

By

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KEYWORDS

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ABSTRACT

In the 20th century, where tobacco smoking continues to be the leading preventable cause of death, an alarming number of people continue to smoke, despite awareness of the implications of exposure for themselves and those around them. Campaigns for the promotion of effective tobacco legislation and awareness are continuously being confronted by the tobacco industry's reluctance to put the health of their consumers before company profits, leading to a ripple effect of misinformation, serious health risks and economic implications, at least for the consumers. Pregnant women are especially a concern because exposure to tobacco smoke affects not only the smoking mother but has serious implications for the health of her unborn child. Some mothers try to quit smoking by using nicotine replacement therapy. However, recent studies implicate nicotine as the causative factor for some of the cigarette smoke associated respiratory diseases. Therefore, the aim of this study was to investigate the effect of: 1) maternal exposure to nicotine (1mg/kg BW/day) during all the phases of lung development, or from the onset of the phase of rapid alveolarisation and, 2) whether copper supplementation (1mg/kg BW/day) will prevent the adverse effects of maternal nicotine exposure, on lung development in the offspring. The latter aim is based on studies in our laboratory which suggested that nicotine may impair lysyl oxidase activity by reducing the lung copper content in the lungs of rats that were exposed to nicotine via the placenta and mother's milk. It was found that although maternal nicotine exposure had no significant effect on the growth parameters of the offspring, it did have an effect on the development of the lung, and indeed compromised the ability of the lung to act as an organ of gaseous exchange. This included alveolar destruction due to damage to the connective tissue framework of the lung, and consequently a decrease in the surface area available for gas exchange. The changes occurred after the lungs reached maturation and resembled microscopic emphysema. Copper supplementation did not prevent the lung from damage caused by maternal nicotine exposure but appeared to lessen the severity of disease progression. The late development of the lung lesions in the offspring suggests that maternal nicotine exposure changes the program that regulates

ageing and maintenance of the lungs, such that the lungs are more susceptible to damage and disease. Pregnant women who smoke, or use nicotine replacement therapy, will therefore increase the risk of the offspring to develop respiratory disease, even if the offspring never smoke themselves.

DECLARATION

I declare that "*An investigation into the effect of maternal exposure to nicotine and copper on neonatal lung development"* is my own work, that it has not been submitted for any degree or examination in any other university and that all resources I have or quoted have been indicated and acknowledged by complete references.

Shantal Lynn Windvogel November 2006

Signed:

DEDICATION

This thesis is dedicated to all my family and friends for their encouragement and support. You have all been my source of inspiration. Thank you especially to my supervisor, Prof. G.S. Maritz, for mentoring me and always encouraging me. Most importantly, I am eternally grateful to our Dear Lord for being my anchor through all the storms and making my dreams a reality.

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LIST OF ABBREVIATIONS

ANOVA	one way analysis of variance
AP	activator protein
AWUV	airspace wall surface area per unit volume
BAL	bronchoalveolar lavage
BMP	bone morphogenetic protein
BW	body weight
cAMP	cyclic AMP
$CaCl_2$	calcium chloride
CC	chest circumference
cm	centimetre
COPD	chronic obstructive pulmonary disease
Cp450	cytochrome p450
Cu^{2+}	copper
DOH	Department of Health
DNA	2'-deoxy-5'-ribonucleic acid
DPX	DPX mountant for histology
DSPC	surfactant diphosphatidyl choline
EGF	epidermal growth factor
FAS	apoptosis stimulating fragment
FGF	fibroblast growth factor
g	gram
G&L	gestation and lactation
GCS	glutamyl cysteine synthetase

γ-GCS	γ-glutamyl cysteine synthetase
GSH	glutathione
H &E	haematoxylin and eosin
HNE	4-hydroxy-2-nonenal
H_2O_2	hydrogen peroxide
HSPG	heparan sulphate proteoglycans
IL	interleukin
kDa	kilodalton
kg	kilogram
L	length of traverses
LOX	lysyl oxidase
Lm	mean linear intercept
Lv	lung volume
m RNA	messenger ribosenucleic acid
m	metre
M	molar concentration
MAGP	microfibril-associated glycoprotein
MAP	mitogen activating protein
mg	milligram
ml	millilitre
mm	millimetre
MMP	macrophage metallomatrix protein
N	number of fields
Na	number of alveoli per unit area

nAChR	nicotinic acetylcholine receptor
NF-κβ	nuclear factor kappa beta
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRT	nicotine replacement therapy
Nv	number of alveoli per unit volume
p	probability
PDGF	platelet derived growth factor
pH	measure of the acidity of a solution
PNE	postnatal exposure
PThrP	parathyroid hormone related protein
Raf	Raf protein kinase
ROS	reactive oxygen species
Sa	internal surface area
shh	sonic hedgehog morphogen
SIDS	sudden infant death syndrome
SP	surfactant protein
TBARS	thiobabituric acid reactive species
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
T*sept*	interalveolar septal thickness
USA	United States of America
Va	volume density of airspaces
Valv	volume proportion of alveoli (mean alveolar volume)
VEGF	vascular endothelial growth factor

Vt	volume density of parenchymal tissue
WHO	World Health Organisation
α	alpha
B	alveolar shape constant
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

CHAPTER 1

Introduction

Tobacco smoking is the leading preventable cause of death (WHO Report, 1999) and by 2020 it is expected to be the 3rd leading cause of death worldwide (Murray and Lopez, 1997). Deaths from lung cancer are in the order of approximately 1.3 million per year (WHO fact sheet, 2006) and tobacco smoking is responsible for 30% of all cancers (Mackay and Eriksen, 2002). Tobacco contains more than 4800 different compounds (Green and Rodgman, 1996), and includes carcinogenic substances such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as well as nicotine and a variety of free radical oxidants such as hydrogen peroxide (Pryor and Stone, 1993).

Tobacco use during pregnancy is associated with a wide range of complications such as pre-term labour, reduced placental blood flow and complications to the foetus that includes reduced birth weight and early respiratory disease such as wheezing in young children (Fergusson et al. 1981; Stein et al. 1999). It has also been linked to increased incidences of spontaneous abortions and sudden infant death syndrome (Di Franza and Lew, 1995).

Nicotine is implicated as the causative substance for the addictive properties of cigarettes, and a recent study by Le Foll and Goldberg (2006) classified it as possessing the typical properties of other drugs of abuse. It is therefore

understandable that people find it difficult to quit, as kicking a drug habit is a problem with most drug addicts. The addictive properties of nicotine are ascribed mainly to its positive-reinforcing effects on the reward pathways of the brain by increasing the activity of dopaminergic neurons (Salokangas et al. 2000), eliciting pleasurable sensations. An increase in withdrawal symptoms is in fact seen with reduced nicotine intake (Epping-Jordan et al. 1998). Nicotine replacement therapy (NRT) is available for use by many individuals who want to stop smoking (Jorenby et al. 1999).

Despite efforts by government to increase public awareness regarding the detrimental effects of tobacco, people continue to be ignorant of its effects, not only on themselves but also on the passive smokers around them. Of great concern are mothers who continue to expose their unborn babies and young children to smoke, more adept at trading the 'high'' of a puff for the health of their children. As tobacco smoking and maternal nicotine exposure by unborn and young children continues to be a health concern, a need exists to describe the deleterious effects of maternal nicotine exposure during lung development and its effects, to gain an understanding of the pathology of tobacco related lung disease.

It is hoped that the results of the study will also be made available to the general public and especially smoking mothers, so as to instil an awareness of the detrimental effects that smoking has on their unborn and young children and also on those around them. This is especially so in these times, as tobacco smoking among young and old is rife and people find it very difficult to quit smoking.

Tobacco smoking as a cancer and respiratory disease promoting xenobiotic, is contributing to a cycle of poverty as tobacco companies in their promotional activities target people in the developing world, and more and more people start smoking at an earlier age. This implies that people will either die or be incapacitated at a younger age, as a resulted of tobacco-related lung disease and death, and ultimately the socio-economic stability of the country will be affected. As tobacco smoking and maternal nicotine exposure by unborn and young children continues, a need exists to describe the deleterious effects of maternal nicotine exposure during lung development and its effects in later life on the respiratory health of the offspring. This is also important so that people, especially mothers who smoke may gain an understanding of the potential risks that they pose to themselves and especially their innocent children. Nicotine in tobacco smoke is implicated as a causative factor in the development of certain tobacco-related respiratory diseases.

The aim of my investigation is to study the effect of maternal nicotine exposure and supplementation with copper on the development of the airways of the rat lung:

a. During the alveolarisation phase of lung development (from the 3rd day of postnatal development and throughout lactation).

b. Throughout the entire period of pregnancy and lactation.

This will give an understanding of when nicotine is most harmful to the development of the lung of the offspring. It will also give a better understanding of the site of action of nicotine and help to develop a strategy to prevent the

harmful effects of maternal nicotine exposure on the development of the respiratory system of the offspring. This might be through the adjustment of the diet or the taking of copper supplements.

In particular, it is the aim of this project to describe:

a. The phase/s of lung development in which nicotine induces changes in lung structure and the consequences thereof to lung function.

b. The long-term consequences of maternal nicotine exposure on the lungs of the offspring.

c. The site and mechanism of action of nicotine.

d. To discuss strategies which may prevent the adverse effects of maternal nicotine exposure on lung development of offspring.

In this study, the offspring were exposed to nicotine or a combination of copper and nicotine via the placenta or mother's milk. Copper was included as a supplement as it is believed that it is needed as a cofactor for lysyl oxidase (Harris, 1976; Harris, 1986), the enzyme responsible for the laying down of collagen and elastin cross-links (Scarpelli, 1990). It is hypothesized that an alteration of lysyl oxidase activity leads to decreased cross-link formation and thus an increase in emphysematous lesions, as well as premature ageing of the lung. This however is probably not limited to the activity of lysyl oxidase, but may be contributed to by a variety of other factors such as the time or phase of developmental exposure and duration of exposure to nicotine. The genes affecting

the development and functioning of the lung may also be affected, as well as the action of nicotinic acetylcholine receptors.

In this study I will use morphometric and morphologic methods to study the impact of maternal nicotine exposure on lung development and maintenance of lung integrity of the offspring in the long term. The morphometric methods involve histological examination of the lung tissue using haematoxylin and eosin and connective tissue staining to determine parameters such as alveolar numbers, internal surface area and connective tissue examination for indications of emphysematous lesions. Morphologic techniques include the examination of the surface area for any surface lesions. A review of the literature introduces the reader to the phases of lung development and is followed by a discussion of the connective tissue components, the effects of maternal smoking and the lysyl oxidase, which is thought to be involved in the pathology of the lesions caused by maternal smoking. Factors affecting lung development are also discussed and inflammation and oxidative stress in the lungs are discussed, all of which are important in exposure of the lung to tobacco smoke.

This study is based on the hypothesis that exposure to maternal nicotine causes *in utero* "reprogramming" of the genes affecting the respiratory system, leading to structural and functional impairments and this leads to changes in the normal health of the respiratory system in the later life of the offspring.

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CHAPTER 2

Literature Review

2.1 Introduction

In comparison to other organs, prenatal development of the lung is important so that it can develop into an efficient gas-exchanger. It must develop to such an extent in utero, that it is immediately ready to function at birth. This is why its entire development extends from the embryonic period through the foetal period, up to birth and afterwards. Pre- and postnatal lung development can be divided into several distinct phases. These phases are crucial to its future role as a gas-exchanger. Interference with these phases has an effect later on in life, in the maturation of the lung and its resistance to disease (Stocks, 1995).

Mesenchyme directs the growth and cytoarchitecture of the lung at the cellular-molecular level by means of various growth and differentiation factors that are hormonally regulated (Torday, 1992). Sanchez-Esteban and co-workers (2002) have associated the thinning of the mesenchyme, which is essential for optimal gaseous exchange at birth, to mechanical stretch, which can be caused by foetal breathing movements. Cyclical mechanical stretch was found to inhibit the proliferation of fibroblasts and stimulate apoptosis of the epithelium during the canalicular phase of lung development, a period when thinning of the mesenchyme normally occurs (Sanchez-Esteban et al. 2002). This implies that apoptosis is a normal part of the developmental process of the lung.

2.2 Prenatal Lung Development

Prenatal growth can be divided into a number of phases, which are ordered sequences of events, taking part at specific times and involving specific events. The phases of prenatal growth are the embryonic, pseudoglandular, canalicular, and terminal sac (saccular) stages (Farrell, 1982). In mammals, unlike other organ systems, the foetus develops in an aquatic environment and the placenta provides not only respiratory but also gastrointestinal, hepatic, polyendocrine and renal functions (Longo, 1987). The lung is remarkable in that it does not become functional until after birth, therefore it must be prepared to function adequately at that time, or the life of the organism may be put at risk.

2.2.1 *Embryonic Phase*

This phase leads to airway formation, occurs directly after conception has taken place and in humans ends after 8th week of gestation (O' Rahilly, 1979). In humans, the lung develops from the laryngo-tracheal groove and lies ventrally along the surface of the endodermal tube. By the 5th week of gestation, after rapid endodermal branching, the trachea is separated from the oesophagus. The lobe pattern and the right and left lungs have also been established by this time (Farrel, 1982). At day 12 of gestation in rats, the lungs develop by endodermal budding from the floor of the caudal pharynx (Ten-Have-Opbroek, 1981). The left and right branches, which give rise to the left and right lungs, are also developed by day 13 of gestation in rats (Rothschild et al. 1996).

2.2.2 *Pseudoglandular Phase*

This phase is the start of foetal development and begins at approximately day 13 of gestation in rats (O'Hare and Townes, 1970). The epithelial cells are low columnar and the loose mesenchymal stroma condenses with mesenchyme. There are few signs of organelle differentiation and bronchial development is completed following division of the bronchial buds by asymmetric dichotomy (Bucher and Reid, 1961). In humans, this phase lasts until the 16th week of gestation and includes the proliferation of the bronchial tree by dichotomous branching (Murray, 1986), eventually leading to the formation of the terminal bronchioles. The closure of the pleural and peritoneal cavities and the differentiation of smooth muscle as well as epithelial cell differentiation also characterize this phase, and the lower conducting airways are formed (Farrel, 1982). The development of connective tissue cells from mesenchymal stem cells in foetal rat lung have been described from as early as day 15 of gestation (Collet and Des Biens, 1975). Here, the authors describe the development from the stem cells of fibroblasts, which are associated with the alveolar epithelium and also myoblasts, associated with the bronchial epithelium. Collagen and elastin fibre development from mature fibroblasts or myofibrobasts have been suggested to show the existence of a common precursor for these connective tissue elements.

2.2.3 *Canalicular Phase*

This phase takes place at approximately days 19 to 20 of gestation in rats (Farrel, 1982). The capillary bed comes into close apposition with the epithelial layer of tubules or ducts. There is an increase in the blood supply and a decrease in

connective tissue, as well as a flattening of the respiratory epithelium that line the airways (Thurlbeck, 1978). The production of surfactant follows the differentiation of type-1 and type-2 alveolar cells. Thin blood-air barriers are also present in places and resemble that found in adults. During weeks 16-26 of gestation in humans, the respiratory bronchioles arise from the terminal bronchioles and these divide into approximately 3 to 6 alveolar ducts (Sadler, 2000).

2.2.4 *Saccular Phase*

Day 21 of gestation (Thurlbeck, 1978) marks the beginning of the saccular phase in rats. During this period of lung development, differentiation of the alveolar epithelium and the production of surfactant continue. Alveolar septa develop after the development of capillaries and the lung's surface area is increased as the saccules, with thick septa, become more prominent. The potential airspaces are filled with foetal pulmonary fluid, which is expelled at the onset of labour and during vaginal delivery. In humans, the formation of primitive alveoli occurs from 26 weeks of gestation to birth with capillaries found lying in close apposition (Sadler, 2000).

2.3 Postnatal Lung Development

Postnatal development of the rat lung occurs in 3 phases, referred to as the phases of lung expansion, tissue proliferation and equilibrated growth, respectively (Burri et al. 1974). At birth, rats have lungs with no alveoli or alveolar ducts (Engel, 1953). They require a period of approximately 3 weeks for their lungs to develop into

mature organs (Burri, 1985). Peripheral airways in newborn rats have smooth walls that do not resemble any adult structures (Scott, 1994). Rat lung contains approximately 70 x 10 ^{6} cells at birth and this increases rapidly to at least 350 x10^{6} cells at 31 days of age (Thurlbeck, 1978). There are more generations of airways beyond the terminal bronchiole in adults than in newborns. Developing airways in the foetus have capillary networks of their own and these are retained as the airways branch and develop in the respiratory epithelium.

2.3.1 *Rapid Lung Expansion*

During the phase of lung expansion, airspace volume in rats increases by approximately 87 %. Lung volume increases proportionally to body weight during the first 10 days of life and thereafter is related to lung volume (Burri, 1974). Primary saccules are surrounded by a double capillary network, thick connective tissue and lined by type-1 and type-2 pneumocytes, the latter of which occur more frequently in newborn than in more mature rats.

2.3.2 *Phase of Rapid Alveolarisation*

During days 4-13 of postnatal growth, the phase of tissue proliferation, also known as the phase of rapid alveolarisation, occurs in rats (Burri et al. 1974). Secondary crests divide the primary saccule, leading to the formation of more definitive alveoli. The secondary septa arise from the primary septa and divide the saccules and transitory ducts into alveolar sacs and alveolar ducts, respectively.

This process is accompanied by the proliferation of myofibroblasts and lipofibroblasts, the former of which synthesizes collagen and elastin (Brody et al. 1983). The surface area per unit volume increases due to the rapid proliferation of alveoli and the corresponding complexity of the lung (Kuhn, 1982).

2.3.3 *Equilibration Phase*

During the phase of equilibrated growth from approximately postnatal days 14 to 20 in rats, the capillary endothelium is a single layer thick but capillary surface area continues to increase. The mean blood-air barrier thickness decreases, along with the interstitium, due to the continued formation of new alveoli and declining proliferation of tissue. There is decreased lung volume expansion, since the lung grows slower than the increase in body weight (Kauffman et al. 1974).

During postnatal growth of the lung from 21 to 131 days after birth, tissue volume increases to the power 2.6, airspace volume to the power 6.2 and capillary volume proportionally to airspace volume, approximately 7.6 times. In the adult rat, conducting airways differ from neonatal airways, whereas terminal bronchi may undergo transformation to gas-exchanging tissue. Bronchi and trachea increase in length and diameter by a factor of 3 and conducting airways are found to grow in proportion to lung growth (Scarpelli, 1990). In contrast to the rapid formation of alveoli during the alveolar period in rats, the increase in the production of alveoli in humans is thought to be almost complete by about 2 years of age (Thurlbeck, 1982). The capillaries are closely associated with the alveoli that contribute to the

formation of a very thin blood-air barrier that is essential for optimal gaseous exchange in the organism.

2.4 Connective Tissue

The extracelullar matrix is an important component of the lung parenchyma and is important in determining the development of the connective tissue framework of the lung (Shifren and Mecham, 2006). Consequently it is important for the development and maintenance of lung structure and function. It may also affect cellular differentiation (Hay, 1981). The main components of the extracellular matrix include collagen, elastic fibres, reticular fibres, noncollagenous glycoproteins as well as proteoglycans and glycosaminoglycans.

The extracellular matrix molecules are large and form a number of different covalent and non-covalent bonds. They interact with each other, with cell surface receptors and can self-assemble into multimolecular structures. Elastin and collagen cross-links may be formed either enzymatically or non-enzymatically. Lysyl oxidase mediates the former series of reactions whereas the latter non-enzymatic cross-links are formed by the glycation of lysine and hydroxylysine residues (Reiser et al. 1992).

2.4.1 *Collagen*

There are at least 16 genetically distinct types of collagen (Berk et al. 2000). The collagen fibrils form either homo- or heterotrimers, consisting of subunits called α-chains. They have a unique amino acid sequence (Gly-X-Y)n, (Bradsky and

Persikov, 2005) with glycine being the most abundant amino acid, filling every third position. The α-chains also contain large amounts of proline and hydroxyproline, with other amino acids filling the rest of the positions. The triple helical structure of collagen is due to the 3 polypeptide chains each consisting of an α-chain. It is this α-chain that consists of the repeating Gly-X-Y amino acid sequences (Linsenmayer, 1991).

Collagen type I consists of thick banded fibrils that have great tensile strength. It forms the basic building block of the extracellular matrix. It is the major determinant of the mechanical properties of bone, skin and lung tissue and is able to withstand stresses and strains as a result of large fibre diameter (Culav et al. 1999). Collagen types I and III are found in the interstitium and collagen type II is found in the cartilage. Type IV is found in basement membranes and does not normally form fibrils owing to a molecular weight of more than 95kDa but can self-aggregate to form multimolecular structures. Collagen types IX and X have a low molecular weight and are small, giving rise to types II and I respectively (Burgeson and Nimni, 1992).

2.4.2 *Elastic Fibres*

These fibres give flexibility to material. They consist of two components, namely insoluble elastin and microfibrils (Ross et al. 1977). Microfibrils appear to direct the laying down of the amorphous component. Tropoelastin has a molecular weight of approximately 70 kDa (Sandberg et al. 1989), forms the main subunit of elastin and is secreted by fibroblasts and smooth muscle cells (Damiano et al. 1984). In studies

by Fornieri et al. (1987), it was shown that the free epsilon groups on elastin provide for the association between elastin and glycosaminoglycans. It suggests that the free epsilon groups prevent spontaneous tropoelastin aggregation and when lysyl oxidase was severely inhibited, the elastin/ glycosaminoglycan aggregates were abnormal.

Elastin consists of non-glycosylated glycine and alanine side chains that are joined to form desmosines and isodesmosines (Francis et al. 1973). The latter two are unique to elastin and have frequently been used to quantify this connective tissue. The cross-links found in elastin are more extensive than those in collagen and glycoproteins have been found in close association to growing elastin fibres (Mecham et al. 1984). Developing elastic tissue consists of fibrillar components that develop first and stains with lead citrate, whereas the amorphous component develops later and stains with silver porphyrin (Albert, 1972). This serves as a method of distinguishing between newly formed and mature elastic tissue.

Studies by Niewoenher and Kleinerman (1977) suggest that the total length of elastic fibres is already present at an early age and no significant differences exist between the length and diameter of elastic fibres in patients with mild emphysema and those with normal lungs. It has been shown that triflouroacetylpeptide anilides are reversible *in vitro* inhibitors of elastases and may protect the lung from destruction and emphysema initiated by human neutrophil elastases (Amour et al. 1996). Cigarette smoke has been associated with the inhibition of the formation of elastin cross-links *in vitro* (Laurent et al. 1983).

2.4.3 *Proteoglycans and Glycosaminoglycans*

Proteoglycans have been found to play a role in the formation of elastin fibres (Pasquali-Ronchetti et al. 1988). Decorin and biglycan are chondroitin/ dermatin sulfate proteoglycans, which have distinct differences in the substrates they bind to (Winnemöler et al. 1991; Kresse et al. 1994). They were found to cause cytoskeletal and morphological changes in fibroblast cells, resulting in increased migration of these cells and a potential contribution to airway remodelling (Tufresson and Westergren-Thorsson, 2003). These proteoglycans assist in the maintenance of the extracellular matrix through interactions with collagen and elastin and have been termed the small leucine-rich proteoglycans (Iozzo and Murdoch, 1996).

In patients with emphysema and fibrosis, a diminished staining for heparan sulfate proteoglycan has been found and in severe cases of emphysema, decorin and biglycan had diminished staining in the peribronchiolar area of the lung (Van Straaten et al. 1999). It has been proposed that interstitial proteoglycans may play a role in the elastic recoil loss associated in patients with smoking-related emphysema and obstruction of the bronchi. The proteoglycan hyaluran has been shown to protect the elastin network from damage caused by neutrophil elastase and other elastases (Cantor et al. 1999).

2.4.4 *Noncollagenous Glycoproteins*

Laminin, found in basement membranes and fibronectin, which is found in both the interstitium and basement membranes, are adhesive glycoproteins that connect the cells to the matrix and interact with the extracellular matrix components. It is also

found in plasma and made in the liver. Fibronectins play a role in cell adhesion and extracellular matrix organisation (Torikata et al. 1985) and may possibly play a role in structural organisation and maturation of the lung (Hein et al. 1990).

Laminin is the most abundant glycoprotein in basement membranes. Heparin sulphate interacts with laminins (Kouzi-Koliakos et al. 1989) and laminins have also shown to play a role in cell adhesion and lung development (Schuger et al. 1990). The latter have also been found to play a role in epithelial mesenchymal interactions and proliferation of epithelial cells (Schuger et al. 1995).

Glycoproteins play a role in the orientation of elastin fibres (Cleary et al. 1983). It was also recently shown that changes in the levels of the microfibril-associated glycoprotein (MAGP) are associated with adult onset of emphysema in tight skin mice (Ito et al. 2006). This implies that interference with the extracellular glycoproteins may not only have an adverse effect on cell adhesion and thus cell communication, but may also result in the development of emphysema. This furthermore implies that interference with the metabolic development of these glycoproteins by smoking or nicotine during lung development may render the lungs more susceptible to damage. It was indeed shown that tobacco-related lung diseases such as emphysema and chronic bronchitis have been linked to excess deposition of connective tissue molecules such as the matrix glycoprotein fibronectin. It was also shown that nicotine stimulates expression of fibronectin in cultured primary lung fibroblasts as well as *in vivo*. In doing so, nicotine promotes tissue remodelling around airways and within lung parenchyma, and this is likely to

present an important mechanism by which tobacco results in abnormal lung structure and function (Roman et al. 2004).

2.5 Lysyl Oxidase

As previously mentioned, lysyl oxidase (Enzyme Commission number 1.4.3.13) is a copper-dependant enzyme, that is essential for the formation of elastin and collagen cross-links (Scarpelli, 1990). It is well known that compliance, which is a measure of the change in lung volume over a given change in transpulmonary pressure, is dependant on surface tension at the air-liquid interface at the blood-air–barrier and the degree of stretch of lung connective tissues (Vander et al. 1994). Interference with the enzyme, either by inhibition of activity, or change in the availability of substrates would ultimately affect the lung's ability to perform as a gas-exchanger.

In studies by Maritz and Burger (1992) and Maritz and Thomas (1994), exposing rats to nicotine not only led to a reduced mechanical distensibility but also in the surface area available for gas exchange by alterations in the fractions of soluble: insoluble elastic tissue. Lysyl oxidase is a copper containing amine oxidase with peptidyl 2, 4, 5 tri-(oxo) phenylalanine (TOPA) at its active centre. The enzyme has been isolated as 4 isoforms each weighing approximately 32kDa but prolysyl oxidase is a 48kDa protein, which undergoes posttranslational modification to its active form (Trackman et al. 1990, 1992 and Romero-Chapman et al. 1991).

Copper facilitates the oxidative deamination of the targeted peptidyl lysyl groups on tropocollagen and tropoelastin, which results in the formation of a peptidyl α-

aminoadipathic-δ-semialdehyde. This product spontaneously condenses with lysyl groups or aldehydes, resulting in the formation of inter- and intrachain cross-links (Rucker et al. 1998). Lysyl oxidase occurs in high concentrations in dense connective tissues such as skin and tendon and is not easily influenced by modulations in dietary copper. The activity of lung and kidney however are more easily influenced by deficiencies in copper since they do not have these high concentrations (Rucker et al. 1996). The genes for lysyl oxidase have been located on chromosome 5 and 18 in humans and mice (Contente et al. 1993; Hämäläinen et al. 1991) and associated with the anti-oncogene ras recession gene (rrg), (Kenyon et al. 1991).

Cadmium chloride exposure leads to the induction of lysyl oxidase in the lung, with a focally fibrotic response (Almassian et al. 1991). Inhibitors of lysyl oxidase, for example ß-aminoproprionitrile, aminoacitonitrite, semicarbazide and isonicotinic acid hydride cause abnormal elastin aggregates. These are permeated by glycosaminoglycans and are directly dependent on the degree of inhibition and independent on animal species (Fornieri et al. 1987).

Lysyl oxidase is associated with the stimulation of the activity of the collagen III promoter as well as cell adhesion and growth control (Csiszar, 2001). *In vitro* studies in foetal rat fibroblasts have shown that cigarette smoke condensate reduced the transcription of lysyl oxidase, inhibited lysyl oxidase promotor activity, decreased lysyl oxidase half-life and was accompanied by decreased transcription of type I collagen and tropoelastin (Gao et al. 2005). The authors suggest the

possibility that lysyl oxidase may be the mechanism by which cigarette smoke induces damage to the extracellular matrix, therefore leading to the development of emphysematous lesions. Male turkey aortae were found to have lower levels of lysyl oxidase activity than females, which may have implications for the greater susceptibility of male turkeys to aortic aneurisms (Narayanan et al. 1982) This implies that male turkeys and possibly other male species are more susceptible to factors affecting lysyl oxidase activity. If this is so, it implies that the lung tissue of male smokers will be less resistant to the effect of smoke on lysyl oxidase.

2.6 Growth Factors

The factors playing a role in the regulation of lung development include fibroblast growth factor (FGF), transforming growth factor-B (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), cyclic AMP (cAMP), glucocorticoids, and the sonic hedgehog morphogen (shh). Nicotine has an effect on endothelial cells, possibly via nicotinic acetylcholine receptors (nAChRs) and induces the release of growth factors such as prostaglandin-derived growth factor (PDGF), (Cucila et al. 2003). Growth factors are also involved in modulating injury-repair responses (Desai and Cordoso, 2002). This implies that growth factors may have an important role to play in the response to injuries caused by cigarette smoking and also in repairing the damage.

2.6.1 *Glucocorticoids*

It is well known that the administration of cortisol to premature babies helps to accelerate lung maturation and prevent respiratory distress syndrome in them

(Smith, 1984). It was proposed that cortisol binds to receptors in the mesenchyme where it stimulates the production of fibroblast pneumocyte factor (FPF), (Smith, 1979). Fibroblast pneumocyte factor then acts on type-2 alveolar cells to produce saturated phosphatidylcholine (SPC), (Post and Smith, 1984). Cigarette smoking acutely increases the blood cortisol level (Steptoe and Ussher, 2006). This might have a beneficial effect on prenatal lung maturation. Nicotine replacement studies show that nicotine is not preventing the drop in cortisol level when people quit smoking (Steptoe and Ussher, 2006). Thus, it is unlikely that nicotine will not have an affect on lung development via this route.

2.7 Maternal and Paternal Smoking and the Impact on Lung Development and Disease in Adults and Children

The World Health Organization (WHO) has estimated that 1 in every 10 people worldwide die as a result of exposure to tobacco smoke (WHO, 2006). Many of these people die in the prime of their lives, when they should be making invaluable contributions to their families and the socio-economy of their countries. An alarming number of pregnant women smoke despite being aware of the harmful consequences of smoking. Tobacco smoking during pregnancy is associated with adverse outcomes on the health of the foetus and infant. These include reduced birth weight, decreased gestational age and decreased crown-heel length (Davies and Albertiny, 1976; Bardy et al. 1993). Smoking is also more prevalent among younger, than older women and is associated with an increased risk of death from SIDS (sudden infant death syndrome), an increase in the risk for asthma and also in the severity of asthma (Cook and Strachan, 1999). There is also a decrease in foetal

breathing movements, which is associated with the increased plasma level of maternal nicotine (Manning and Feyerabend, 1976), and leads to hypoplasia and structural immaturity of foetal lungs (Harding, 1997). Tobacco smoke contains over 4800 different components (Green and Rodgman, 1996), many of these substances having carcinogenic potential and include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco specific carcinogen. NNK and nicotine both share common metabolic pathways and are oxidised by the p450 cytochromes (Cp450). The different cytochromes differ in rats and humans (Keyler et al. 2003).

Nicotine is the chief alkaloid in tobacco smoke (Benowitz et al. 1983) and people who smoke have an average nicotine intake of about 35mg per day (Benowitz and Jacob, 1984), taking into account that the range lies between 0.3 and 2mg nicotine per cigarette. Luck et al. (1985) found that the levels of nicotine were higher in the foetus than that occurring in smoking mothers, whereas cotinine levels were similar or lower than that found in maternal serum.

Studies in rhesus monkeys in which nicotine was administered during pregnancy at 1mg/kg BW/day, showed that the expression of nicotinic α-7 receptor subunits and the expression of collagen was increased (Sekhon et al. 1999). This corresponded with increased α-7 receptor expression in the larger airways and blood vessels. In addition, these findings were accompanied by lung hypoplasia and increased numbers of type-2 cells (Sekhon et al. 1999).

Smoking has been listed as the major cause of death in the United States of America (NIDA Research Report Series, 2000) and in South Africa an alarming number of pregnant women smoke cigarettes (Guthrie et al. 2001) despite being made aware of the consequences of tobacco smoking. These figures raise tremendous concerns among the world's 'health conscious' at least, as it holds serious negative implications for the health of all people, both smokers and those exposed to side–stream smoke.

In Cape Town, South Africa, a study conducted to show the prevalence of tobacco smoking among pregnant women indicated that 'Coloured' and 'White' women had the highest rates amounting to 32.4 and 31.2% respectively, while 'Blacks (4%) ' and 'Indians (3%) 'had lower rates (Guthrie et al. 2001). It is important that pregnant and lactating mothers who smoke understand that they are not only putting themselves and the lives of those exposed to their side-stream smoke at risk. They are also putting their unborn and young children at risk to a wide range of respiratory and other health problems, including premature death.

Maternal smoking has been found to have a greater effect on the health of the offspring than paternal smoking and in a dose-dependant manner. This may probably be as a result of the fact that mothers spend more time in the company of their newborn and young children while fathers are traditionally the breadwinners who have to go out and work to provide for their families. Women have typically been found to be social smokers who tend to group together to smoke. Smokers in general, are often reluctant to reveal their true smoking status.

2.8 Emphysema

2.8.1 *Definition*

Pulmonary emphysema is defined as an enlargement of the airways distal to the terminal bronchiole and without obvious fibrosis (Snider et al. 1985).

2.8.2 *Causes*

The two main causes of pulmonary emphysema are acquired (as a result of cigarette smoke) and hereditary (due to a deficiency in α-1-antitrypsin).

2.8.3 *Mechanisms*

Morphogenetic mechanisms for the development of emphysema include the development of pores in the alveolar walls, possibly due to the enlargement of alveoli by dilation of the alveolar ducts and retraction of the alveolar walls as the septa decrease in length (Kuhn et al. 1976).

α-1-Antitrypsin is a 52kDa glycoprotein synthesized in the liver and is often deficient or absent in patients with hereditary emphysema. α-1-Antitrypsin is a protease inhibitor and an imbalance thus exists between proteases and antiproteases, leading to breakdown in the connective tissue framework of the lung in patients with hereditary emphysema. This causes widespread and progressive destruction of the airways, causing a mainly panlobular (panacinar) emphysema (Coakley et al. 2001). The pattern of destruction in centroacinar (centrolobular) emphysema is mainly in the central parts of the acinus, close to the respiratory bronchioles.

Centroacinar emphysema develops mainly as a result of chronic exposure to cigarette smoke (Higgins, 1991). In a study by Carnaveli et al. (2003), cigarette smoke induced apoptosis of lung fibroblasts as measured by DNA damage. They associated it with increased levels of oxidative stress. Calabrese et al. (2005) have shown that apoptosis may play a major role in end-stage emphysema. It has been hypothesized that an imbalance occurs between the numbers of actively proliferating cells as measured by the epithelial turnover, and that of apoptotic cells. The apoptotic index was found to be significantly higher in emphysematous and α-1-antitrypsin deficient patients in their study. Patients who were α-1-antitrypsin deficient also had a higher T-lymphocyte count than smokers and controls, with smokers having a higher transforming growth factor ß1 (TGF-ß1) expression pattern than α-1-antitrypsin deficient and control groups.

The discovery of α-1-antitrypsin deficiency (Laurell and Eriksson, 1963) and its association with increased protease activity by the discovery that papain instillation in rats caused emphysema (Gross et al. 1964) led to the hypothesis that emphysema is caused by an imbalance between proteases and antiproteases. Subsequently, many researchers have focused their efforts on the induction of emphysema with elastases, with papain and porcine pancreatic elastases being the most widely used. α-1-Antitrypsin inhibits pancreatic and leukocytic elastases (Castillo et al. 1979). In papain-induced lung injury in rats, internal surface area (Sa) decreased and the mean linear intercept (Lm) increased as the animals aged, in comparison to control rats (Johanson and Pierce, 1973).

An attempt was made to understand the pattern of emphysema in C57 and pallid mice based on the α-1-antitrypsin deficiency of the animal. Pallid mice are different to C57 mice in that they have low levels of circulating α-1-antitrypsin. Pallid mice developed a pattern of emphysema that was diffuse (panlobular) whereas C57 mice had a more centrilobular emphysema (Takubo, 2002).

Smoking causes emphysematous lesions due to imbalances in the activity of proteases and antiproteases (Tetly, 1993; Janoff, 1985) as well as oxidants and antioxidants (Riley and Kerr, 1985). It is possible that there may be alterations in the repair process (Jeffrey, 2001; Chow et al. 2003). Cigarette smoke causes an inflammatory response in which macrophages and neutrophils are recruited to the site of epithelial cell injury and perhaps as a result of chemicals in the smoke condensate (McKusker, 1992).

Niewoenher and colleagues (1977) found that the total length of elastic fibres are established by age 10 years in humans and remain constant throughout life. The fibre diameter in the normal adult lung also remains constant. Other studies have also indicated that the collagen and elastin content of emphysematous lungs remain similar to that of normal lungs (Pierce et al. 1961). Pulmonary emphysema is associated with destruction of the elastic fibres in the lung (Turino et al. 1980) and also with the remodelling of collagen and elastin (Snider et al. 1992; Fukuda et al. 1989). This implies that emphysema is caused by breaks in the fibre and shortening of fibres. If this is so it will reduce the elastic recoil of the lungs and adversely affect the role of elastin and collagen in maintaining lung shape and

structure. Consequently, lung function will be adversely affected. This may result in thickened septa as observed by Vlahovic and colleagues (1999). Some studies have shown that dissolution of the amorphous component of elastic fibres occur after the administration of papain. Structural remodelling of the lung and regeneration of the microfibrillar skeleton is hypothesized to be part of the mechanism involved in the repair process (Johanson and Pierce, 1973). It is possible that the repair process involved in the pathogenesis of emphysema may be impaired, hence contributing to the pathogenesis of emphysema. Elastin and collagen deposition in emphysematous lungs is altered with the deposition of thickened collagen and disrupted layers of elastin, which have perforations and suggests a process of remodelling for both connective tissue elements (Finlay et al. 1996; Finlay et al. 1997). This supports other studies by Kuhn et al. (1976) in which there is increased deposition and remodelling of collagen.

In studies by Sulkowski and colleagues (1994), papain was injected into male rats and ultrastructural changes were observed in the alveolar epithelial cells. As emphysema developed, the alveolar epithelium underwent accompanying changes. In the first week after injection destructive changes in the alveolar epithelium predominated and 7 days after instillation of the protease, an increased number of type-2 cells were observed and ascribed to be possibly the result of a regenerative mechanism whereby type-2 cell conversion into type-1 cells could occur, thus leading to an attempt at restoration of the gas exchange region for gaseous exchange. An influx of macrophages was also noted 7 days after the instillation of papain and corresponds to the increase in type-2 cell formation.

Kuhn et al. (1976) have shown that while the elastin and collagen content of emphysematous lungs remains relatively normal after administration of pancreatic elastase, the lung's repair mechanisms to restore the destructive changes that accompany emphysema were inadequate as the newly formed elastin fibres were of abnormal configuration. Nagai and Thurlbeck (1991) have also shown that collagen deposition in emphysematous lungs is indicative of a continuous remodelling process. The pathology of the disease progression in respiratory damage normally follows a common pattern of events, which include inflammation, and accumulation of interstitial fluid, followed by proliferation of type-2 cells and interstitial fibroblasts. As already discussed, the inflammatory process involves the recruitment of polymorphonuclear lymphocytes, macrophages and neutrophils. The proliferation of type-2 cells may be attributable to the fact that since type-2 cells are commonly transformed into type-1 cells, it follows that proliferation of type-2 cells is a way in which the lung aids in restoring the surface area available for gaseous exchange (Ulich et al. 1994).

Lysyl oxidase is responsible for the stabilisation of collagen and elastin cross-links and its levels were found to decrease during copper deficiencies, such as during exposure to cigarette smoke (Chen et al. 2005). Decreased lysyl oxidase activity thus leads to the defective re-synthesis of elastin (Osman et al. 1985). This thus alters the synthesis of the elastin component of the connective tissue framework of the lung. It may be that a complex remodelling process is responsible for the structural alterations that take place in the elastin and collagen

framework of the lung during the pathogenesis of emphysema (Snider et al. 1992; Fukuda et al. 1989).

2.9 Inflammation and Oxidative Stress

Oxidative stress is thought to occur as a result of an imbalance between the ratios of oxidants to antioxidants. Oxidative stress along with the inflammatory events in the lung and the repair process involved in the lung in patients with emphysema are thought to contribute to the manifestations of the disease symptoms.

Inflammatory cells such as neutrophils and macrophages are released in response to epithelial cell injury or smoke and release excessive proteolytic enzymes, which cause destruction of alveolar tissue (McKusker, 1992). Previously, neutrophils were associated with the destruction of the elastin framework because of its higher elastinolytic activity per cell than macrophages in the lungs of smokers (Janoff, 1985). Meyer et al. (1998) have proposed that neutrophils release elastases that are in part responsible for the inflammation that takes place in the ageing lung. This seems to be part of the normal ageing process but the phenomenon has also been prevalent in patients with chronic obstructive pulmonary disease (COPD) and particularly in emphysematous lungs. In the latter other inflammatory cells such as macrophages have been implicated in the release of substances which would appear to act as proteinases. This process, along with apoptosis as well as innate mechanisms present in the lung, provide a way in which the organism adapts itself to potential threats or situations which may compromise the general health of the organism.

Exposure to cigarette smoke may trigger certain events that could possibly mimic those that would have occurred as a result of normal development or ageing. This may be as a result of the sharing of common mechanisms or pathways to the inflammatory process. It could also be exaggerating host defences to the inflammatory response and apoptosis, thus causing more detrimentally long-term effects. More emphasis has however been placed on the role of macrophages in the pathogenesis of emphysema in recent investigations because it was found that these inflammatory cells occur in high numbers in the lungs of smokers (Stockley and Burnett, 1993; Niewoenher, 1974) and accumulate in the centriacinar zones of the lungs which are normally associated with cigarette smoke induced emphysema (Hoidal and Niewoehner, 1982). Macrophages also play a role in sequestering neutrophils during the inflammatory process. The release of metalloproteinases from both macrophages and neutrophils are actively involved in the ageing lung, as well as that exposed to oxidative stress. Studies by Ludwig et al. (1985) have shown that cigarette smoking is associated with an increase in the number of polymorphonuclear leukocytes in the alveolar septum and is not intimately associated with the development of emphysema. This suggests that a multitude of factors may be involved in the pathogenesis of emphysema. The proteases and free radicals released from the inflammatory cells may cause changes in the structure and function of collagen, elastin, proteoglycans and α-1-antitrypsin (Vlahovic et al. 1999).

Nicotine has been found to increase the release of the superoxide anion and may play a role in exacerbating damage to the cardiovascular and respiratory system.

The hydroxyl radical can be formed according to the Fenton reaction (Fenton, 1894) follows:

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH$$

Nicotine sequesters Fe^{2+} and inhibits the Fenton reaction, thus the generation of the hydroxyl radical is decreased, leading to the excess accumulation of free radicals (Alvarez et al. 2002). Nicotine also decreases superoxide dismutase, glutathione reductase and catalase, leading to increases in hydrogen peroxide and the superoxide anion, potentiating free radical formation (Ashakumary and Vijayammal, 1996). *In vivo* studies by Ramp et al. (1991) have shown that nicotine inhibits alkaline phosphatase and decreased collagen synthesis, whereas it increased the synthesis of DNA in osteoblast-like cells.

Ofulue and Ko (1999) have found that the breakdown of lung elastin may be attributed more to alveolar macrophages than as a result of neutrophils. Macrophage metallomatrix protein 12 (MMP12) has been suggested to be an important protease for the development of cigarette smoke induced emphysema in mice (Hautamaki et al. 2004). Neutrophil elastase is released by activated neutrophils. Cathepsins L and S are elastolytic serine proteases MMP 2 (gelatinase A), MMP9 (gelatinase B) and MMP 12 (macrophage metalloprotein 12) are the predominant MMP's capable of elastolysis. Russell et al. (2002) showed that the contribution of MMP to elastolysis increased, whereas serine proteases decreased and cysteine proteases remained constant in alveolar

macrophage mediated elastolysis. MMP 9 and MMP 12 are responsible for the majority of macrophage-derived elastolysis in pulmonary emphysema (Ohnishi et al. 1998; Finlay et al. 1997; Fitzgerald and O'Connor, 1997). Studies by Minematsu et al. (2001) found that the polymorphism of MMP acts as a genetic factor for the development of smoking–induced pulmonary emphysema.

TNFα, IL-8 and IL-1 acts in response to oxidative stress by recruiting neutrophils and cause activation of transcription factors such as activator protein 1 (AP 1) and nuclear factor κβ (NF-κβ), (Rahman and MacNee, 1998). The metalloproteases contained in macrophages do not only have the ability to digest the elastin framework but also play a role to inhibit α-1-antitrypsin (Banda et al. 1980). Macrophages release cytokines such as TNFα, IL-1, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), which may stimulate the proliferation of cells involved in inflammation and repair (Kelley, 1990).

Kirkham and colleagues (2003) found an increased number of macrophages adhering to acrolein or 4-hydroxy-2-nonenal (HNE), lipid peroxidation products. This suggests that not only are macrophages activated, but they are also attracted to the site of lipid peroxidation via the initiation of a positive feedback loop, further exacerbating the effects of cigarette smoke induced lipid peroxidation. There is thought to be an association between fibroplasias in induced emphysema and other pathological states in pulmonary tissue involving increased activity of alveolar macrophages. Type-2 cells appear to be involved in these processes and support the inflammatory repair hypothesis in the development of emphysema

(Sulkowska and Sulkowski, 1997). Alveolar epithelial cells may contribute proteinases that lead to the destruction of the alveolar septa, since they express a collagenase MMP that has collagenase activity (Imai et al. 2001).

An increase in pulmonary vascular endothelial growth factor (VEGF) expression in the alveolar walls, parenchymal lining and the small diameter of pulmonary vessels in chronic obstructive pulmonary disease patients may reflect a partly unsuccessful attempt to stimulate tissue repair mechanisms caused by tobacco induced injury. VEGF and receptors Flt1 and Flk1 may be involved in the vascular airway remodelling process in an autocrine and/or paracrine manner in patients suffering from chronic obstructive pulmonary disease (Kranenburg et al. 2005).

A possible role has been proposed for the contribution of mast cells in the inflammatory response in the lungs of smokers (Kalendenian et al. 1988). Mast cells are found lining the alveolar walls (Warburton et al. 1986) and the release of neutrophil chemotactic factors is thought to exacerbate the pathology of lung injury as a result of neutrophils elastases (Schwartz, 1985).

Extracellular matrix changes in patients who suffer from pulmonary emphysema include reduced staining for proteoglycans such as the interstitial proteoglycans, decorin and biglycan and the heparin sulphate proteoglycans. Heparin sulphate proteoglycans (HSPG) are normally found interacting with basement membrane components thus contributing to the stability and integrity of the membrane.

Decreased staining patterns for HSPG, as in patients with emphysema and fibrosis may thus be indicative that the integrity of the membrane has been affected (Van Straaten et al. 1999; Dunsmore and Rannels, 1996). Loss of the interstitial proteoglycans, decorin and biglycan may be associated with loss of elastic recoil and airway collapse which occurs in patients with emphysema (Hogg et al. 1994) since they are normally associated with fibrillar collagens (Schönerr et al. 1995). Reduced heparan sulphate levels were also proposed to cause the altered cytokine activities normally observed in emphysema patients.

Cigarette smoke contains approximately 10^{20} free radicals with every inhalation (Pryor and Stone, 1993). Cigarette smoke oxidants such as $\cdot NO$ and $\cdot O_2^-$ which contribute to the formation of $\cdot OH$ free radical and H_2O_2, a facilitator of free radical formation, may be responsible for damage done to tissue, DNA, lipids and proteins and may contribute to ageing (Church and Pryor, 1991; Pryor and Stone, 1993). Free radicals possess a free electron (free radical), which may cause oxidative damage, but this can be prevented by the donation of an electron and stabilization of the molecule by antioxidants, thereby neutralizing the reactive oxygen species. Metallothionine acts as a scavenger of free radicals (Satao and Brenner, 1993).

Damage caused by polynuclear phagocytes and monocytes include the release of reactive oxygen species (ROS) such as superoxide anions, or other oxidant generating enzymes such as myeloperoxidase, which catalyse hypochlorous acid (reactive oxygen intermediate) formation or the release of proteases (Weiss, 1989;

Sibile and Reynolds al, 1990). Reactive oxygen species may damage the extracellular matrix components of the lung (Janoff et al. 1987) if the oxidant/ antioxidant balance is not maintained and may cause widespread destruction of the extracellular matrix, as in emphysema. The release of reactive oxygen species from neutrophils and macrophages further add to oxidative injury (Schalberg et al. 1992; Ludwig and Hoidal, 1982).

The epithelium lining the airways of smokers appears to be more permeable than that of non-smokers (Jones et al. 1980). This suggests that the epithelium in smokers may have an increased susceptibility to damage that can be caused by oxidants and other substances. Morrison and colleagues (1999) have shown that cigarette smokers have an acute increase in permeability and the number of neutrophils in their airspaces, which is associated with an evidence of increased oxidative stress due to an increase in lipid peroxidation products, thiobarbituric acid-reactive species (TBARS).

2.10 Glutathione

Glutathione (GSH) is found in the fluid lining of the epithelium of the lung. It is an important antioxidant that is responsible for the detoxification of free radicals, oxidants and other compounds normally involved in oxidative stress (Rahman et al. 1995; Li et al. 1994). The sulphydryl group on glutathione helps to protect cells against the potential agents of oxidative stress such as oxidants and electrophillic compounds (Meister and Anderson, 1983).

The human lung forms an important storage location and thus a source of glutathione (Cook et al. 1991). The rate limiting substrate in the formation of glutathione is cysteine (Meister and Anderson, 1983). In its oxidized form, cysteine is increased during periods of oxidative stress and may cause an increase in glutathione synthesis (Deneke et al. 1989). Glutathione is able to offer protection to lung cells against oxidative damage, with the aid of a transport system into these cells (Hagan et al. 1986; Susanto et al. 1998; Cross et al. 1994).

In acute smokers, glutathione levels are depleted, whereas in chronic smokers glutathione levels are increased. The mechanism for this process is as yet unknown but it appears possibly as a result of the altered expression of the rate-limiting enzyme in glutathione formation, γ-glutamyl cysteine synthetase (γ-GCS), (Rahman and MacNee, 1999). Glutathione thus has a crucial role to play in the prevention of cigarette smoke induced lung injury.

2.11 Nutrition and Oxygen Demand

Massaro et al. (2002) have proposed that endogenous programmes of destruction and regeneration of the gas exchange units may regulate the structure function relationship in the lungs of adult mammals. The proposal emanates from the fact that:

1. The oxygen consumption of an organism is proportional to its surface area that is available for gaseous exchange.

2. Oxygen consumption decreases with caloric restriction, with an associated reduction in the need for the larger gas exchange surface area.
3. Proteases break down lung tissue during caloric restriction and hence maintain energy by reducing the need to maintain a larger gas exchange surface area, at the same time providing an energy source.
4. Re-feeding results in a concomitant increase in oxygen consumption, hence there is an increased need once again for a larger gas exchange surface area.

To test their hypothesis they subjected adult mice to 2/3 caloric restrictions for 2 weeks with subsequent re-feeding for 3 weeks. They found that the alveolar numbers and alveolar surface area were significantly reduced by 55% and 25 %, respectively but returned to normal values with re-feeding. It was therefore concluded that endogenous programs exist in adult mammals that regulate destruction and regulation of the gas exchange surface area. Furthermore, lung size is a function of the organism's need for oxygen. When the oxygen needs of the organism increases as a result of physical activity (Weibel, 1979) and high altitude (Hugonnaud et al. 1977), the structure of the lung changes in response to increase demands for oxygen. Massaro and colleagues (2004) have proposed a link between the destruction of alveoli in patients with COPD and calorie-restricted individuals. It was suggested that the endogenous methods that aid in survival during food scarcity switch the use of glucose as preferred substrate for the lung to lipid metabolism. The metabolism of lipids provides a new source of glucose to the brain

and destruction of the alveolar network provides amino acids for gluconeogenesis and muscle tissue. Shortly after the onset of calorie restriction, natural killer cells were found to play potential roles in initiating the events leading to destruction of the alveoli, perhaps due to the secretion of granzymes. Granzyme A, released by natural killer cells seems to cause destruction of the extracellular matrix (Lieberman, 2003). Granzyme B, besides its role in the destruction of alveoli, plays a role in the activation of apoptosis. The TNF ligand was also increased after 2 hours of caloric restriction. The author suggested that COPD and caloric restriction share common mechanisms for alveolar destruction.

2.12 Vitamins and Emphysema

2.12.1 *Vitamin A*

Vitamin A is found in the body as an alcohol (retinol), aldehyde (retinal) and an acid (retinoic acid). Retinoic acid is mainly responsible for normal growth and differentiation of epithelium and plays a role in embryonic development (Marks et al. 1996). It plays a role in the maintenance of the alveolar epithelium, normal lung development and lung maturation (Takahashi et al. 1993).

Emphysema is irreversible. Researchers are investigating possible mechanisms to not only prevent the disease from developing, but also to reverse the condition. Studies by Massaro and Massaro (1997) with vitamin A, showed positive results. However, in a study by Meshi et al. (2002) guinea pigs exposed to cigarette smoke had lungs with lesions resembling human centrilobar emphysema and

retinoic acid did not reverse the emphysematous lesions in these animals. Li and colleagues (2003) found that cigarette smoke-exposed rats had reduced vitamin A levels in the lung, serum and liver. This was associated with inflammation and emphysematous lesions. Since cigarette smoking is associated with decreased elastin staining, increased breakdown and decreased vitamin A concentration in emphysema, this may be explained by the fact that retinoic acid increases the transcription of elastin (Liu et al. 1993; McGowan et al. 1997).

Decreased levels of surfactant diphosphatidyl choline (DSPC), probably due to decreased levels of retinal at thresholds of about 18μg/dl were thought to affect surfactant phospholipid synthesis. From this it was deduced that delays in the maturation of the lung may occur even as a result of mild vitamin A deficiencies and is not limited to severe deficiencies.

Vitamin A deficiency could also occur in developing countries as a result of overall malnutrition and in developed countries as a result of dietary influences. Retinoic acid was found to stimulate alveolarisation in neonatal rats and adult rats suffering from elastase induced emphysema (Aoki, 2003). In a study by Lucey et al. (2003), it was found that in contrast to other studies where retinoic acid was found to stimulate alveolar development in elastase–induced emphysema, their elastase-induced emphysematous mice failed to show any restoration of airspace size, elastin expression or α-collagen mRNA. This may be as a result of species differences to all-trans-retinoic acid treatment and the mice used were also from an inbred strain whereas the rats used were heterogeneous. The latter group might

therefore better reflect the genetic diversity of humans suffering from emphysema or COPD. Differences in the rate of body growth have also been proposed to account for the varied response to treatment with all-trans-retinoic acid between the two groups of animals, with mice being faster growers than rats.

In contrast to studies by Lucey et al. (2003), Maden and Hind (2004) showed that mice treated postnatally with dexamethasone to disrupt alveolar development, had retinoic acid receptors, retinoid binding proteins and synthesizing enzymes for retinoic acid, which 'peaked' postnatally. The surface areas for gaseous exchange per unit of body weight and lung structure were found completely restored. The time and duration of treatment with retinoic acid between the two studies and its relation to the 'insult' may have played a role in the observed outcomes. There have been some degree of success in the treatment of humans with vitamin A in the treatment of emphysema (Mao et al. 2002) and studies in rats should thus be encouraged as a way of elucidating the possible treatment options for patients with emphysema.

Long-term vitamin A deficiency has been associated with growth retardation, foetal malformation and death (Wilson et al. 1953; Takahashi et al. 1975). In a study by Frey et al. (2004), a 60 % vitamin A deficiency was associated with lower body weights compared to controls. In the 1st postnatal week there was also an associated delay in lung maturation, which was reflected by a decreased lung volume and surface area as well as an increased thickness of the blood air barrier and increased type-2 cell volumes. In the 3rd postnatal week a decrease in

interstitial volume and increased maturity of the vascular system was observed in these animals. It was concluded that this mild deficiency mainly caused a delay in lung and body growth and maturation but did not result in any serious abnormalities. This should however still be taken into consideration as important findings, since it is well known that influences in the development of an organism can manifest itself as disease or reduced function in later life.

Liebeskind et al. (2000) reported that all-trans-retinoic acid, when administered exogenously and especially when used in combination with vitamin D enhanced the stimulation of lung fibroblasts and alveolarisation via a platelet derived growth factor (PDGF)–related autocrine mechanism.

Dietrich and colleagues (2003) have shown that when compared to non-smokers, smokers had lower levels of antioxidants in their plasma. A higher γ-tocopherol level was found for smokers, which may suggest other as yet unknown mechanisms for the effects of cigarette smoke in the contribution of oxidative damage. Rautalahti and colleagues (1997) have shown that supplementation with α-tocopherol or β-carotene did not offer any beneficial effects from symptoms in patients with chronic obstructive pulmonary disease but did offer some protection in elderly chronic smokers. In a review by Kelly (2002) it was hypothesized that smokers respond differently to supplementation with β-carotene and supplementation with β-carotene and other vitamins did not offer beneficial outcomes in smokers. It was unsure whether β-carotene supplementation added to

the effects of smoking in inducing cancer and was not supportive of it being added as a modifier of decreased susceptibility to diseases such as cancer.

2.12.2 *Vitamin C (Fig. 2.1)*

Vitamin C is able to act as a scavenger of reactive oxygen species (Halliwell, 1996) and may thus be an important supplement to smokers. Cigarette smoking has been associated with an increased need for vitamin C (Schectman, 1993) as its levels are normally lower in smokers than non-smokers (Kurata et al. 1998). It may therefore act as an antioxidant. Humans do not synthesise ascorbic acid because of the loss of functionality in the gene encoding the final step in its synthesis lacks activity (Nishikimi and Yagi, 1991) whereas rats are capable of synthesizing ascorbic acid (Krasnov et al. 1998). Ascorbic acid is thought to play a role in the regulation of gene expression of anti-oxidative enzymes, xenobiotic metabolising enzymes and ascorbic acid recycling enzymes (Veta et al. 2003). Ascorbic acid plays a role as an antioxidant in the cytosol and on plasma membranes where it may act as a reducing agent of the α-tocopheroxyl free radical. The result is the formation of α-tocopherol (Scarpa et al. 1984), which may help in the protection of the plasma membrane against lipid peroxidation.

Constantinescu et al. (1993) have shown that vitamin C assists in the recovery of vitamin E, the latter which also acts as an antioxidant against radicals produced during lipid peroxidation of cell membranes. Vitamin C does this by acting as a direct reducer of the generated tocopheroxyl radicals (Packer et al. 1979). The condensate, which forms on the epithelial lining of the lungs, creates a source of

free radicals that may last longer than that obtained from the gas phase of cigarette smoke.

Vitamin C supplementation was found to increase the reduced forced expiration normally found in nicotine treated monkeys. It also decreased the normally increased levels of surfactant protein B (SPB). The decrease in elastin content observed by nicotine exposure was also reduced by supplementation with vitamin C (Proskocil et al. 2005).

Studies have been performed to determine whether vitamin C can also act as a pro-oxidant. In a review of various studies by Carr and Frei (1999) it was postulated that since most previous studies have shown that vitamin C acts as an antioxidant rather than a pro-oxidant, results obtained showing its role as a prospective pro-oxidant were probably due to inefficient experimental design and methodology. Studies by Van Wyk (1995) have shown that on its own, vitamin C treatment in rats produced effects on radial alveolar counts similar to those that were treated with nicotine but it was reversed by 21 days of age. The percentage of abnormal alveolar attachments and the destructive index was higher than in the control rats. This may add in excess vitamin C acting as a pro-oxidant since it causes reduction of Fe^{3+} to Fe^{2+}. This causes a reaction with hydrogen peroxide to form the hydroxyl radical (Yu, 1994). Vitamin C may thus act as pro-oxidant if not administered with care. Vitamin C has also been found to convert the β-carotene radical to its stable form, β-carotene (Black, 1998). It is thus proposed that though vitamin C may be beneficial in

preventing the damage caused by smoking cigarettes, it should be taken in moderation.

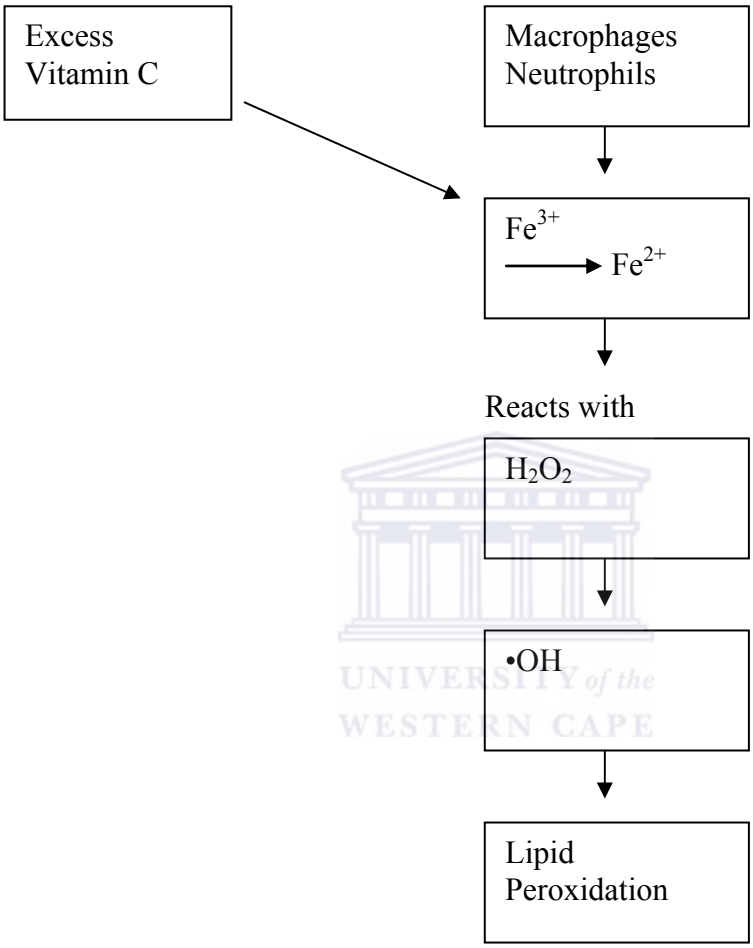

Fig. 2.1. Interaction of excess vitamin C and the development of lipid peroxidation.

2.12.3 *Vitamin E (Tocopherol)*

The consumption of cigarettes is associated with increased lipid peroxidation (Brude et al. 1997) and in products of lipid peroxidation such as 4-hydroxy-2-neonal, which is thought to play a role in signalling processes during lung inflammation. This adds to the imbalance of the expression of oxidant and antioxidant genes, favouring the former. Vitamin E (α-tocopherol) decreases lipid peroxidation in smokers (Hoshino et al. 1990) and lipid peroxidation tends to increase after the cessation of smoking and prior to supplementation with Vitamin E. In one study, its levels in smokers was found to be decreased as compared to non-smokers and could not be corrected by short-term intensive supplementation (Pacht et al. 1986). A more long-term supplementation with vitamin E may however be considered as a part of the therapy for reducing the damage caused by cigarette smoke induced lipid peroxidation of cell membranes. Further research however should be conducted to confirm the therapeutic benefits of the vitamin as supplement.

2.13 The Foetal Origins Theory (Barker Theory)

'Programming' refers to the phenomenon that takes place at certain critical phases of development in which insults such as under-nutrition and exposure to tobacco smoke cause permanent alterations in the structure and function of tissues and organs (Barker et al. 1994). The timing of the insult is important, as there are times in *in utero* development when organs undergo rapid cell division and undergo critical phases of remodelling. During these phases of rapid cell division the organ is most susceptible to a change in the program that determines its

development which may make the organ more susceptible to disease in later life (Stocks, 1995).

In a study by Roseboom et al. (2001), it was suggested that maternal malnutrition could give rise to disease in later life but may not necessarily be associated with reduced birth weight as suggested by Barker and colleagues (1992). Their study provides a model in which under-nutrition during pregnancy leads to chronic diseases such as diabetes and coronary heart disease, with the timing of the malnutrition once again determining the outcome of the disease. Many other studies have also focussed on the impact of early nutritional influences on disease outcomes in later life (Barker et al. 1990; Fall et al. 1998; Eriksson et al. 2001; Barker et al. 1993).

In a study by Barker and colleagues (1992), the relationship between weight at birth and childhood respiratory infection was studied and correlated with subsequent lung function in adulthood as well as death resulting from chronic obstructive pulmonary disease. They found that males with low birth weight had a higher incidence of impaired lung function in adulthood. Lung infections during childhood also contributed to reduced lung function and morbidity rates from chronic pulmonary disease were increased when accompanied by low body weights at birth or during the first year of life. The investigators propose that this may be due to programming of the lung during critical periods of development as a result of adversities *in utero.* This leads to reduced birth weight and impaired lung function in later life. Other studies in animals (Hoet and Hanson, 1999)

indicate that despite unaltered birthweight, nutritional perturbations during pregnancy are still associated with adverse outcomes in later life. This essentially means that low birth weight does not necessarily have to be an indicator of maternal nutritional influences and these influences can have adverse outcomes in the health of the offspring in later life, whether evident as low birth weight or not.

Data by Snoek and colleagues (1990) show that when foetuses received inadequate amounts of protein, they had reduced numbers of pancreatic cells and correspondingly lower secretion of insulin, which is indicative of a potential for the development of diabetes. Low birth weights have been associated with the increased onset of type-2 diabetes in adults (Hales et al. 1991; Curhan et al. 1996). Barker and colleagues (1989) showed an association between low birth weights and coronary heart disease in later life, referred to as the "foetal origins of coronary heart disease". Other studies showed similar links between nutritional influences during early life and later risk of coronary heart disease (Eriksson et al. 2001).

Low birth weight has also been associated with the incidence of stroke and hypertension (Martyn et al. 1996; Eriksson et al. 2000; Law et al. 1996). Studies by Ankarberg (2003) found that exposure to nicotine during a critical period of neonatal brain development had an effect in adult life on learning and memory. The cause of programming may also be extended to include factors other than nutritional influences, for example, it is thought that foetal programming may lead

to alteration in gene expression and cause effects in the lifetime of the individual (Wu et al. 2004).

2.14 Motivation

Information from the literature clearly shows that smoking has an adverse effect on the protection mechanisms of the lung. It also shows that maternal smoking increases the incidence of respiratory disease in the offspring. Some of the respiratory diseases are associated with maternal nicotine intake. Nicotine intake not only occurs during smoking, but also during nicotine replacement therapy (NRT). Therefore, this project is designed to investigate the effect of maternal nicotine exposure:

a. during all the phases of lung development and

b. from the onset of the phase of rapid alveolarisation,

to establish whether exposure at different phases of lung development will have a different outcome in the long-term.

Since it is unlikely that smokers will kick the habit when pregnant, or will use NRT, it is important to develop a strategy to prevent the adverse effects of maternal nicotine exposure on lung in the offspring. Preliminary studies suggest that copper supplementation might protect the lung against the harmful effects of maternal nicotine exposure. The second aim of this study is therefore to determine whether maternal copper supplementation will ensure normal development of the neonatal lung and maintenance of the lungs in the long term. Morphometric and morphologic techniques will be used.

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CHAPTER 3

Materials and Methods

3.1 Animals

White virgin Wistar rats were utilized in this study. Animals were housed at the Department of Medical Biosciences of the University of the Western Cape, where an in-house breeding programme was maintained. Animals were fed a stock diet consisting of Epol rat cubes and water, which was consumed *ad libitum*. Room temperature was maintained at 22± 1° Celsius and a day-night cycle of 12 hours was maintained. Animals were mated overnight and then randomly assigned to control and experimental groups. Each group consisted of at least 6 dams.

3.2 Ethical Clearance

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

3.3 Treatment

The daily nicotine intake of human males and females who smoke tobacco, varies between 10.5 and 78.6 mg (Benowitz and Jacob, 1999). Assuming that 90% of the nicotine is absorbed on inhalation (Gleason et al. 1963), the nicotine intake of a 60kg female will be between 0.16 and 1.18 mg per kg body weight (BW) per day. The dose of 1 mg nicotine per kg BW/day used in this study is therefore within the range of intake of habitual smokers. Since nicotine readily crosses the placenta

and occurs in the mother's milk (Luck and Nau, 1984), the foetal and neonatal rats would be expected to receive nicotine via the placenta and mother's milk.

The study was divided into 2 components:

a. Animals that were exposed to nicotine, and

b. Animals that were exposed to a combination of nicotine and copper.

The nicotine-exposed animals were divided into 2 groups:

a. Pregnant rats exposed to nicotine from day 3 after mating up to weaning on postnatal day 21. The foetuses and neonates were thus exposed to nicotine via the placenta and mother's milk during all the phases of lung development.

b. Rats exposed to nicotine from postnatal day 3 until weaning on postnatal day 21. The offspring of this group were therefore exposed to nicotine via mother's milk only. Exposure starts from 1 day before the onset of the phase of rapid alveolarisation. The day of birth was designated day 0.

The animals exposed to a combination of nicotine and copper were also treated as described above. The copper supplementation, in the form of a solution of copper sulphate was 1mg/kg BW/day. The control animals received saline. The nicotine, copper and saline were injected subcutaneously once a day at about 10:00 am. Animals were killed 24 hours after the last exposure. The offspring therefore only

received nicotine or copper via the placenta and/or mother's milk. After weaning no nicotine was given to the offspring.

3.4 Removal of Lung Tissue

Rat pups were sacrificed on postnatal days 14, 21 and 42. This was done by an overdose of an intraperitoneal sodium pentobarbital injection at a dosage of approximately 0.5ml per kg BW. Animals were treated humanely at all times during sacrifice. At least 6 rat pups were selected from each of at least 5 litters.

3.5 Intratracheal Instillation

Gehr and Crapo (1978) have reported that fixation of lung tissue by intratracheal instillation is for the most part a simple, reliable method, as it gives complete unfolding of the alveoli, preserves the vascular bed and shows minimal shrinkage. The trachea was surgically exposed and the diaphragm punctured in order to remove air from the lungs. The trachea was canulated and ligated to prevent loss of fixative. The fixative (pH 7.4) consisting of a 4% paraformaldehyde-2% glutaraldehyde solution in phosphate buffered saline (phosphate buffered saline, pH 7.3) was allowed to run into the lungs, whilst a transpulmonary pressure gradient of 25 cm fixative was maintained for approximately 30 minutes. The canula was then quickly and carefully removed and the ligature secured to ensure that no fluid escaped. The entire lung was removed by careful dissection and the trachea was cut off dorsal to the ligature. Lung tissue was then placed in fixative for 24 hours before histological processing.

3.6 Measurement of Lung Volume

The lung volume was measured by the fluid displacement method of Scherle (1970). A beaker containing physiological saline was placed on a scale and weighed. The lung was immersed into the beaker with the aid of a forceps at a level of buoyancy and the initial weight (beaker + saline) subtracted from the final weight (beaker + saline + tissue) to obtain the lung volume. The specific gravity of physiological buffered saline is 1.0048 and therefore no correction was made to adjust lung volume. The lung was then placed in sample vials filled with buffered formalin at a pH of 7.2.

To ensure unbiased sampling, the stratified random sampling technique was used to sample the lungs for morphometric and morphologic analysis of tissue samples. In this technique the lung of the organism of interest is divided into equally sized strata or levels from cranial to caudal, the number of strata increasing with the size of the lung (Gehr and Crapo, 1998). 5 To 8 randomly selected fields were examined for each lung lobe.

3.7 Histological Processing and Staining Techniques

3.7.1 *Secondary Fixation, Dehydration and Wax Impregnation*

3.7.1.1 *Reagents*

Buffered formalin:

Formalin	200ml

Sodium chloride	17g
Sodium di-hydrogen phosphate	7g
Di-sodium hydrogen phosphate	13g
Distilled water	1.8l

The tissue was cut unto thin slices, placed in embedding cassettes and fixed and processed in a Histokinette^{TM} tissue processor using various solutions, in the order shown in table 3.1. The total duration of secondary fixation and wax impregnation was 22 hours. Histological techniques were based on established histological methods (Culling, 1974).

3.7.2 *Microtomy*

The tissue was removed from the cassettes and embedded in wax and allowed to set. The tissue blocks were trimmed and sections of 4 μm were cut for haematoxylin and eosin (H&E) staining and transferred onto microscope slides, by allowing the wax ribbon to float in a warm water bath. Sections of 4 and 20 μm were cut to stain for elastic tissue. Tissue and tissue blocks that were inadequately processed or embedded were excluded from the study. Sections that broke up easily when placed in the water bath were also excluded from the study.

1. 70% alcohol	3 hours
2. 90% alcohol	3 hours
3. Absolute alcohol 1	1 hour
4. Absolute alcohol 2	1 hour
5. Absolute alcohol 3	2 hours
6. Absolute alcohol 4	2 hours
7. Xylene 1	1½ hours
8. Xylene 2	2½ hours
9. Wax bath 1	3 hours
10. Wax bath 2	3 hours

Table 3.1. Histological tissue processing for light microscopy

3.7.3 *Haematoxylin and Eosin Staining*

1. The sections were placed in graded solutions of solvents and alcohol in order to de-wax (table 3.2).

2. The slides were rinsed in water for 5 minutes.
3. They were stained in haematoxylin for 15 minutes.
4. In order to remove the excess stain, they were thoroughly washed in tap water for 2-3 minutes.
5. The slides were then decolourised in acid-alcohol solution (0.5-1% HCL and 70% alcohol) for a few seconds. The blue stain of haematoxylin changed to red by the action of the acid.
6. The blue colour was regained and decolourisation stopped by washing in alkaline running tap water for ± 5 minutes.
7. Slides were stained in 1% aqueous eosin for 1 minute.
8. The surplus stain was washed off in running water.
9. The slides were microscopically examined. Cytoplasm and muscle fibres were deep pink and collagen fibres were a lighter pink. Red blood cells and eosinophil granules were bright orange-red.
10. Slides were dehydrated in alcohol and cleared in xylene.
11. The slides were viewed under a light microscope for evidence of inadequate preparation. Those sections that did not adhere to the slide or were not adequately stained were excluded from the study.
12. The slides were mounted in synthetic resin medium of DPX.

1. Xylene 1	5 minutes
2. Xylene 2	5 minutes
3. 100% Ethanol 1	3 minutes
4. 100% Ethanol 2	3 minutes
5. 90% Ethanol	3 minutes
6. 80% Ethanol	3 minutes
7. 70% Ethanol	3 minutes

Table 3.2. De-waxing of tissue sections

3.7.4 *Verhoeff's Method for Staining of Elastic Fibres*

3.7.4.1 *Staining Solution*

10% Alcoholic haematoxylin	2.5ml
Absolute alcohol	2.5ml
10% Ferric chloride	2ml
Lugol's iodine	2ml

a. *Lugol's Iodine:*

Iodine	1g
Potassium iodide	2g
Distilled water	100ml

b. *Van Gieson:*

Saturated aqueous picric acid	100ml
1% acid fuschin	10ml

3.7.4.2 *Staining Method*

1. The sections were taken down to distilled water.
2. The stain was poured on the slides for approximately 20 minutes.
3. The slides were washed in water and the bottom of the side was cleaned.
4. The slides were differentiated in 2% ferric chloride and checked microscopically.
5. The slides were rinsed in 96% alcohol for 5 minutes.
6. The slides were rinsed in water.
7. The slides were counterstained with Van Gieson for 3 minutes.
8. They were then rinsed in water.
9. The slides were dehydrated in alcohol, cleared in xylene and mounted with DPX mountant.

3.7.5 *Shrinkage*

Investigators have previously shown that ethanol caused shrinkage of only 2% for lung tissue as compared with the usage of acetone (Bastacky et al. 1985). Data should be corrected for shrinkage alterations in the structure of the tissue, as shrinkage would have an effect on morphometric measurements such as mean linear intercept. In order to ensure accurate measurements, tissue blocks were trimmed at right angles before calculating shrinkage. A calibrated eyepiece

micrometer was then used to measure the linear shrinkage of the tissue blocks and found to be approximately 3.2 ± 0.3%.

3.8 Morphometric and Morphologic Methods

3.8.1 *Morphometric Techniques*

Morphometric techniques included:

a. Mean Linear intercept (Lm)

b. Interalveolar septal thickness (Tsept)

c. Microscopic emphysema (AWUV)

3.8.1.1 *Total Number of Alveoli per Lung (Na)*

The alveolar number (Na) of the lungs was determined as described by Angus and Thurlbeck (1972). Tissue samples were taken randomly from the upper, middle and lower lung lobes. At least 6 tissue samples were taken per lung for processing for morphology and morphometry. Na was calculated as follows:

Na = Lv.Va/ Valv,

Where:

Lv = lung volume (ml)

Va = alveolar air volume density (%)

Valv = alveolar volume (ml)

3.8.1.2 *Volume Density (Va)*

The alveolar air volume density (Va) and alveolar tissue volume density (Vt) was determined by using the point counting technique at 100x magnification. A 121-point eyepiece graticule was used. For the purposes of this study, an alveolus was defined as an airspace either completely enclosed by respiratory epithelium, or having a smooth rounded contour for more than one third of a projected circle with the remaining boundary formed by an imaginary line between the distal ends of secondary septa.

A 10x eyepiece and a 10x objective were used to obtain a total magnification of 100x. Two blocks were taken from the upper lobe, 1 from the middle lobe and two from the lower lobe. Non-parenchyma included tissue which had a diameter of >1.1mm.

The following alveoli were included:

a. Those that were found within the graticule.

b. Those that touched the lower and right borders of the graticule.

The alveoli that were excluded from the count included:

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

b. In addition, fields containing non-parenchymatous tissue were excluded from the counts. At least 25 randomly selected non-overlapping fields were analysed and 75 fields per animal were counted.

3.8.1.3 *Mean Alveolar Volume (Valv)*

Valv was measured according to the following formula:

Valv = $Lm^3 x \pi/3$ (Boros et al. 1997).

Mean alveolar volume gives an indication of the size of the alveolus and hence the volume of air occupying it. For alveoli with larger alveolar volumes, it is expected that the total surface area for gaseous exchange in the lung be reduced because it also implies the possibility of less alveolar surface area for gaseous exchange and less alveoli.

3.8.1.4 *Mean Linear Intercept (Lm)*

Mean linear intercept, is the average distance between the walls of an alveolus. It is an index of alveolar wall size. The Lm will thus increase with an increase in alveolar size such as in alveolar wall destruction (Thurlbeck, 1967; Graves and Colebatch, 1980). Dunnill (1962) has described total surface area of the air-tissue interface according to the following formula:

$Sa = 4\lambda vp^2f^2 / Lm$

This indicates that surface area (Sa) and mean liner intercept (Lm), are inversely related. Thus, an increase in Lm, as evidenced by destruction of alveolar walls is indicative of a decreased surface area available for gaseous exchange. The method of Weibel and Knight (1964) was employed to determine Lm.

Lm = N x L/m

Where:

N = mean number of fields counted,

L = length of the traverses, (i.e. the cross hair length added to the vernier length, totalling 2.02mm) and

m = the sum of all the intercepts.

The number of alveolar intercepts (m) was counted at 100x magnification using an eyepiece micrometer. Approximately 6 points were used to determine the mean linear intercept per slide preparation.

Alveolar walls that contributed to the intercept count included those that:

a. Touched but did not intercept the left side of the vertical line

b. Touched but did not intercept the upper side of the horizontal line.

c. Intercepted the cross hairs.

Cuts into and out of blood vessel walls were allocated counts of half an intercept each.

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

a. Touched without crossing the right border of the vertical arm and

b. Touched without crossing the lower border of the horizontal arm

Structures including airways or blood vessels that were greater than 1.1mm in diameter were also excluded from the counts.

3.8.1.5 *Interalveolar Septal Thickness (Tsept)*

T*sept* is the distance between adjacent alveoli or thickness of the alveolar wall between adjacent alveoli. The point counting method and linear intercept method described by Weibel (1963) was used. The number of points falling on the alveolar septum and number of alveolar intercepts were determined using the Weibel no. 1 graticule at 100x magnification. At least 6 fields per slide were used. The T*sept* was calculated 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

3.8.1.6 *Internal Surface Area (Sa)*

The internal surface area available for gaseous exchange (Sa) was measured according to the following formula:

Sa = 4 x Lv/ Lm (Weibel, 1963).

3.8.1.7 *Thickness of Elastic Fibres*

The method as described by Gehr and Crapo (1988) for the determination of the harmonic mean thickness of the blood-air-barrier was used to determine the thickness of the elastic fibres in the lung parenchyma. Tissue sections of 20 μm were made and stained for elastic tissue. Measurements were made at a final magnification of 50 000 x. A multipurpose test grid with 32 lines and 64 points was used to measure the thickness of the elastic tissue fibres in the alveolar walls. The thickness of 50 fibres was determined for each of the control and experimental groups. The thickness of the fibres was calculated as follows:

$$T_h = 2/3\{n/\sum/ (1/L)\}/M$$

Where:

n = number of measurements (i.e. intercepts of thicknesses measured)

L = length of intercept

M = final magnification at which measurements were made.

2/3 = adjustment for possible overestimates which usually occur due to obliqueness of sectioning.

3.8.1.8 *Microscopic Emphysema*

The definition of emphysema has classically been stated as an enlargement of the airspaces distal to the terminal bronchiole, without obvious fibrosis (Snider et al. 1985). The ageing lung can be defined as an enlargement of the distal airspaces with increasing age and without any associated tissue loss (Escolar et al. 1994).

This means that the airspace wall surface area per unit volume (AWUV) increases with increasing age. Emphysematous lungs may be defined as having a mean AWUV below the 95% prediction limit (Gillooly and Lamb, 1993). Measurement of AWUV can detect the presence of microscopic emphysema since macroscopic emphysema is normally only present when there has already been a significantly high amount of tissue destruction. Since airspace size increases with age (Anderson et al. 1964; Snider et al. 1985; Pinkerton et al. 1982) and the definition of emphysema includes associated tissue loss whilst that of the senile lung excludes it (Snider et al. 1985), it is important that one is able to accurately distinguish and diagnose between the two conditions.

As a person ages, the shape and structure of the lungs also change (Anderson et al. 1964). Macroscopic techniques have been largely inadequate in pointing out early emphysema because even though the size of the airways may be < 1mm in diameter, the presence of focal abnormalities is still indicative of emphysema. AWUV in this study was measured according to the method employed by Gillooly and colleagues (1991) which they reported as being efficient for the detection of early-onset emphysema. The method involves the calculation of the mean linear intercept, which was obtained as described previously (see mean linear intercept, Lm). It was then calculate according to the following formula:

AWUV= 2/Lm (mm^2/mm^3)

3.8.2 Morphologic Methods: Scanning Electron Microscopy

3.8.2.1 *Principle*

The procedure employed is based on a principle that involves dehydration of the wax-embedded tissue samples with varying concentrations of acetone and critical point drying followed by sputter coating of the tissue with gold.

3.8.2.2 *Equipment*

a. Hitachi™ HPC-2 Critical point drier

b. Edwards™ S150B Sputter coater

c. Hitachi™ x650 Scanning electron microscope

d. Acetone (Analytical grade)

e. Xylene (Analytical grade)

f. Absolute alcohol that was diluted with distilled water to achieve the desired concentrations.

g. Pill bottles

3.8.2.3 *Method*

a. *Dehydration:*

1. The wax-embedded samples that had been used for light microscopy were cut into 3mm^3 tissue chips using a sharp scalpel.

2. The tissue chips were placed in labelled cassettes that were then transferred to a Shandon™ tissue processor (table 3.3).

1. Xylene	60 minutes
2. Xylene	30 minutes
3. 100% Alcohol	30 minutes
4. 95% Alcohol	15 minutes
5. 90% Alcohol	15 minutes
6. 80% Alcohol	15 minutes
7. 70% Alcohol	15 minutes
8. Distilled H_2O	30 minutes
9. Distilled H_2O	30 minutes

Table 3.3. Wax removal of tissue chips

3. The tissue was transferred from the labelled cassettes down to acetone along a series of concentrations of acetone (table 3.4).

1. 70% Acetone	30 minutes
2. 80% Acetone	30 minutes
3. 90% Acetone	15 minutes
4. 95% Acetone	15 minutes
5. 100% Acetone	15 minutes
6. 100% Acetone	15 minutes

Table 3.4 Dehydration of tissue chips

b. *Critical Point Drying:*

Dehydrated tissue was placed on coded pieces of filter paper in small compartments on a metal holder. This holder was closed and put into a critical point drier, which was operated according to manufacturer's instructions. After critical point drying the sample was placed on stubs, which was appropriately labelled and placed in a desiccator before being sputter-coated.

c. *Sputter Coating:*

The samples were transferred to a sputter-coater that was operated according to the manufacturer's instructions. Specimens were then viewed in a scanning electron microscope.

3.8.3 Statistical Analysis (Morphometry)

Results were analysed on the "Graph Pad Instat" and "Prism" statistical analysis programme using standard error bars and a one-way analysis of variance test (ANOVA) for unpaired data and the Student-Newman Kuel's test for pairwise comparisons. A probability level of $P<0.05$ was chosen as significant to the study. Results were recorded as means ± standard error of means.

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CHAPTER 4

The Effect of Maternal Exposure to Nicotine on the Growth of the Offspring

4.1 Introduction

The foetal origin of adult disease theory (Barker, 1995) implies that any foetal undernutrition during critical periods of intrauterine development may lead to permanent alterations in the health of the offspring in later life. Previous studies showed that nicotine caused a significant reduction (> 40%) in uterine and placental blood flow (Birnbaum et al. 1994). Phillip and colleagues (1984) also showed that maternal smoking during pregnancy led to a reduction in utero-placental blood flow and led to lower birth weights, as well as body length (Milner et al. 1999; Wen et al. 1990). The reduction in placental blood flow was also related to the number of cigarettes smoked. Mothers who smoked more therefore had more placental vasoconstriction. It is therefore conceivable that the nutrient supply to the foetus will be lower in smokers, which may result in slower foetal growth and thus lower body weight at birth. However, it was concluded by Birnbaum and co-workers (1994) that vasoconstriction alone as a result of nicotine administration during the last trimester of gestation does not necessarily reduce nutrient supply to the foetus and does not affect foetal growth in rats.

Although Milner and co-workers (1999) found that the static compliance of the lungs of boys, and the airway conductance in girls was reduced, they did not find evidence that maternal smoking affected foetal lung growth.

Tobacco smoke contains over 4800 different components (Green and Rodgman, 1996). Nicotine is the major alkaloid in tobacco smoke (Benowitz et al. 1983) and it is well known that maternal nicotine exposure exerts deleterious effects on the health of offspring. Nicotine is primarily absorbed via the alveoli but it can also be absorbed via the buccal mucosa and the skin and hair, amongst other means (Armitage, 1974).

Nicotine has been implicated as the cause of low birth weight in infants and prematurity and increased incidences of lower respiratory illness such as asthma (Cliver et al. 1995; Hanrahan et al. 1992; Gilliland et al. 2003). In a study of foetal growth in human twins, *in utero* nicotine exposure as a consequence of smoking resulted in a significant reduction in birth weights when compared to controls and also showed a reduced gestational period in these twins (Salihu et al. 2005). It is therefore conceivable that nicotine in tobacco smoke as well as nicotine taken in during nicotine replacement therapy (NRT) may have a negative impact on the health of the offspring.

Chronic exposure to low doses of nicotine (1.5 to 3ml/kg BW /day) did not affect the gross structural development of the offspring of Fischer 344 rats (Peters and Nagan, 1982). Other studies (Ong et al. 2002) have shown that maternal smoking

caused a reduction in growth during gestation and size at birth but was accompanied by considerable catch up growth in later life. In their studies males have also been shown to have more rapid and earlier catch-up growth than females and this has been attributed to the earlier production of sex hormones in males, as compared to females. As male rats age, growth hormone secretion starts to resemble the constant low level of growth hormone secretion as observed in females (Kamataki et al. 1985). In studies by Schulte-Hobein and colleagues, (1992) infants exposed to maternal smoking during pregnancy weighed less at birth than those of non-smokers but at 12 months of age no significant differences were found in their body weights. However, at present no information as to the effect of nicotine (or smoking during pregnancy) on lung growth in relation to body growth is available.

The aim of this chapter was therefore to:

a. Determine the effect of maternal nicotine exposure during gestation and lactation as well as,

b. from the onset of alveolarisation, on growth of the offspring.

The growth and development of the lung tissue as well as growth of the lung in relation to an increase in body weight as the animals aged was also determined.

4.2 Results

4.2.1 *The effect of maternal exposure to nicotine and supplementation with copper on body weight (BW) and lung volume (Lv), as well as on the BW/Lv ratios of growing rats.*

The body weight (Fig. 4.1a) of the control animals increased 4.8-fold ($P<0.001$) from 26.99 ± 0.40 g on postnatal day 14 to 130.20 ± 4.04 g on postnatal day 42. In animals exposed to nicotine during both pregnancy and lactation (G&L) as well as those exposed to nicotine from the onset of the phase of alveolar formation (PNE), that is 3 days after birth, body weight increased from 25.90 ± 1.37 g to 122.79 ± 6.45 g (4.74-fold: $P<0.001$), and 28.28 ± 1.37 g to 122.94 ± 7.87 g (4.35-fold: $P<0.001$), respectively. No differences in body weight were observed between the control and experimental animals on each of the postnatal days. There were no significant differences in the body weights of control, nicotine (G&L as well as PNE) on days 14, 21, and 42 (Fig. 4.1a) after birth.

Although the body weights, (Fig. 4.1 b) of the 42-day old control male rats (130.83 ± 7.88 g) tended to be higher than that of the females (114.98 ± 9.18 g), it was not significant. The body weights of the 42-day old nicotine-exposed male rats was at 126.90 ± 7.63 g, not different from that of the control male rats. However, the BW of the control female rats was higher ($P<0.005$) than the 99.93 ± 3.89 g of the nicotine-exposed female rats.

The lung volumes (Fig. 4.2 a) of the control rats increased 3.82-fold from 1.27 ± 0.06 ml on postnatal day 14 to 4.85 ± 0.16ml on postnatal day 42. The Lv of the G&L rats increased 4.32-fold from 1.13 ± 0.07 ml to 4.89 ± 0.16 on postnatal day 42 ($P<0.001$). The Lv of the PNE rats increased 3.9-fold ($P<0.001$) from 1.17 ± 0.06 ml on postnatal day 14 to 4.63 ± 0.22 on postnatal day 42. No difference in lung volume and the rate of increase was observed between control and experimental animals. The Lv (Fig. 4.2 b) of the 42-day old male control rats (4.88 ± 0.27 ml), female control rats (4.56 ± 0.20 ml), male nicotine-exposed rats (4.86 ± 0.18 ml), and the nicotine- exposed female rats (4.34 ± 0.18 ml), were the same ($P>0.05$). The BW/Lv ratios were also not affected by maternal nicotine exposure (Fig. 4.3).

4.2.2 *The effect of maternal nicotine exposure (G&L and PNE) on the chest circumferences (CC) and the CC/Lv ratios of growing rats.*

The chest circumference (CC) of the control animals increased 1.6-fold between postnatal days 14 and 42 (74.77 ± 2.97 to 121.34 ± 4.21 mm), and the G&L and PNE animals, 1.67- and 1.69-fold respectively ($P<0.01$). The CC of the G&L and PNE rats on postnatal days 14 and 42 was not different ($P>0.05$) from that of the control animals of the same ages (Fig. 4.4 a). Since the Lv and CC of the control, G&L and PNE animals were the same for each of the age groups, the CC/Lv ratio was also not affected by maternal nicotine exposure during gestation and lactation or from the onset of the phase of rapid alveolarisation (Fig. 4.4 b).

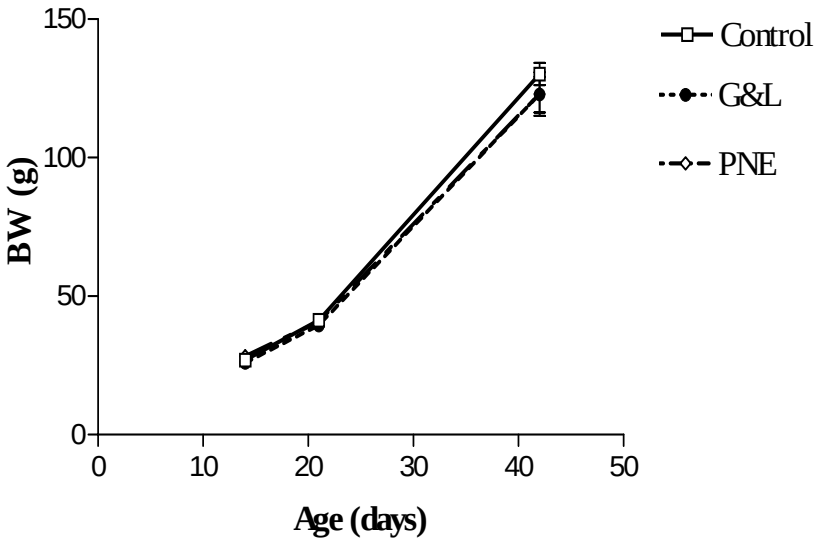

Fig. 4.1 (a). The effect of maternal nicotine exposure during different phases of lung development on neonatal body weight (BW).

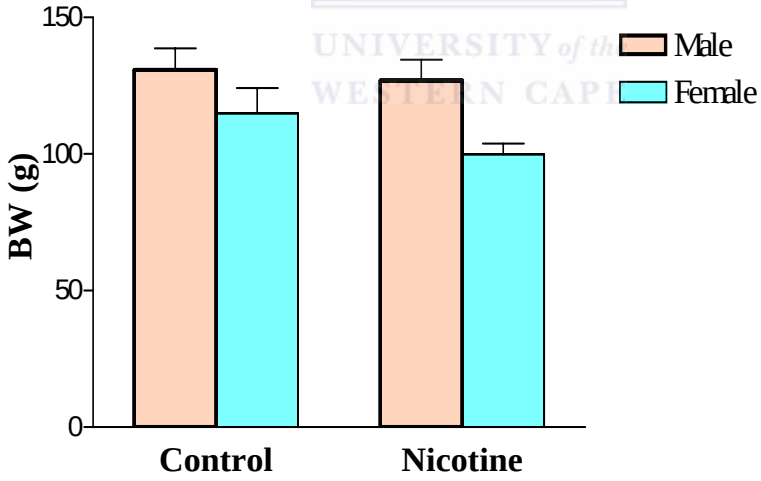

Fig. 4.1 (b). The effect of nicotine on the body weights of 42-day old control rats and rats exposed to nicotine during gestation and lactation.

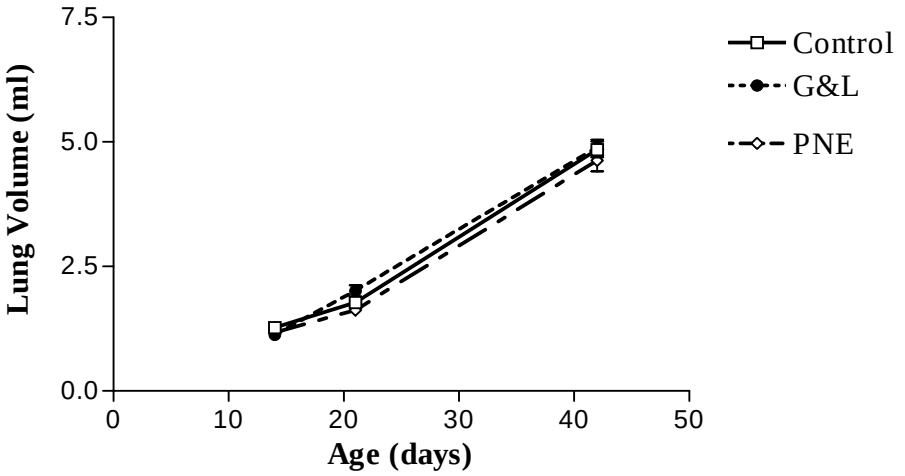

Fig. 4.2 (a). The effect of maternal nicotine exposure during different phases of lung development on lung volume.

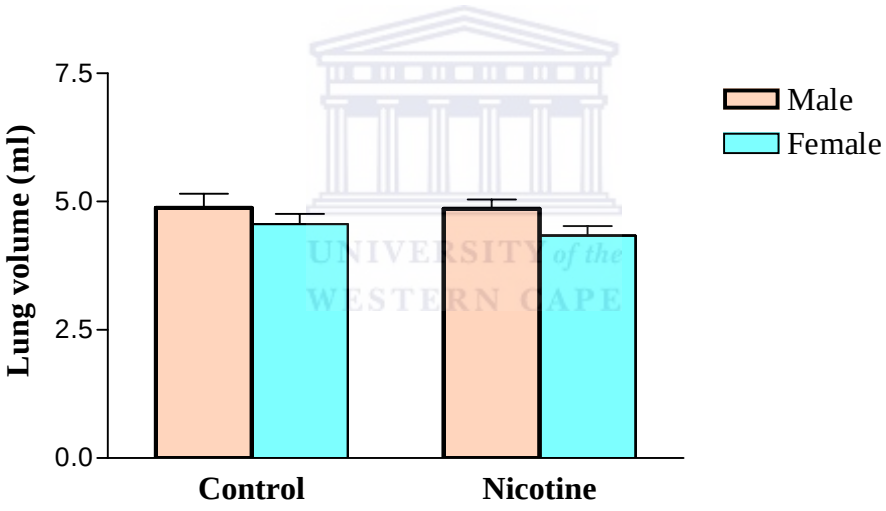

Fig. 4.2 (b). The effect of maternal nicotine exposure on lung volumes of 42-day old control, and rats exposed to nicotine during gestation and lactation.

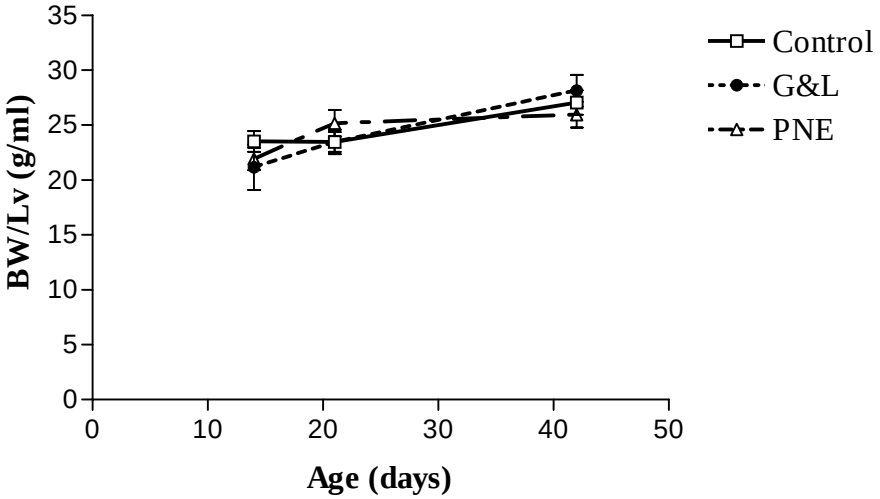

Fig. 4.3. The effect of maternal exposure to nicotine during gestation and lactation and at the onset of rapid alveolarisation on the BW/Lv ratio.

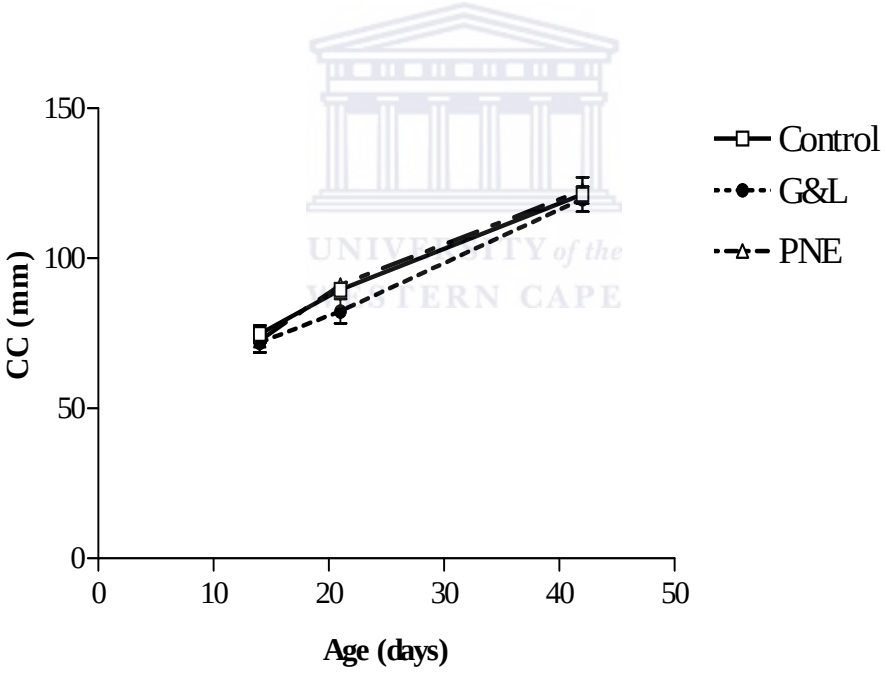

Fig. 4.4 (a). The effect of maternal nicotine exposure during different phases of lung development on the chest circumference (CC) of the offspring.

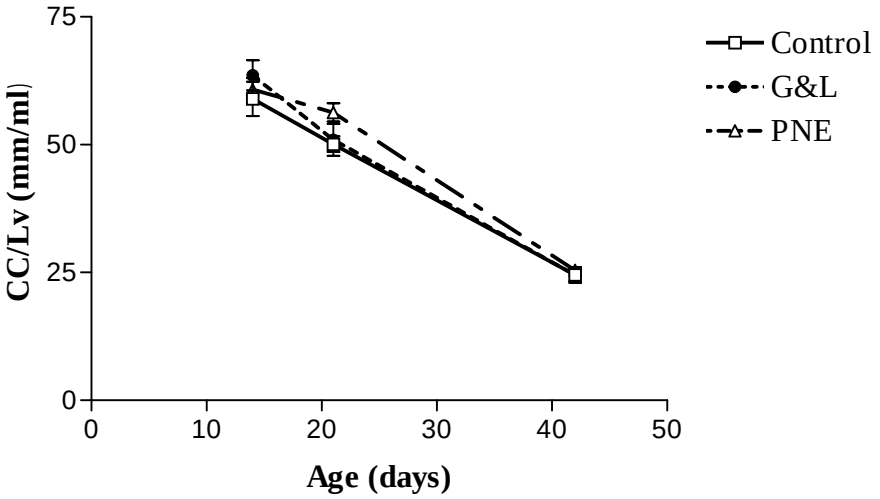

Fig. 4.4 (b). The effect of maternal exposure to nicotine during different phases of lung development on the neonatal chest circumference/lung volume, (CC/Lv) ratio.

4.2.3 *The effect of maternal exposure to nicotine on the volume density of the airspaces (Va) and parenchymal tissue (Vt) of the growing rat offspring.*

The volume density (Va) of the airspace of the lung parenchyma, and the volume density of the parenchymal (Vt) tissue (table 4.1) shows that on postnatal day 14 the Va of the G&L rats was lower ($P<0.05$) than that of the control rats of the same age. Consequently the Vt was higher ($P<0.05$) than that of the control rats. No differences were observed between the 14-day old control and PNE rats or between the control rats and experimental rats at the other age groups.

Age	Control			G&L			PNE		
	Va	Vt	Va/Vt	Va	Vt	Va/Vt	Va	Vt	Va/Vt
14	83.81±0.96	16.19±0.96	5.18	80.00±0.69	20.00±0.69	4.00	84.98±0.42	15.02±0.61	5.66
21	85.78±0.84	14.24±0.84	6.02	86.23±0.66	13.77±0.65	6.26	86.52±0.46	13.76±0.46	6.29
42	88.12±0.33	11.89±0.33	7.41	89.19±0.36	10.82±0.36	8.24	87.30±0.54	12.70±0.54	6.87

Table 4.1. The influence of maternal nicotine exposure on the alveolar air (Va) and alveolar tissue (Vt) volume of the offspring as a percentage of total lung volume.

4.3 Discussion

Lung growth is tightly controlled, but there may be several factors affecting its development into an effective gas-exchanger (Fig. 4.5). This is reflected by the development of the lung being determined by the oxygen demand of the body (Massaro and Massaro, 2002). From the literature it appears that interference with lung growth may render the lungs more susceptible to respiratory disease in later life (Landau, 2006). Adequate nutrition is essential for foetal and neonatal development as it supplies all the building blocks for tissue growth, and the energy to support growth. Inadequate nutrition will therefore result in stunted foetal and neonatal growth, and will result in newborns that are small for gestational age. It is suggested that poor foetal nutrition not only results in a smaller body but also results in foetal adaptations that 'programme' future propensity to adult disease (Lucas et al. 1999).

It was shown that enhanced nutrition increases the internal surface area of the lungs, but not septation, whereas decreased nutrition during foetal life seems to impair septation and reduce surface area, but not the final alveolar size (Massaro and Massaro, 1997). Restriction of protein intake during foetal development decreases somatic growth as well as reducing lung volumes (Kalenga et al. 1995). However, the latter authors also showed that specific lung volumes, that is, volume per body weight were less affected by malnutrition than the rest of the body. Re-feeding resulted in catch-up growth, with normalisation of specific lung volume, suggesting that the potential for recovery is present despite severe foetal malnutrition (Kalenga et al. 1995).

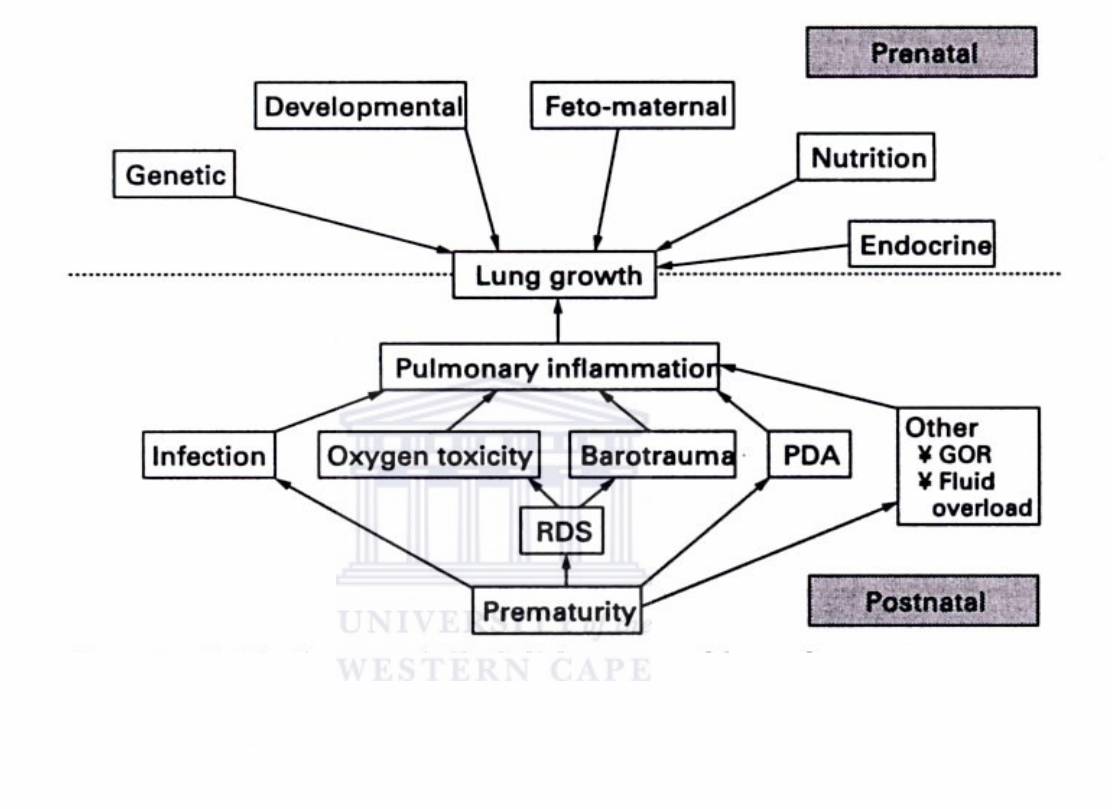

Fig. 4.5. Proposed model of lung growth.

Teratogenesis is usually associated with congenital disease and is also associated with disruptions of organogenesis during the embryonic period. Studies by Barker (1995) showed evidence of certain adult onset diseases that may result from conditions in the uterus prior to birth. These diseases have been associated with foetal growth rather than to changes at the embryonic stage of foetal development. The data of this project showed that the volume density of the alveolar air of lungs of the 14-day old control rats, was higher than that of the G&L rats due to a higher volume density of the parenchymal tissue of the latter group. However, after postnatal day 14, thinning of the alveolar walls of the G&L animals occur so that on postnatal day 42 no differences were observed. Interestingly, exposure to nicotine from the onset of the phase of rapid alveolarisation had no effect on the volume densities of the parenchymal tissue of the offspring. The proportion of alveolar air in control rats at day 14 was higher than that in rats exposed to nicotine during the entire phase of gestation and lactation. The higher volume density of the parenchymal tissue of the 14-day old G&L rats might be due to an increased cellularity or extracellular matrix. Previous studies indeed indicated that maternal nicotine exposure induced an increase in type-2 cell numbers in the lungs of the offspring (Maritz and Thomas, 1995). By day 42 however, no significant differences were seen in the proportion of air and tissue in the three groups of rats, which indicate that after weaning and nicotine withdrawal the volume density of the parenchyma of the nicotine-exposed rats returned to normal.

Undernutrition can permanently reduce the number of cells in those organs whose critical periods of growth coincide with the lack of nutrients (Widdowson and McCance, 1975). Undernutrition will result in stunted growth and it was shown by Barker et al. (1989) and Osmond et al. (1993) that children weighing less than 2.5kg at birth, died from heart disease more frequently than those born with higher weights. In previous studies (Phillip et al. 1984; Birnbaum et al. 1994) it was shown that nicotine could cause constriction of blood vessels and thus reduce blood flow in the placenta. It implies that nutrient supply will also be reduced for the period of vasoconstriction. This further implies that maternal nicotine during pregnancy may result in slower foetal growth and may result in lower birth weight of the offspring. However, in this study it was shown that maternal nicotine exposure during different phases of foetal and neonatal development had no effect on the weight of the male offspring. It is therefore unlikely that the nutrient supply via the placenta was affected by nicotine.

In conclusion, it appears that maternal nicotine exposure had no effect on the growth of rats at the different phases of lung development. It is therefore unlikely that any changes in the long term in lung structure and function of the nicotine-exposed rats, can be attributed to an inadequate nutrient supply to the developing foetus in the uterus.

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CHAPTER 5

Chronic Maternal Nicotine Exposure during Different Phases of Lung Development: The Development of the Lung Parenchyma in the Offspring and Response to Nicotine Withdrawal

5.1 Introduction

Maternal smoking is associated with an increased incidence of respiratory disease in offspring (Fergusson et al. 1981; Stein et al. 1999). Researchers furthermore showed that chronic exposure of rats to whole cigarette smoke during pregnancy induced a slower pace of septal growth and thus of alveolarisation in the lungs of the offspring (Collins et al. 1985; Szüts et al. 1978). Nicotine in tobacco smoke is implicated as the causative substance. Some studies illustrated that maternal smoking results in the accumulation of nicotine (Jauniaux et al. 1999) and cotinine (Bardy et al. 1993) in the foetal tissues and an extensive epidemiological study demonstrated a positive correlation between the concentration of nicotine in the maternal blood and foetal growth retardation (Maritz and Burger, 1992). Nicotine also accumulates in the respiratory tract of the foetus (Jauniaux et al. 1999). Previous studies showed that maternal nicotine exposure during gestation and lactation indeed inversely interferes with energy metabolism in the developing lung (Maritz and Dennis, 1998), as well as with structural development of the lung (Wall et al. 1985). In some epidemiological studies, a relationship is drawn between diseases of the distal airways of children of smoking parents and chronic bronchitis and emphysema of the same individuals

in adulthood (Farrell, 1982). Research also indicates that maternal nicotine exposure during gestation and lactation renders the lungs of the offspring more susceptible to damage (Maritz et al. 1993).

Growth and development is slow during the embryonic phase, but from the foetal phase it proceeds rapidly until after birth (Barker, 1996). During phases of rapid growth and thus of rapid cellular proliferation, the cells and organs are most vulnerable for the effects of external factors that are foreign to the body including poor nutrition. Lung development can be divided into several phases (Farrel, 1982). One of these phases, the phase of rapid alveolarisation, is characterized by rapid cell division. This “critical window” of development of the lung is essential for the development of the lung into an efficient gas-exchanger. It is therefore plausible that exposing the developing lung to nicotine at this late stage of lung development may also result in changes in lung structure and its efficiency as a gas-exchanger.

The aims of this chapter were therefore to not only determine:

a. the influence of chronic maternal nicotine exposure 1) during gestation and lactation on lung development, but also 2) from the onset of the phase of rapid alveolar formation, and
b. whether nicotine withdrawal after weaning will result in a reversal of any changes in the lung parenchyma.

5.2 Results (Morphometry)

5.2.1 *Internal Surface Area (Sa), (Fig. 5.1)*

Fig. 5.1 shows that the Sa of the control animals increased from 535.30 ± 38.40 cm^2 on postnatal day 14 to 1946.50 ± 64.58 cm^2 on postnatal day 42. The Sa of the lungs of the G&L and PNE animals increased from 556.04 ± 18.90 cm^2 and 576.29 ± 29.60 cm^2 respectively on postnatal day 14 to 1733.12 ± 96.70 cm^2 and 1542.80 ± 63.00 cm^2 respectively on postnatal day 42. On postnatal days 14 and 21 there were no differences ($P>0.05$) in the Sa of the lungs of the control and experimental animals and between the G&L and PNE animals. At postnatal day 42, the Sa of the control lungs were bigger ($P<0.001$) than that of the G&L and PNE animals. The Sa of the G&L and PNE offspring were not different at postnatal day 42 ($P>0.05$). The Sa of the lungs of the control animals increased by 1422.29 cm^2 between postnatal days 14 and 42. During the same period of time, the Sa of the G&L rats increased by 1177.08 cm^2. This is 245.15 cm^2 less ($P<0.01$) than that of the control animals. The Sa of the lungs of the PNE animals increased by 966.69 cm^2 between postnatal days 14 and 42. This was 426.6 cm^2 less ($P<0.001$) than the increase in the control animals. Since the BW of the animals that were exposed to nicotine was not different from the control animals (see chapter 4), a correction for BW will not change the difference in the Sa between the control animals and those that were exposed to nicotine.

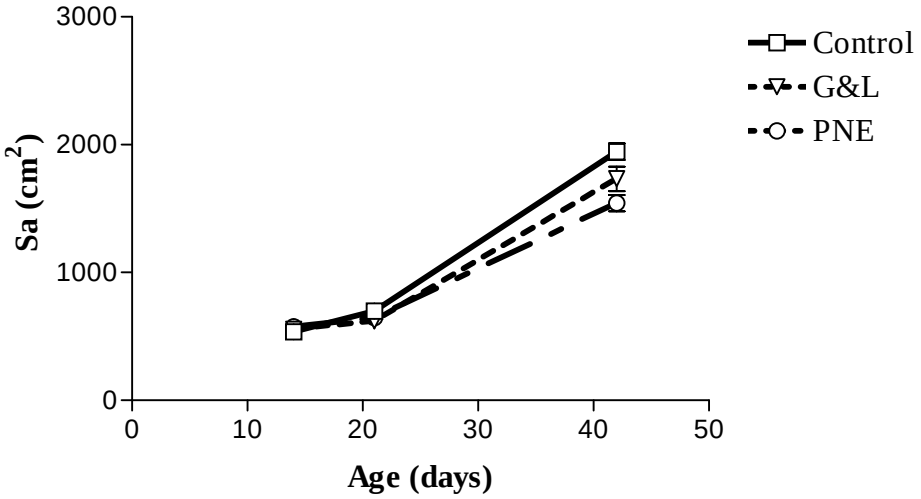

Fig. 5.1. Influence of maternal nicotine exposure on the Sa of the lungs of the offspring.

5.2.2 *Airspace Wall Surface Area per Unit Volume (AWUV), (Fig. 5.2)*

From figure 5.2 it can be seen that AWUV of the 14- and 21-day old control, G&L and PNE rats was not different (P>0.05). However, on postnatal day 42 the AWUV of the lungs of the control animals was at 22.26 ± 0.62 mm^2/cm^3, higher (P<0.01) than the 18.65 ± 0.54 mm^2/cm^3 of the G&L and the 16.69 ± 0.27 mm^2/cm^3 of the PNE rats. As for the Sa of the G&L and PNE rats, the AWUV also tended to be higher for the PNE animals compared to that of the G&L animals. However, as for Sa, there were no significant differences (P>0.05) in the AWUV of the lungs of the G&L rats compared to that of the PNE rats.

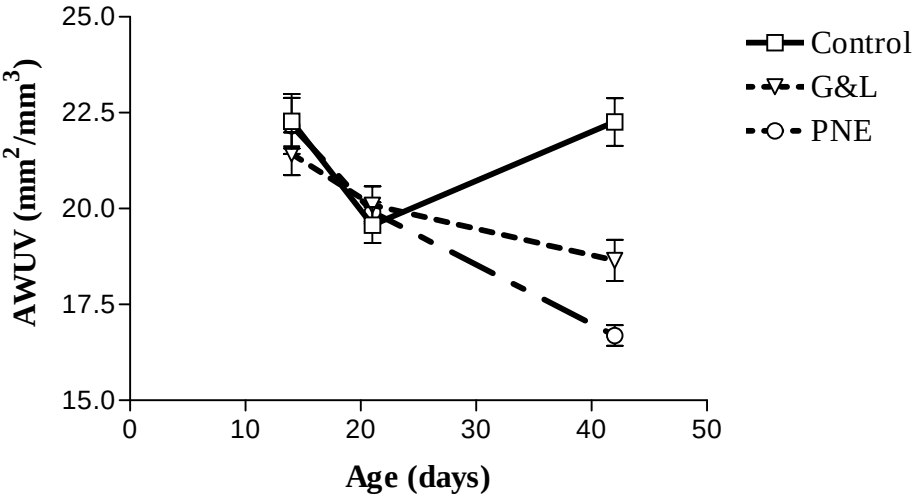

Fig. 5.2. Influence of maternal nicotine exposure on the AWUV of the lungs of the offspring.

5.2.3 *Alveolar Volume (Valv) and Alveolar Number (Na), (Fig's. 5.3, 5.4)*

The Valv of the 14- and 21-day old control rats were 10.99 ± 0.35 x 10^4 µL and 11.25 ± 0.21 x 10^4 µL respectively (P>0.05). The Valv of the14-day old G&L and PNE rats were at 11.42 ± 0.75 x 10^4 µL and 9.52 ± 0.79 x 10^4 µL, the same (P>0.05) as that of the control animals. However, at postnatal day 21 the Valv of the lungs of the G&L animals was at 13.86 ± 1.35 x 10^4 µL, 21.5% larger than that of the 14-day old G&L rats and 23.3% larger than that of the control animals of the same age. The Valv of 10.61 ± 0.38 x 10^4 µL of the PNE rats was not different from that of the control rats. The Valv of the lungs of the G&L and PNE rats increased to 14.71 ± 1.38 x 10^4 µL and 13.38 ± 0.85 x 10^4 µL respectively between postnatal days 21 and 42, which was 48.4% and 35% respectively larger, than the 9.91 ± 0.61 x 10^4 µL of the 42-day old control rats. At postnatal day 42, the Valv of the lungs of the G&L rats was not significantly different from that of the PNE rats.

The Na of the 14-day old control rats were 1.18 ± 0.08 million and increased to 1.44 ± 0.11 million on postnatal day 21 (P<0.01). This represents an increase of 22.03%. The Na of the G&L rats increased by 45.6%, from 0.96 ± 0.03 million on postnatal day 14, to 1.40 ± 0.08million on postnatal day 21. On postnatal day 42 the Na of the lungs of the control rats was 4.03 ± 0.29 million, which was 34% higher (P<0.001) than the 2.66 ± 0.22 million of the lungs of the G&L rats and 19.6% higher (P<0.01) than the 3.24 ± 0.18 million of the PNE rats. It is interesting to note that from postnatal day 21 up to postnatal day 42, the Na of the control rats increased by 59.9% per week while that of the G&L and PNE rats increased by 30% and 47.3% respectively.

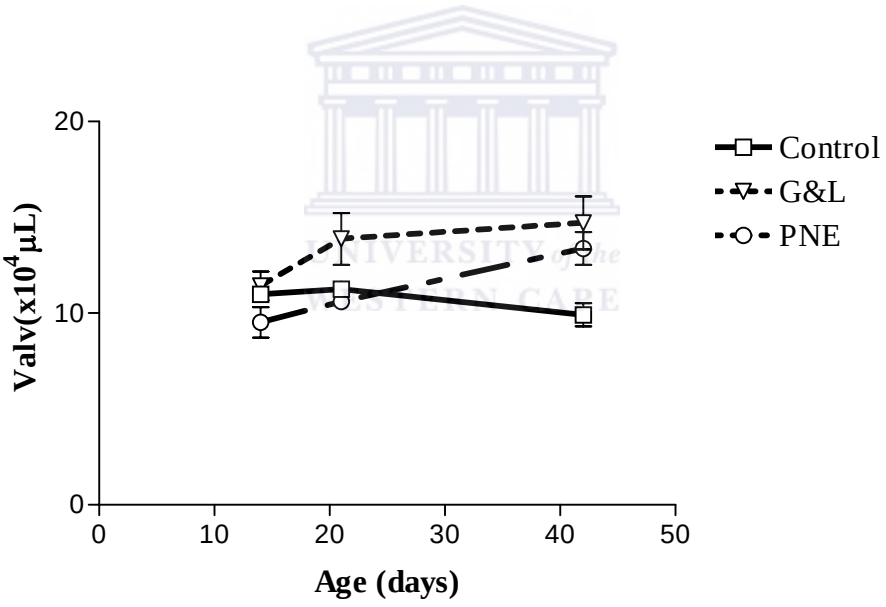

Fig. 5.3. Influence of maternal nicotine exposure on the Valv of the lungs of the offspring.

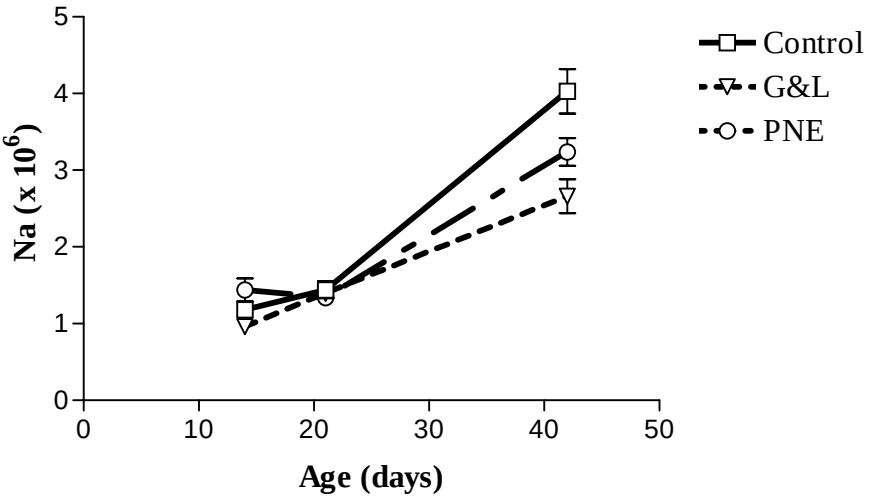

Fig. 5.4. Influence of maternal nicotine exposure on the Na of the lungs of the offspring.

5.2.4 *Thickness of Alveolar Walls (Tw), (Fig.5.5)*

The data summarized in Fig. 5.5 shows that the Tw of 2.68 ± 0.13 µm of the control animals on postnatal day 14 was not different from that of the 14-day old PNE rats (2.92 ± 0.09 µm), but thinner ($P<0.01$) than the 3.34 ± 0.16 µm of G&L rats. On postnatal day 42 there were no differences in Tw between control (2.47 ± 0.08 µm), PNE (2.99 ± 0.09 µm), and G&L (2.42 ± 0.09 µm) rats.

5.3 Results (Morphology)

From figure 5.6A – F it is clear that the alveolar walls of the 14-day old control rats (A) were thinner than that of the 14-day old G&L rats (B). In contrast, the alveolar walls of the PNE rats (C) were not different from that of the control rats of the same age. In addition, the alveolar walls of the 14-day old control and PNE rats showed more complexity than that of the G&L rats of the same age. However, on postnatal day 42 the parenchymal tissue of the G&L (E) and PNE (F) showed

deterioration of the alveolar walls. From figure 5.7 B it is clear that the alveolar walls of the 14-day old rat pups that were exposed to nicotine during gestation and lactation were more cellular than those of control and PNE rats of the same age group. No apparent differences in cellularity occurred between control and nicotine-exposed rats at postnatal day 42.

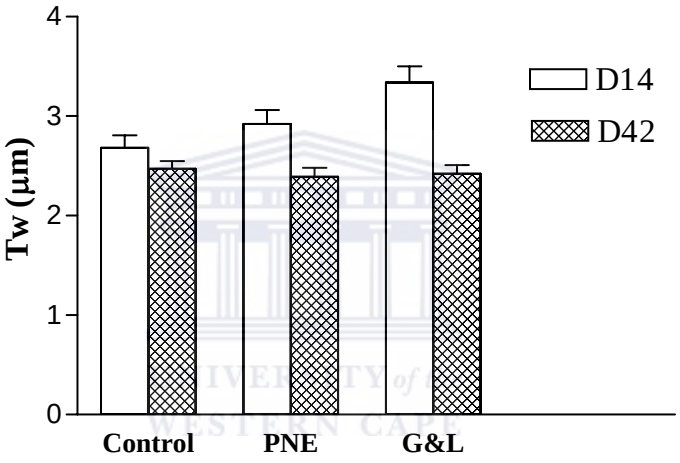

Fig. 5.5. The influence of maternal nicotine exposure on the alveolar wall thickness of the lungs of the offspring.

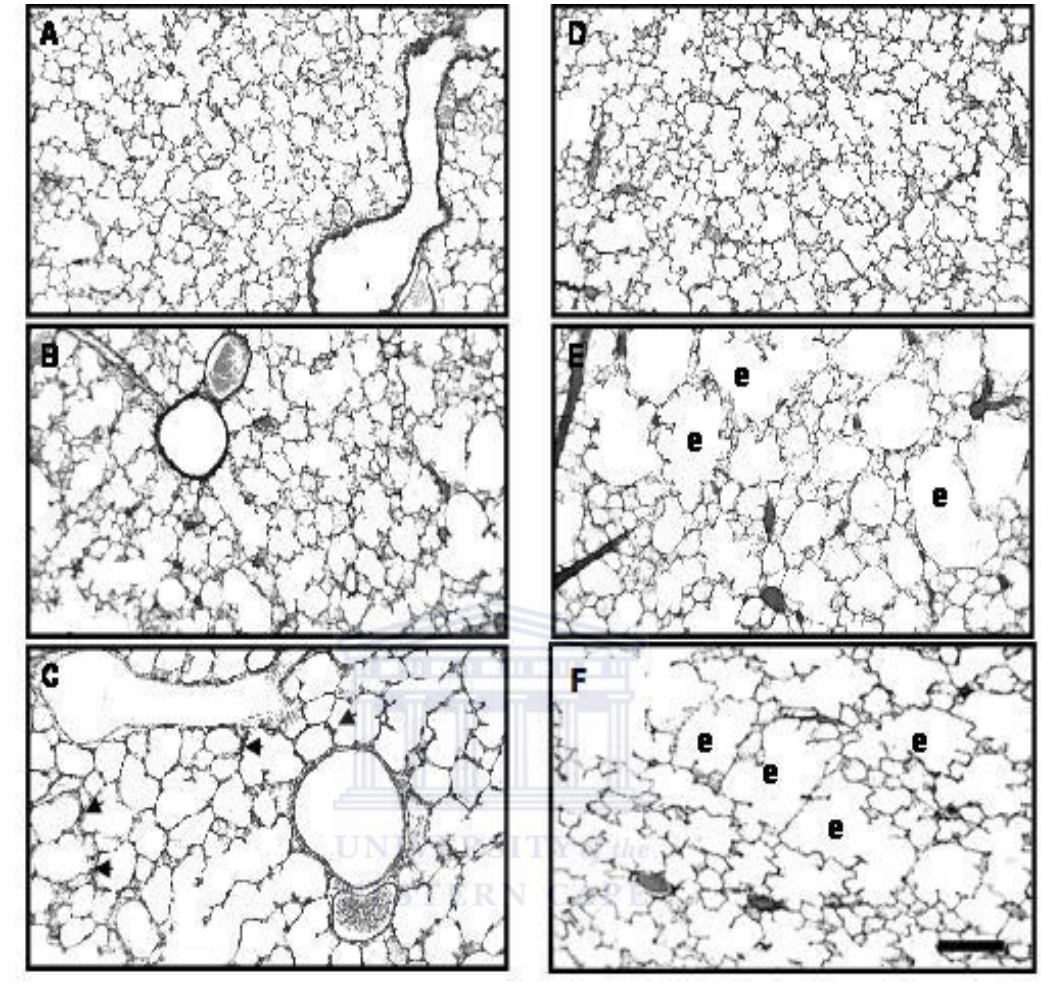

Fig. 5.6. The influence of maternal nicotine exposure during gestation and lactation (G&L), or from the onset of alveolar formation on the parenchyma of the lungs of the offspring. A - C are light micrographs of the lung parenchyma of 14-day old control (A), PNE (B) and, G&L (C) rats. D-F are representative of the corresponding 42-day old control (D), PNE (E) and G&L (F) lungs. The alveolar walls of the 14-day old G&L rats were thicker (arrow heads) than that of the control and PNE animals. At postnatal day 42 the lung parenchyma of the G&L and PNE animals showed signs of emphysema (e). (Bar = 20 µm)

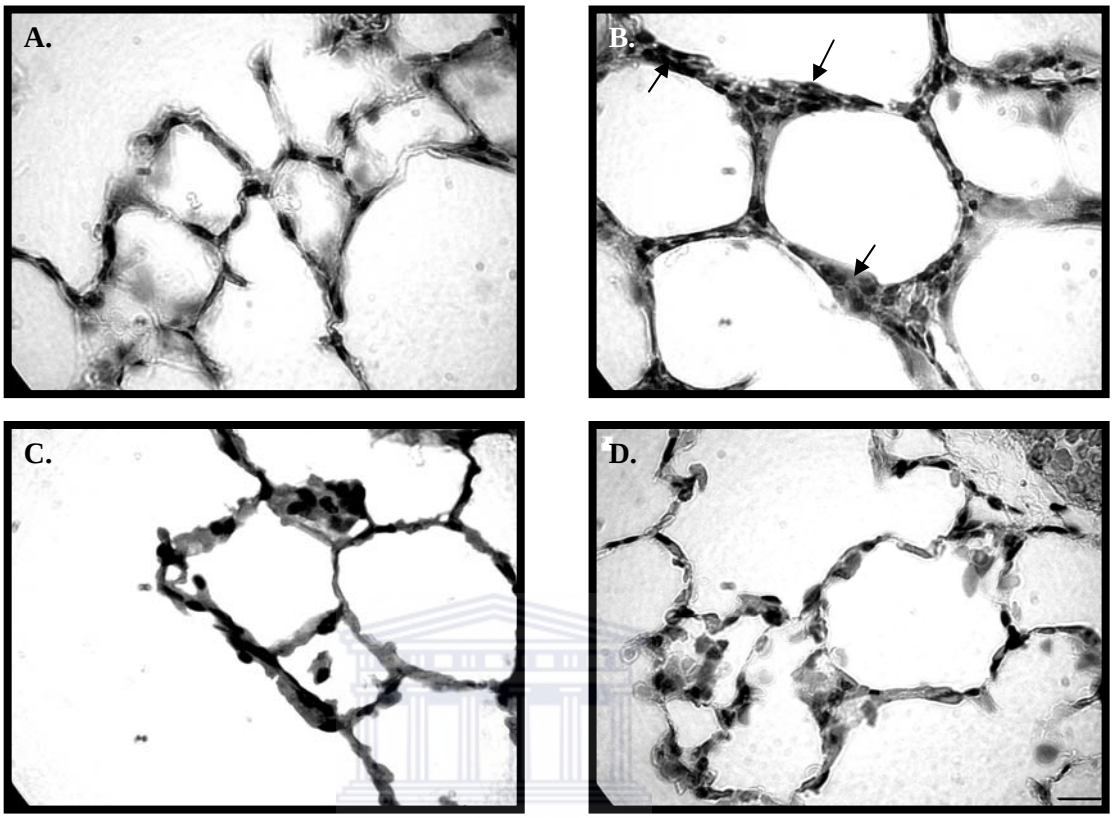

Fig. 5.7. Light micrographs of alveolar walls of 14-day old control (A) and G&L (B) rat lungs. The alveolar walls of the 14-day old rats were more cellular (arrows) than that of the control rats. At postnatal day 42 there was no difference between control (C) and G&L (D) rats. (Bar = 16 µm)

Figure 5.8 A–F represents the walls of the respiratory bronchioles of the 14- and 42-day old control (A and B), G&L (C and D) and PNE (E and F) rats. From the images it is clear that the epithelial cells of the respiratory bronchioles of all the rats on postnatal day 14 were flattened with flattened nuclei. At postnatal day 42, the epithelial cells of the respiratory bronchiole of the lungs of the control rats had the same characteristics as those of the 14-day old control rats. In contrast to this,

the bronchiole of the G&L and PNE rats have both flattened and cuboidal epithelial cells. The nuclei of the cuboidal cells were rounded. These epithelial cells occurred on the luminal surface of the wall and it appeared as if these cells were being sloughed off.

Scanning electron micrographs (SEM) of the lungs of the 42-day old rats show well-developed alveoli in the lungs of the control rats (Fig. 5.9). The alveoli of the lungs of the rats that were exposed to nicotine via the placenta and mother's milk, in other words during all phases of lung development, are flattened. Alveolar fenestrations are also evident (Fig. 5.10). The lungs of the PNE rats show greater alveolar complexity. Alveolar fenestrations also occur but to a lesser extent. Localised areas of flattened alveoli can be seen (Fig. 5.11).

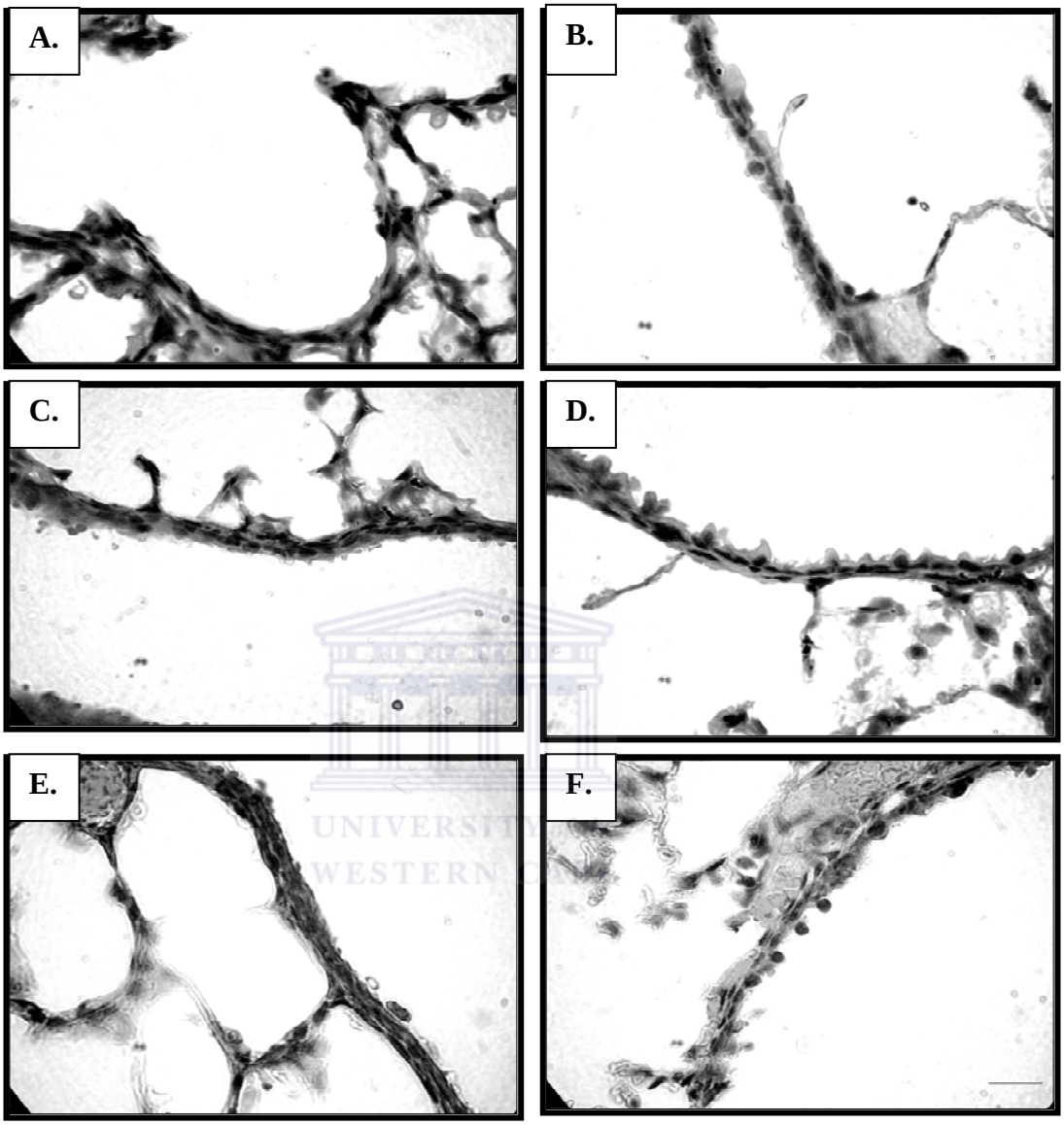

Fig. 5.8. Respiratory bronchioles of 14-day old control (A), PNE (B) and, G&L (C) rats. No differences occur between the control and nicotine exposed animals. The epithelial cells are not cuboidal with flattened nuclei (arrows). The respiratory bronchioles of the 42-day old PNE and G&L (E & F) rats have more cuboidal cells (arrows) than the control (D) lung. The epithelial cells of the G&L lungs appear to be sloughing off (arrows). (Bar = 16 µm)

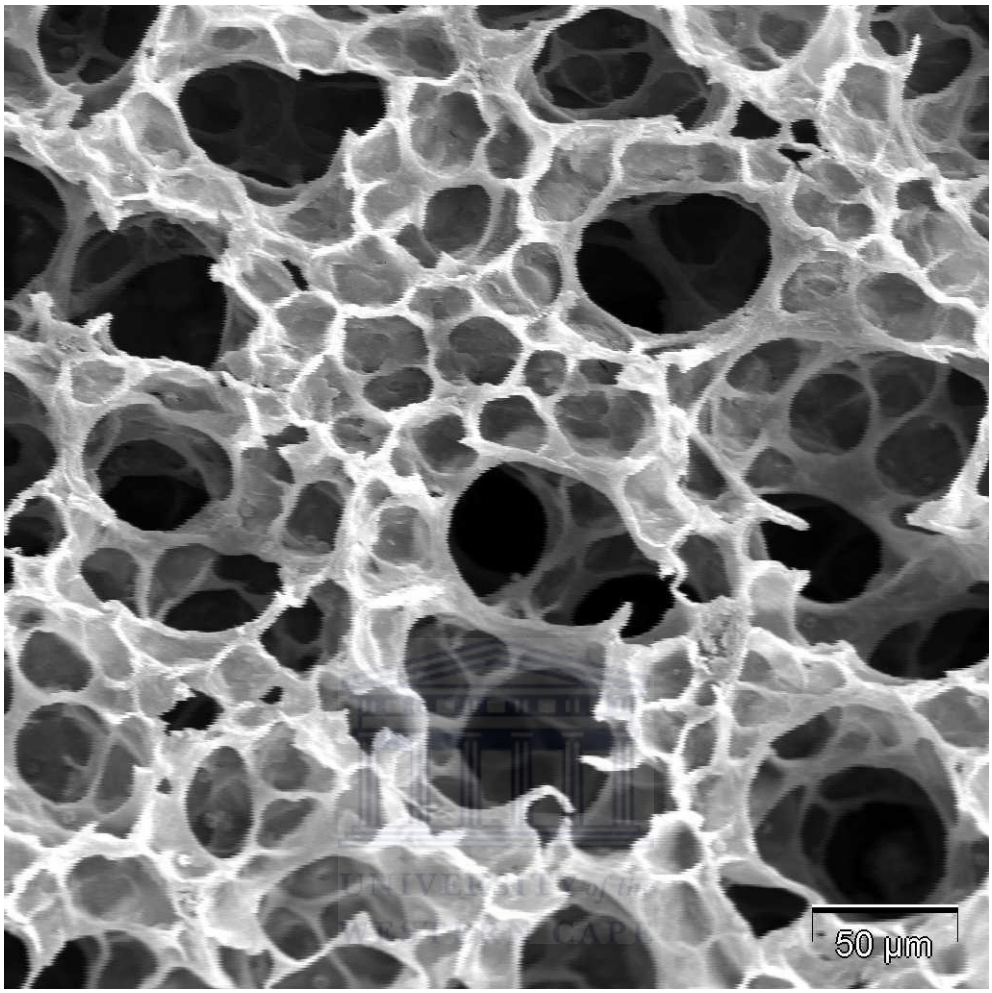

Fig. 5.9. Scanning electron micrograph of the lung of 42-day old control rat lung. The parenchyma shows a high degree of complexity. No alveolar damage is evident. Note the high level of complexity of the lung parenchyma.

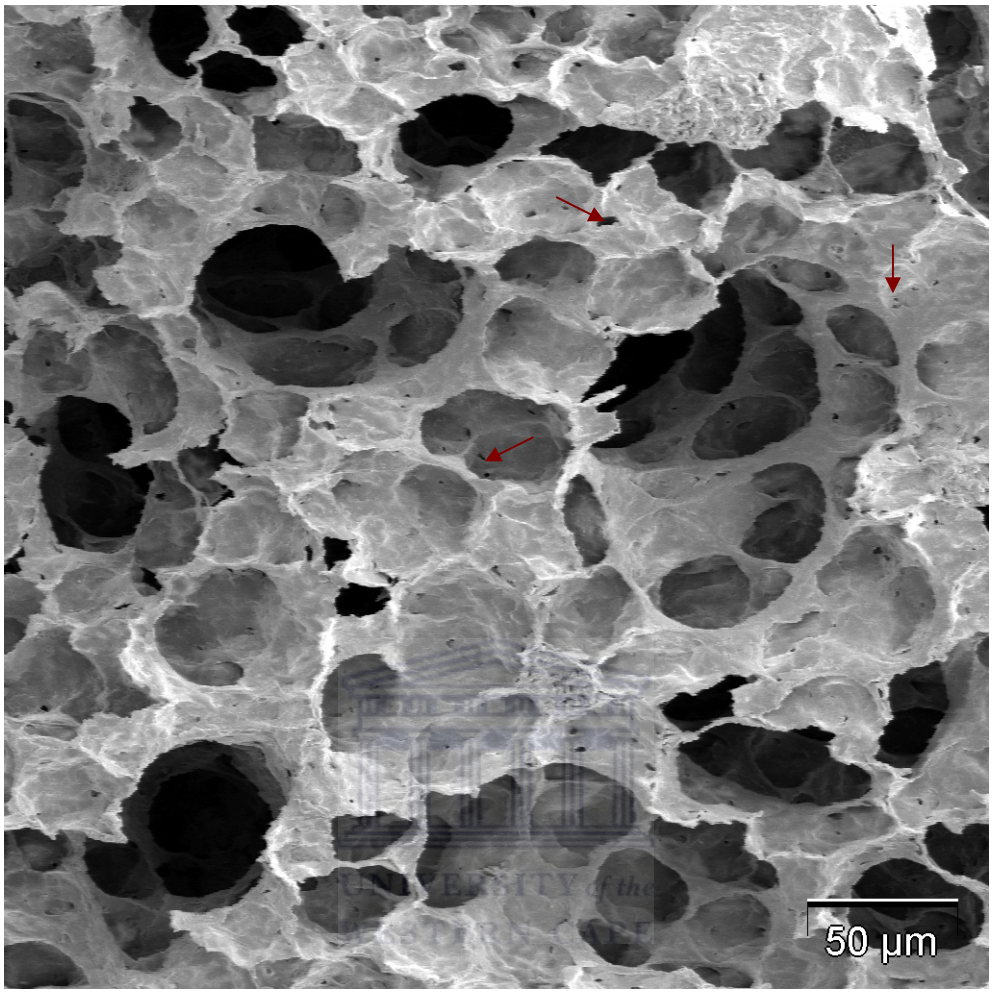

Fig. 5.10. Scanning electron micrograph of the lung tissue of a 42-day old rat exposed to nicotine during all the phases of lung development up to weaning on postnatal day 21 (G&L). The alveoli are shallow and show fenestrations (arrows).

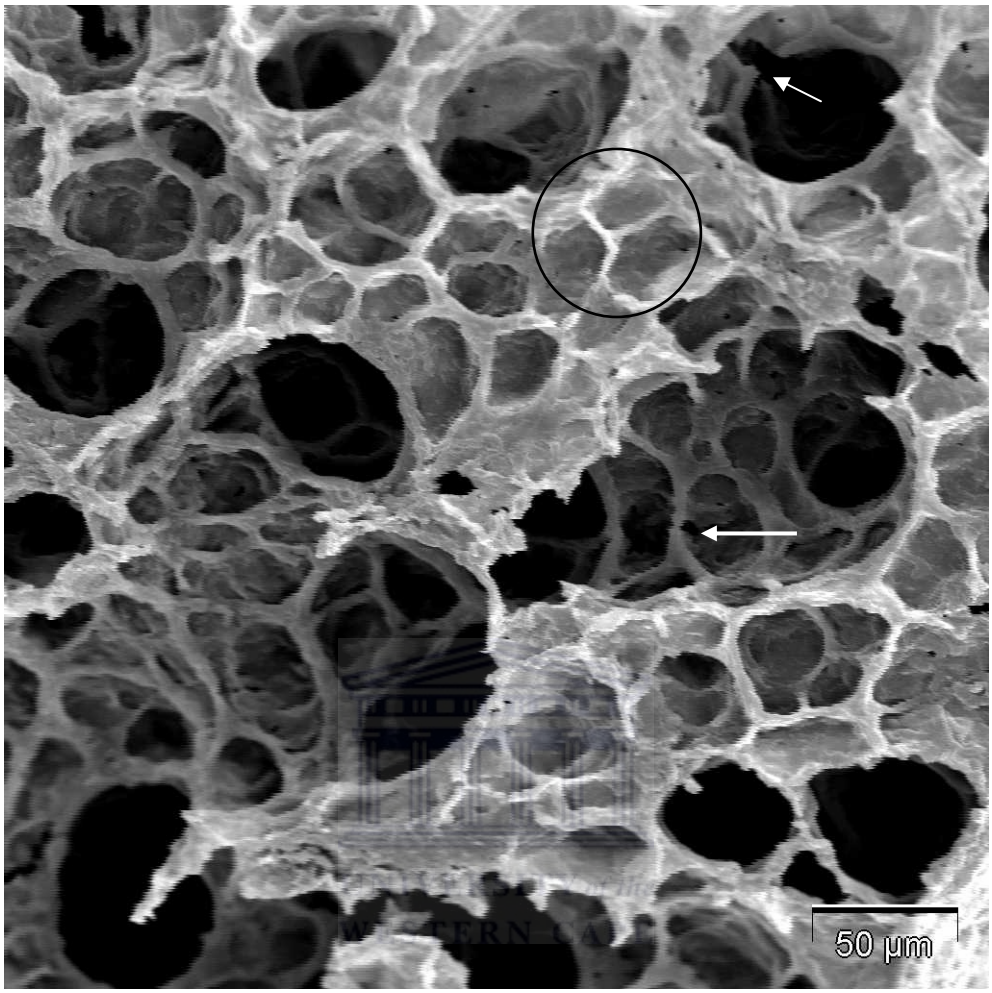

Fig. 5.11. Scanning electron micrograph of 42-day-old rat lung exposed to nicotine from 1 day before the onset of alveolarisation, on postnatal day 3 (PNE). Lung parenchyma shows a high degree of complexity. Localized fenestrations (arrows) occur, as well as areas of localised flattening of alveolar walls (circle).

5.4 Discussion

In this study nicotine was administered subcutaneously to 2 groups of rats. In one group the mother received nicotine as a single injection per day during gestation and lactation (G&L), in other words, during all the phases of lung development. In the second group the mother received nicotine from postnatal day 3, which is one day before the onset of the phase of rapid alveolarisation in the rat pup. For both groups the exposure to nicotine was terminated at weaning on postnatal day 21. The single subcutaneous injection per day implies that the blood and milk nicotine levels of the mother reached high levels after the injection and gradually decreased as the day progressed. Previous studies show a recovery of 79 to 93% of the nicotine content in the breast milk of smoking mothers (Page-Sharp et al. 2003). It is therefore conceivable that in our experiments the increase in nicotine content of the mother's milk after a single injection was of the same magnitude. This implies that infants drinking mother's milk shortly the mother smoked or after exposure of the mother to nicotine, as in my study, will be exposed to higher nicotine concentrations as opposed to taking in milk some time after the exposure. As in a previous study, (Maritz and Windvogel, 2003) it is again confirmed here that a single exposure per day of the female rat to nicotine during gestation and lactation, or only from postnatal day 3, had no effect on BW and Lv (see chapter 4) of the offspring. This implies that the nutrient supply via the placenta and mother's milk of rats exposed to nicotine was sufficient to sustain normal growth of the offspring and that the increase in Lv and BW was proportional. It is therefore unlikely that any changes in the structural complexity of the neonatal

lung due to maternal nicotine exposure can be attributed to a lack of nutrient intake by the foetus or neonate.

The development of the mammalian lung is classically divided into the embryonic, pseudoglandular, saccular/terminal sac, and alveolar phases. This is followed by the phase of equilibrated lung growth, which is the final stage of lung maturation (Burri et al. 1974). During late gestation and the early postnatal period, the development of the lung is programmed to develop the gas exchange area to ensure the efficient supply of oxygen to the organism and thus to satisfy the energy demands of the body. The surface area for gas exchange increases as the animal grows by the formation of new alveoli through septation (Burri, 1974). This implies an increase in the complexity of the lung parenchyma. Any interference by a substance such as nicotine with the process of alveolar development, or damage to the alveolar septa during the foetal and neonatal phases of lung development which are characterized by rapid cell division, may therefore reduce the effectiveness of the lung as a gas-exchanger, even after lung growth and maturation has stopped. It is also possible that interference with lung development during certain critical windows of time may change the “program” that directs lung growth and development and so increase its susceptibility to disease in later life (Massaro and Massaro, 2004).

Septation is developmentally regulated, and failure to septate at the appropriate time is not followed by a delayed spontaneous catch-up of septation to restore alveolar numbers to normal, for the age of the animal (Massaro et al. 2003). This implies that delaying the formation of septa will result in permanently less, and

most likely larger alveoli in the lungs. This also implies that the surface area available for gas exchange will be permanently smaller. Factors that affect airway growth early in development appear to cause changes in the lung structure and function that persists into adulthood (Stick, 2000).

It is interesting to note that on postnatal day 14, when rapid alveolarisation in rat lung is completed, the internal surface area, alveolar volume and alveolar numbers of the nicotine-exposed offspring were no different from those of the control animals. However, it is clear that the alveolar walls of the 14-day old neonates that were exposed to nicotine during all the phases of lung development were thicker than that of the control rats, as well as those that were exposed to nicotine only from the onset of the phase of rapid alveolarisation. This appears to be due to a higher cellularity of the alveolar walls of the lungs of these animals. No differences were apparent in alveolar wall thickness or alveolar wall cellularity at postnatal day 42. The increased cellularity on postnatal day 14 indicates that the thinning of the alveolar walls was slower in these animals than in the lungs of the control and PNE animals. Thinning of the alveolar walls during lung maturation is due to apoptosis of the interstitial fibroblasts (Bruce et al. 1999). It is known that nicotine suppresses apoptosis (Maneckjee and Minna, 1990; Heusch and Maneckjee, 1998). Since these animals presumably received the same amount of nicotine via the mother's milk, it is conceivable that the thinning of the alveolar walls of both groups of animals should be slower. Contrary to expectations, only the alveolar walls of those rats exposed to nicotine during all the phases of lung development show a slower thinning of the alveolar walls. This finding is

interesting but difficult to explain. It is possible that the longer period of nicotine exposure enhanced the capacity of the mother and the developing lung to metabolize nicotine faster and thus to prevent its effect on the thinning of the alveolar walls. It is also possible that the exposure of the G&L rats to nicotine during the earlier phases of lung development induced a change in the "program" that regulates thinning of the alveolar walls, by slowing down the apoptosis of the interstitial fibroblasts in the alveolar walls. It is also possible that the intake of nicotine via mother's milk is too low to suppress apoptosis.

At postnatal day 21 the Sa of the nicotine-exposed animals was the same as that of the control animals. This implies that maternal nicotine exposure had no influence on the formation of secondary septa during the phase of rapid alveolarisation. However, at postnatal day 42, the Sa of both G&L and PNE rats were smaller than that of the control animals of the same age. From the data of this study it is clear that the effect of late exposure to nicotine via the mother's milk, namely from the day before the onset of the phase of rapid alveolarisation, had a less severe effect on the lung parenchyma as for those animals that were exposed during all the phases of lung development. This is illustrated by higher complexity of the parenchyma of the lungs of the 42-day old PNE rats compared to that of the G&L rats.

The importance of the Sa to O_2 uptake (VO_2) is supported by the presence among animals of a direct linear relationship between Sa and VO_2. This match has been achieved despite the higher body mass-specific VO_2 of smaller organisms

compared to large organisms, by a greater subdivision of the alveolar surface and not by a larger relative lung volume in small organisms. This highly conserved relationship between alveolar formation and VO_2 suggests the presence of a similarly conserved mechanism of control of the onset, rate, and cessation of alveolar formation and alveolar size (Massaro and Massaro, 2002). Since the Sa decreases only after the completion of the phase of rapid alveolarisation, it is also clear that maternal nicotine exposure had no apparent effect on the control of alveolar formation during this phase of lung development. Therefore, the smaller Sa in the 42-day old nicotine-exposed rats was due to either a slower formation of alveoli after postnatal day 21, that's is during the phase of equilibrated growth, or due to gradual destruction of the lung parenchyma. If this is so, it implies that the Na of the nicotine-exposed rats will be lower than in the lungs of the control rats of the same age.

In this study I also showed that the Na of the control animals as well as the animals exposed to nicotine via the placenta and/or mother's milk only, increased after postnatal day 14. The mechanism of alveolar formation during this phase of lung development is unclear. It is interesting to note that the increase in Na in the period of equilibrated growth of lung development was faster in the lungs of the control animals than in the lungs of the nicotine-exposed animals. Since the Na of the control and nicotine-exposed animals was the same on postnatal day 21, it implies that the effect of maternal nicotine exposure on alveolar formation and complexity was exerted mostly during the post-septation phase of lung development, in other words during the phase of equilibrated growth. It is also

clear that the exposure of the offspring to nicotine via the placenta and mother's milk and thus during all the phases of lung development in the rat, had a more pronounced effect on alveolar numbers in the post-septation phase than those animals that were exposed to nicotine only from the onset of the phase of rapid alveolar formation. There is no direct information as to the reason for the slower rate of alveolar formation during this phase of lung development in the lungs of the nicotine-exposed animals. It is more likely that the slower increase can be attributed to a destruction of alveoli as seen on scanning electron micrographs.

Morphometric data furthermore show that the lungs of the 42-day old G&L as well as the PNE animals developed microscopic emphysema. This is confirmed by light micrographs of the lungs of these animals. The light micrographs also showed that in the epithelial lining of the respiratory bronchiole of the 42-day old rats exposed to nicotine, a combination of squamous type epithelial cells as well as cuboidal epithelial cells were present, whereas on postnatal day 14, all cells were of the squamous type. The cuboidal cells appeared to be sloughed off. If this is so, it is plausible that the turnover of the epithelial cells in the respiratory bronchiole of the nicotine-exposed animals was higher than in the control animals of the same age. This is supported by previous research in our laboratories, which showed an increased turnover of alveolar epithelial cells in the lungs of rats exposed to nicotine via the placenta and mother's milk (Maritz and Thomas, 1994). The impact of maternal nicotine exposure on the epithelial lining of the respiratory bronchioles on lung structure in the longer term and thus respiratory health is not known.

Several studies showed that a number of genes have been candidates for determining the susceptibility to pulmonary emphysema (Finlay et al. 1997; Brooke et al. 1989; Hautamaki et al. 1997; Smith and Harrison, 1997). It was indeed shown that disruption of the *klotho* gene resulted in the gradual development of pulmonary emphysema in mice. In these mice alveolar formation was initially normal. However, as the mice matured the mean linear intercept, which represents the size of the alveoli, became gradually bigger due to gradual destruction of the alveolar walls (Suga et al. 2000). It is therefore plausible that maternal nicotine exposure, even as late as at the onset of rapid alveolarisation, changed the genes that are responsible for the control of lung development and the maintenance of lung integrity after birth and as the lungs mature and age. It is therefore conceivable that maternal nicotine exposure "programmed" the lungs of these rats to develop emphysema despite the fact that only the mother was directly exposed to nicotine. This will have an adverse effect on the function of the lung as a gas-exchanger. The data furthermore implies that nicotine replacement therapies during gestation and lactation may have an adverse effect on lung development in the offspring and render it more susceptible to respiratory disease in the long term.

From the above I conclude that maternal nicotine exposure, even when it commences during the late phases of lung development, resulted in a reduced surface area available for gas exchange by 1) slowing the rate of alveolar formation after the phase of rapid alveolarisation, and 2) causing gradual destruction of the lung parenchyma. These changes seem to be due to changes in the "program" that control lung development, maturation and ageing and do not

appear reversible as they persist even after nicotine withdrawal. Consequently, it renders the lungs of the offspring more susceptible to damage.

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CHAPTER 6

Is Maternal Copper Supplementation during Alveolarisation Protecting the Developing Lung against the Adverse Effects of Maternal Nicotine Exposure?

6.1 Introduction

It was shown that elastic tissue plays an important role in alveolar formation during lung development (Emery and Mithal, 1960). Earlier studies illustrated that the copper content of the lungs of 14-day old rats exposed to nicotine via the placenta and mother's milk was 1.6 times less than that of the control rats of the same age, despite the copper content of the food being the same for all animals (Maritz et al. 2000). Data from the lungs of the copper deficient rats suggest that that when cross-linking of connective tissue proteins, especially elastin, is defective the lungs develop fewer alveolar ducts and alveoli, resulting in abnormally large alveoli (Fiske and Kuhn, 1970; O'Dell et al. 1978). Lysyl oxidase plays a key role in the cross-linking of elastin and the conversion of soluble elastin into insoluble elastin (Scarpelli, 1990). Copper is an essential component of lysyl oxidase (Harris, 1976; Harris, 1986) and therefore, a deficiency of copper could be expected to result in a metabolic defect, such as an increase in the soluble component of the protein and a decrease in the insoluble component (Buckingham et al. 1981). Cross-linking of elastin may be crucial in further partitioning of the primitive alveolar sacs during alveolarisation and it has been shown for some organs, particularly at specific periods of organogenesis (e.g. formation of the aorta or transition from a saccular to an alveolar lung), that

any impairment of the lysyl oxidase activity is critical (Dubick et al. 1985; Reiser et al. 1992). It is therefore plausible that a copper deficiency at the alveolarisation phase of lung development will have an adverse effect on this phase of organogenesis in the lung and that copper supplementation close to the phase of rapid alveolarisation may prevent this.

The aims of this study were therefore to determine whether maternal copper supplementation during the same period of lung growth and development would prevent the adverse effects of maternal nicotine exposure on the development of the lungs of the offspring.

6.2 Results (Morphometry)

6.2.1 *Internal Surface Area (Sa), (Fig. 6.1) and Sa:BW Ratio, (Fig. 6.2)*

The Sa of the control animals gradually increased from 535.30 ± 38.23 cm^2 on postnatal day 21 to 1946.50 ± 64.6 cm^2 on postnatal day 42. The Sa of these animals increased by 162.3 cm^2/week, between postnatal day 14 and 21 and between postnatal days 21 and 42, the Sa of the nicotine-exposed rat pups increased by 71.1 cm^2, from 576.20 ± 29.68 to 647.30 ± 20.11 cm^2. The Sa of those rats exposed to both nicotine and copper increased by 136.7 cm^2, from 592.40 ± 30.90 to 729.10 ± 30.10 cm^2. On postnatal days 14 and 21, the Sa of the rat pups exposed to nicotine only or to both nicotine and copper was the same ($P>0.05$) as that of the control animals. From postnatal day 21 to postnatal day 42, the Sa of the lungs of the nicotine-exposed animals and those exposed to both nicotine and copper,

increased by 298.5 and 348.1 cm^2/week, respectively. Consequently, on postnatal day 42, the Sa of the nicotine-exposed rat pups was at 1542.8 ± 63.53 cm^2, 20.74% or 403.70 cm^2 smaller ($P<0.001$) than that of the control rat pups. The Sa of the lung tissue of the rats exposed to both nicotine and copper was at 1773.40 ± 69.71 cm^2, not different from the control, but 13.00%, or 230.6 cm^2, higher ($P<0.05$) than that of the nicotine-exposed animals. This shows that nicotine suppressed lung expansion in the rat pups exposed to nicotine from the onset of the phase of rapid alveolarisation and that copper supplementation during this phase of lung development prevented the slower rate of lung expansion.

The specific Sa (Sa: 100 g BW ratio) of the 14-day and 21-day old control rat pups (206.93 ± 12.04 cm^2/100 g: 169.34 ± 4.39 cm^2/100 g), nicotine-exposed rat pups (202.80 ± 8.11 cm^2/100 g: 154.96 ± 6.99 cm^2/100 g), and those that were exposed to both nicotine and copper (211.60 ± 8.96 cm^2/100 g: 184.62 ± 8.18 cm^2/100 g) was the same ($P>0.05$). The Sa/100 g BW of the 42-day old nicotine-exposed rat pups was at 131.01 ± 6.57 cm^2/100 g, lower ($P<0.05$) than the 150.00 ± 6.35 cm ^{2}/100 g ($P<0.001$) of the control and 149.32 ± 7.02 cm^2/100 g of the animals exposed to both nicotine and copper. The Sa/100g BW of the control animals was not different from that of the animals exposed to both nicotine and copper ($P>0.05$). This can be attributed to a slower increase in the Sa of the nicotine-exposed rat pups after postnatal day 14.

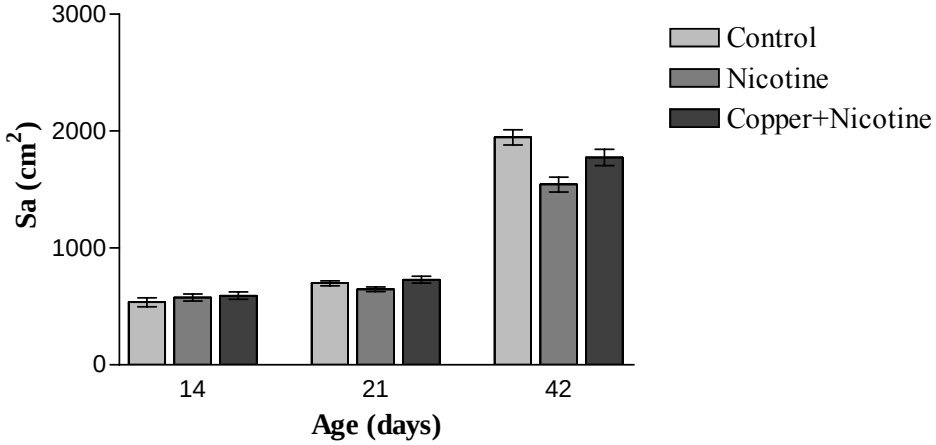

Fig. 6.1. The influence of maternal exposure to nicotine or both nicotine and copper after birth, on the internal surface area (Sa) of the offspring's lungs.

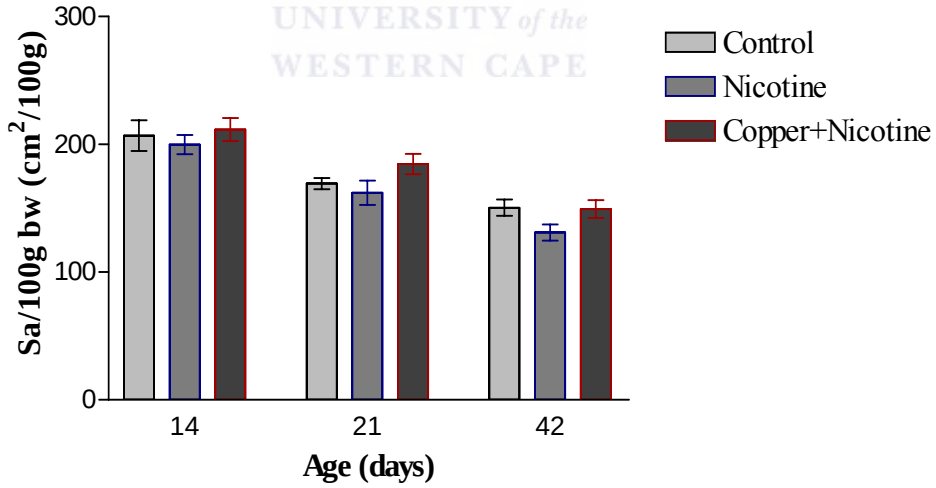

Fig. 6.2. The effect of postnatal maternal exposure to nicotine and supplementation with copper on specific Sa (Sa/100g BW).

6.2.2 *Alveolar Volume (Valv), (Fig. 6.3)*

The Valv of 14- and 21-day old control (9.40 ± 0.86 x 10^4µL and 11.28 ± 0.78 x 10^4 µL), nicotine-exposed rat pups (8.52 ± 0.79 x 10^4µL and 10.61 ± 0.38 x 10^4µL) and those exposed to both nicotine and copper (8.49 ± 0.67 x 10^4µL and 10.41 ± 0.49 x 10^4µL) were the same (P>0.05). From postnatal day 21, the Valv of the nicotine-exposed rat pups increased from 10.61 ± 0.38 x 10^4µL on postnatal day 21 and to 18.14 ± 0.85 x 10^4µL on postnatal day 42 (P<0.001). On the other hand, between postnatal days 14 and 42 (10.37 ± 0.47x 10^4µL), the Valv of the control animals remained unchanged. Although the Valv of the 14- and 21-day old rat pups exposed to both nicotine and copper was not significantly different from that of the 14- and 21-day old control and nicotine-exposed rat pups, it was smaller at 13.38 ± 1.04 x 10^4µL on postnatal day 42 (P<0.001) than the 18.14 ± 0.85x 10^4µL of the 42-day old nicotine-exposed rat pups.

6.2.3 *Alveolar Number (Na), (Fig. 6.4)*

Calculation of the alveolar number (Na) showed that the Na in the lung tissue of the 14- and 21-day old control rat pups (1.18 ± 0.08 million and 1.44 ± 0.11 million), rat pups exposed to nicotine (1.34 ± 0.03 million and 1.34 ± 0.07 million) and those exposed to both nicotine and copper (1.41 ± 0.98 million and 1.61 ± 0.13 million) were the same (P>0.05). Between postnatal days 14 and 42, the Na in the lungs of the control animals increased 3.5-fold (P< 0.001) to 4.08 ± 0.21 million. This represents an increase in Na of 0.73 million alveoli per week between postnatal days 14 and 42. During the same period of lung growth and development, the Na of the nicotine-exposed rat pups increased 2.4-fold or 0.48

million per week (P<0.001) to 2.27 ± 0.18 million. The Na of those rat pups exposed to both nicotine and copper during lactation increased 2.5-fold or 0.71 million (P<0.05), to 3.54 ± 0.33 million on postnatal day 42. The data shown here indicate that the rate of alveolarisation was higher (P<0.001) in the control animals than in both the nicotine-exposed group and the group exposed to both nicotine and copper. However, the rate of alveolarisation of the lungs of the rat pups exposed to both nicotine and copper was slightly faster than in the lungs of the animals exposed to only nicotine, so that on postnatal day 42, the number of alveoli of the animal exposed to both nicotine and copper was higher (P<0.01) than that of the nicotine-exposed animals.

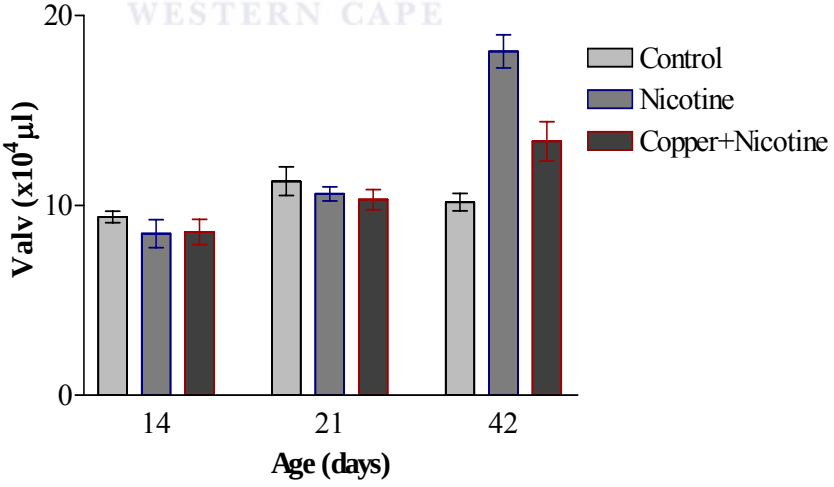

Fig. 6.3. The influence of maternal exposure to nicotine or to both nicotine and copper during lactation on the alveolar volume (Valv) of the offspring.

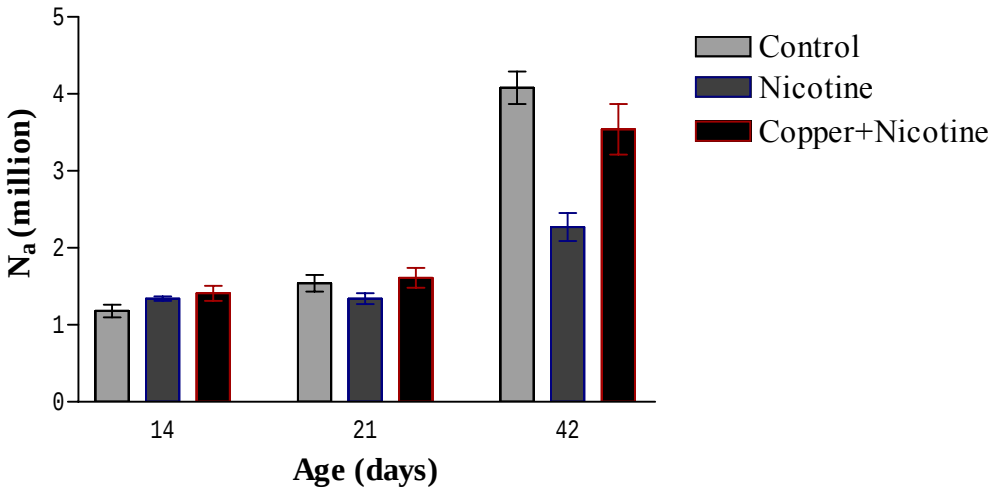

Fig. 6.4. The influence of maternal exposure to nicotine or to both nicotine and copper during lactation, on the alveolar number (Na) of the offspring.

6.2.4 *Airspace Wall Surface Area per Unit Volume (AWUV), (Fig. 6.5)*

The AWUV of all 3 groups of animals showed a gradual decrease in the AWUV as the animals aged. On postnatal day 14, the AWUV of the control rat pups was at 22.28 ± 0.71 mm^2/mm^3, slightly higher ($P<0.01$) than the 19.57 ± 0.47 and 20.09 ± 0.25 mm^2/mm^3 of the 21- and 42-day old control animals. This represents a decrease in AWUV of 9.8 % ($P<0.05$) between postnatal days 14 and 42. The AWUV of the 14-day old nicotine-exposed rat pups was at 22.16 ± 0.73 mm^2/mm^3, the same as that of the control animals of the same age. However, on postnatal day 21, it was 10.1% lower ($P<0.05$) than on postnatal day 14, and on postnatal day 42, the AWUV of the nicotine-exposed animals was at 16.69 ± 0.27 mm^2/mm^3, 24.7% lower ($P<0.001$) than that of the 14-day old nicotine-exposed animals. It was also 16.9% lower ($P<0.001$) than that of the 42-day old control animals. The AWUV of

20.08 ± 0.33 and 21.51 ± 0.65 mm^2/mm^3, respectively, of the 14-and 21-day old rat pups exposed to both nicotine and copper was the same (P>0.05) as for the control and nicotine-exposed rat pups of the same age. The AWUV of these rat pups decreased to 18.56 ± 0.48 mm^2/mm^3 on postnatal day 42, which was lower (P<0.01) than the AWUV of the control animals, but higher (P<0.01) than that of the animals exposed to nicotine only.

6.2.5 *Septal Thickness (Tsept), (Fig. 6.6)*

The T*sept* of the 14-day old rat lung was 2.68 ± 0.13 μm. The T*sept* of the 14-day old PNE rat pups was at 2.92 ± 0.14 μm not different from that of the control animals. Maternal copper supplementation from one day before the onset of the phase of rapid alveolarisation on postnatal day 4, was also not affecting the impact of maternal nicotine exposure during the same phase of lung development. This is illustrated by the fact that the Tw of the 42-day old PNE animals (3.39 ± 0.09 μm) was the same (P>0.05) as the 3.27 ± 0.09 μm of the PNE rat pups that received both copper and nicotine. It is interesting to note that thickening of the alveolar septa occurred after weaning on postnatal day 21.

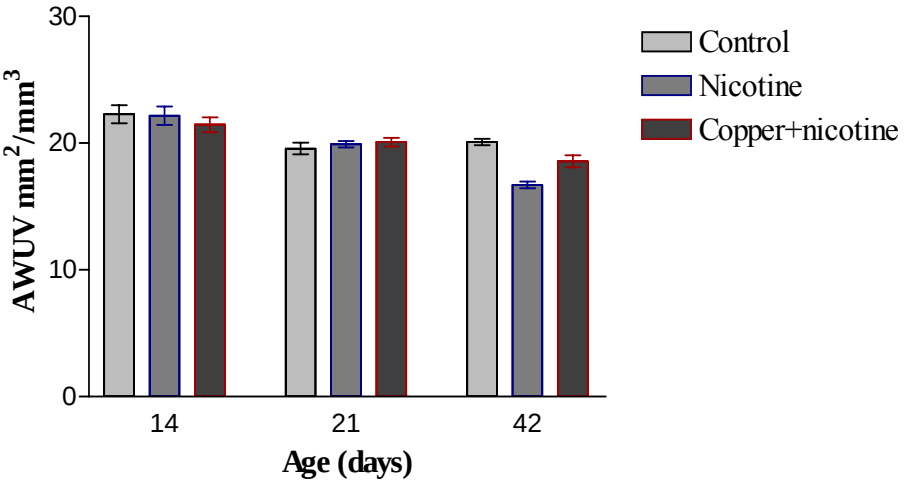

Fig. 6.5. The influence of maternal exposure to nicotine or to both nicotine and copper after birth, on the airspace wall surface area per unit volume (AWUV) of the offspring.

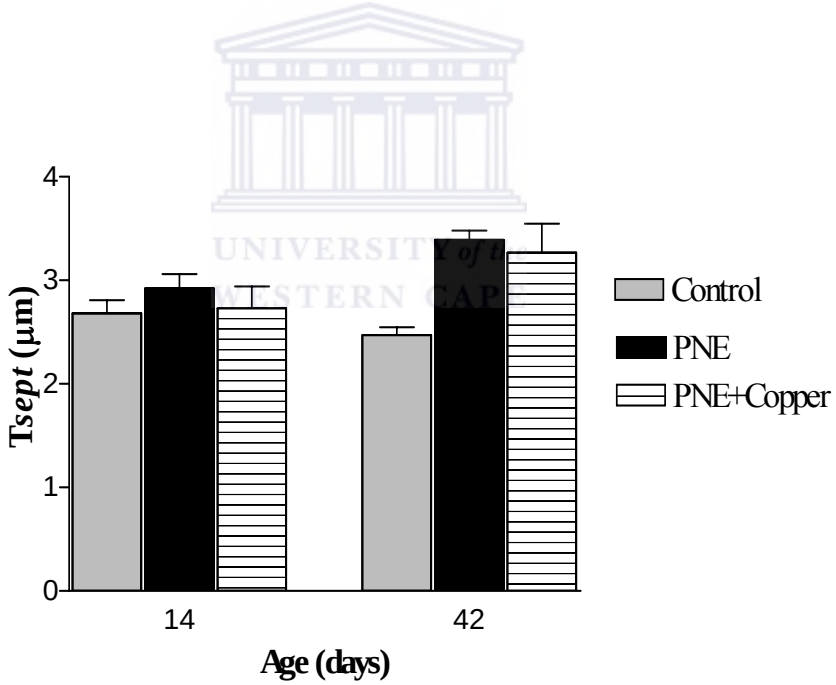

Fig. 6.6. The effect of maternal exposure to nicotine or copper and nicotine on the alveolar wall thickness (*Tsept*) of the offspring.

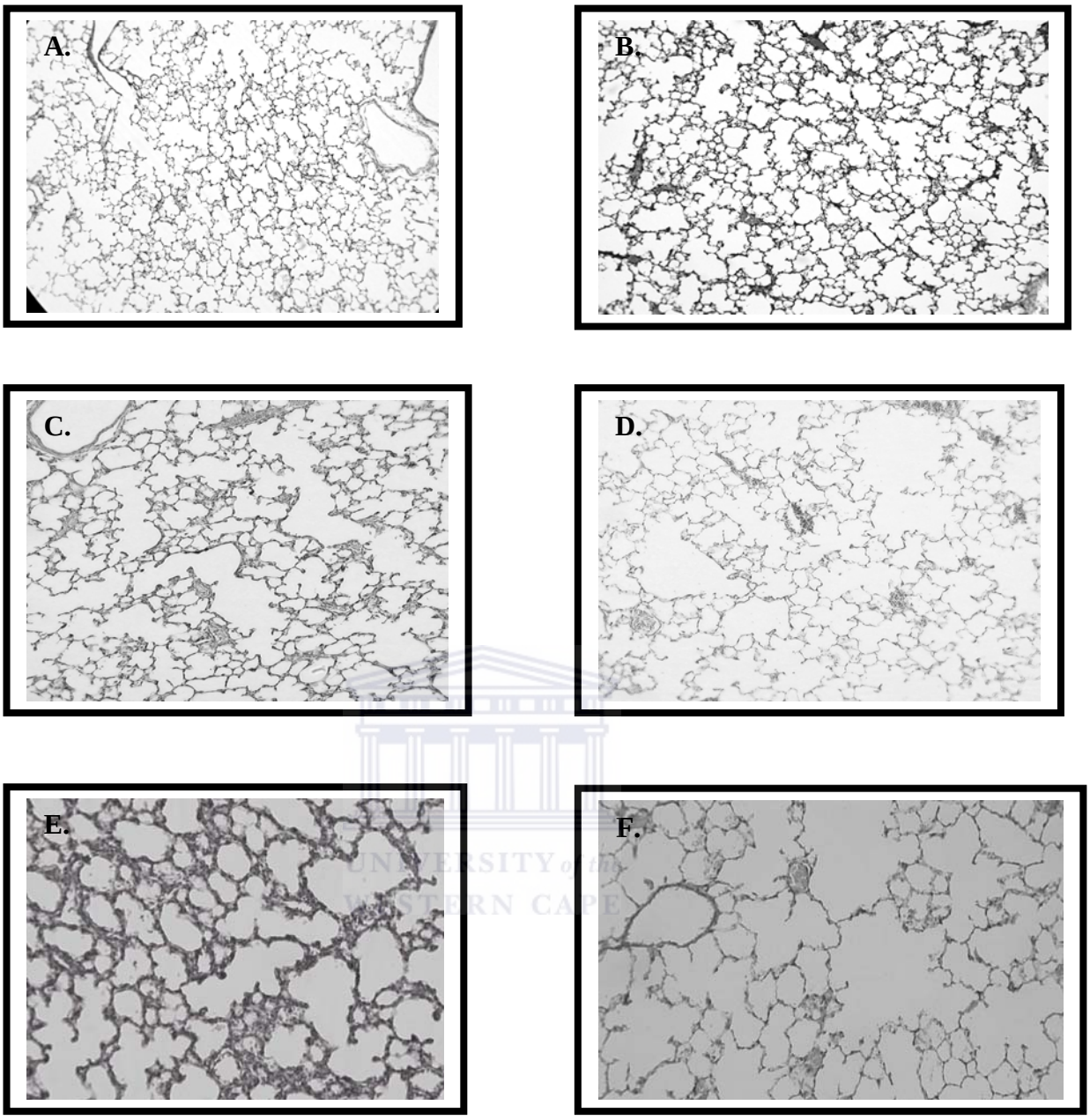

Fig.6.7. Light micrographs of lung parenchyma of 14-day old control (A), G&L (C) and PNE (E) lung parenchyma, and lung parenchyma of 42-day old control (B), G&L and (D) PNE (F). The G&L and PNE rats were exposed to copper via the placenta and/ or mother's milk. Bar = (16 µm)

6.3 Discussion

The development of the mammalian lung is classically divided into the embryonic, pseudoglandular, canalicular, saccular/terminal sac, and alveolar phases. This is followed by the phase of equilibrated growth, which is the final stage of lung maturation (Burri et al. 1974). During late gestation and the early postnatal period, the development and maturation of the lung is aimed at developing the gas exchange area in order to ensure the efficient supply of oxygen to the organism and thus to satisfy the demands of the body. The surface area available for gaseous exchange increases as the animal grows by the formation of new alveoli (Burri, 1974). Any interference with this process of alveolar development, or damage of the alveolar septa during the foetal and neonatal stages of lung development by a substance such as nicotine, may therefore reduce the effectiveness of the lung as a gas exchanger, even after lung growth and maturation have stopped.

Copper plays an important role in lung development (Fiske et al. 1970; O'Dell et al. 1978). Lysyl oxidase, a copper-dependant enzyme, plays a central role in the formation of secondary septa and thus lung maturation (Harris, 1976; Harris, 1986; Buckingham et al. 1981; Dubick et al. 1985; Reiser et al. 1992). It can therefore be expected that a decrease in the copper content of the developing lung will result in a slower lung development or that copper supplementation may enhance lung maturation. In a recent study it was shown that maternal copper supplementation indeed enhanced lung maturation in the offspring (Maritz et al. 2000). It was also shown that maternal nicotine exposure during gestation and

lactation resulted in a lower copper content of the lungs of the offspring and a concomitant decrease in the number of alveoli and internal surface area available for gaseous exchange. This was prevented by maternal copper supplementation during gestation and lactation and clearly shows that copper is indeed important, in the development of the lung into an efficient gas exchanger.

Comparing the data of this study with a previous study in which the mother was exposed to nicotine during gestation and lactation, it was illustrated that maternal nicotine exposure from the onset of alveolaristion only, resulted in a lower Na in the lungs of the offspring.

It is also clear that the rate of alveolar formation after the period of rapid alveolarisation, which occurred between postnatal days 4 and 13 (Burri, 1974), was slower in the lungs of the nicotine-exposed rat pups than in the controls. Even after weaning on postnatal day 21, the pace of alveolar formation was slower in the lungs of those rats exposed to nicotine via the mother's milk than in the lungs of the control animals. Since alveolar formation at this stage of lung development is essentially due to the formation of secondary septa (Burri, 1974), it implies that the formation of secondary septa was retarded despite the fact that the animals were not exposed to nicotine after weaning. Because the rate of alveolarisation of the nicotine-exposed offspring, even after nicotine withdrawal, was slower than that of the control rats, it is unlikely that the Na in the lungs of these animals will reach the same level as that of their control counterparts. However, copper supplementation during gestation and lactation not only prevented the adverse

effects of maternal nicotine exposure on alveolar formation in the lungs of the offspring, but also increased the rate of alveolar formation in these rat pups.

It is clear however, that although maternal nicotine exposure at the onset of the phase of rapid alveolarisation suppresses alveolar formation to the same extent as for those animals that were exposed to nicotine during all the phases of lung development, copper supplementation must start earlier to fully prevent the adverse effects of maternal nicotine exposure on alveolar formation in the lungs of the offspring.

Because the lung volume of the nicotine-exposed rat pups, as determined by the water displacement technique, was not affected by maternal nicotine exposure, and because the Na of the nicotine-exposed rats were lower than that of the control animals, it follows that the Valv of the lungs of these animals should be larger than that of the control animals. It was indeed illustrated in this study that the Valv of the nicotine-exposed rat pups was bigger than that of the control rat pups of the same age. On the other hand, the Valv of the control rats remained the same after completion of alveolarisation on postnatal day 14. In contrast to the control lung, the Valv of the lungs of the nicotine-exposed rat pups increased as the animals aged. The Valv of those rats exposed to both copper and nicotine, like those of the animals exposed to nicotine only, also increased, but at a slower rate, so that on postnatal day 42, the Valv of the animals exposed to both nicotine and copper was only larger than those of the control animals but significantly smaller than that of the rat pups exposed to only nicotine. This clearly shows that copper

supplementation during lactation, from late saccular development, prevented the adverse effects of maternal nicotine exposure on neonatal lung development as well as protected it against the long-term effects of maternal nicotine exposure on the lung parenchyma.

The internal surface area (Sa) of the lung available for gas exchange is determined by the Na of the lung, as well as the surface area of the alveoli available for gas exchange. It is therefore clear that, based on their larger Na, the Sa of the lungs of the control animals will be bigger than in the lungs of the nicotine-exposed rat pups. The slower rate of alveolarisation in the lung of the nicotine-exposed rat pups, and thus the slower increase of the surface area available for gas exchange, also explains the gradual increase in the difference in the Sa between control and nicotine-exposed animals as the animals aged.

The fact that the Sa of the lungs of the control rats increased despite the fact that the Valv remained the same implies that the increase in the Sa during lung growth and development is primarily due to the formation of the secondary septa and thus an increase in Na. This means that inhibition of the formation of secondary septa as a consequence of maternal nicotine exposure will almost certainly result in a permanently smaller Sa. After completion of alveolarisation, the lung volume and Sa increase as a result of stretching of the existing alveoli. It is however, unlikely that the Valv, and thus the lung volume of the nicotine-exposed animals, will increase after postnatal day 42 to such an extent that the Sa of the nicotine-exposed animals will catch up with that of the control animals. As for

alveolarisation, copper supplementation greatly reduced the alveolar enlargement demonstrated for those animals exposed to nicotine only. The exact mechanism whereby copper prevented the adverse effects of maternal nicotine exposure on neonatal lung alveolar formation and enlargement is not known.

An increase in Valv due to gradual destruction of the alveolar walls and consequently a decrease in Na is associated with the development of emphysema. However, the decrease Na and the increase in the Valv in the lungs of the nicotine-exposed rat pups alone are not necessarily indications of the onset of microscopic emphysema. Additional evidence, such as AWUV, is therefore required to establish whether maternal nicotine exposure during lactation indeed induced microscopic emphysema in the lungs of the offspring.

In a study by Gillooly and Lamb (1993), it was shown that the AWUV in humans decreased linearly with advancing age after lung development stopped. The limits for normality for the mean AWUV should be 95% or more than the mean AWUV for a particular age group. If the difference within an age group is less than 95% of the baseline value for that age group, it is a sign of early emphysema (Gillooly and Lamb, 1993). In the present study, the AWUV of the control animals decreased slightly between postnatal days 14 and 21, the AWUV of the animals exposed to nicotine from the end of the saccular phase of lung development and thus from the onset of the phase of rapid alveolarisation, were at ~ 95% from the baseline control values for each age group, within the normal range for these age groups and therefore not showing any sign of microscopic emphysema. However,

3 weeks after weaning on postnatal day 21, and thus 3 weeks since the last exposure to nicotine via the mother's milk, it was 83.08% of the control values and thus below the normal limits for each age group, and therefore reflects a generalized increase in the airspace size, a clear sign of microscopic emphysema. In this study, it was shown that maternal nicotine exposure during gestation and lactation also resulted in an AWUV that is ~ 83% of the control values (Chapter 5). This implies that maternal nicotine exposure from the end of the saccular phase of lung development had the same impact as maternal nicotine exposure during all the phases of lung development, starting with the embryonic phase.

Copper supplementation from the end of the saccular phase of lung development resulted in an AWUV of 92.38% on postnatal day 42 and thus also below the normal limits for this age group. This is in contrast to the findings of recent research that clearly showed that copper supplementation during gestation and lactation, and thus during all phases of lung development, prevent the induction of microscopic emphysema (Maritz et al. 2000). This implies that maternal nicotine exposure from the end of the saccular phase of lung development in the offspring slowed down the process whereby microscopic emphysema was induced, but not preventing it. It is therefore necessary to investigate the effect of copper supplementation at an earlier phase of lung development to establish when copper supplementation will be effective in preventing the induction of microscopic emphysema due to maternal nicotine exposure.

The rat pups received nicotine and copper via the mother's milk only. From about postnatal day 13, the rat pups began to eat solid food in addition to milk. As the animals aged they ate more solid food and the milk intake gradually decreased and thus also the intake of nicotine via the mother's milk. After weaning on postnatal day 21, the neonates received no milk and thus also no nicotine. The half-life of nicotine is about 90 minutes (Russell and Feyerabend, 1978), therefore it is conceivable that the nicotine will be quickly eliminated from the tissue of the neonates. Despite the withdrawal from nicotine, the alveolar region of the lungs gradually decreased after weaning as indicated by the development of microscopic emphysema. It is not known why microscopic emphysema only develops after weaning and thus after nicotine withdrawal. It might be that the process is too slow to show any evidence at postnatal days 14 and 21. It is also possible that the mother's milk contains micronutrients or a combination of micronutrients, which protect the lungs of the offspring against the effects of maternal nicotine exposure. However, no evidence in support of this suggestion exists. It is however clear that maternal nicotine exposure interferes with the lung development of the offspring in such a way that the process of lung deterioration gradually progresses, rendering the lungs more susceptible to damage and disease, even after all the nicotine is eliminated for the tissues of the animal. The effect of maternal nicotine exposure is prevented by early copper supplementation and slowed when copper supplementation starts just before the onset of alveolarisation.

The mechanism(s) whereby maternal nicotine exposure impact on foetal and neonatal lung development, and the long-term consequences thereof on lung

structure and function, is not clear. It has been suggested (Schuller et al. 2000) that *in utero* exposure of pulmonary neuroendocrine cells to cigarette smoke, and thus to nicotine too, contribute to the development of paediatric lung disorders via 2 different mechanisms, namely, (1) the direct effects of released 5-hydroxytryptamine (5-HT) in response to α_7 nicotinic receptor stimulation on fibroblast growth, and (2) the indirect effects of 5-HT on pulmonary neuroendocrine cell numbers via the activation of the Raf-1/MAP kinase pathway, resulting in even more cells to release 5-HT (Russell and Feyerabend, 1978), where 5-HT stimulates fibroblast growth (Schuller, 1989). It is also suggested that binding of nicotine to this receptor will result in the accumulation of collagen in airway and alveolar walls (Sekhon et al. 2002). However, the slower rate of alveolarisation and the development of emphysema-like characteristics in the long term cannot be explained by this mechanism, as the stimulation of fibroblast growth would have prevented the decreased rate of secondary septal formation and thus the lower Na of the lungs of the nicotine-exposed rat pup. Furthermore, a previous study showed that maternal nicotine exposure during gestation and lactation had no effect on the total collagen content on the lungs of the offspring (Maritz and Dolley, 1995). In addition, the gradual deterioration of the lungs as the animals aged also argues against these suggestions, because the half-life of nicotine is only 90 minutes, and deterioration of the lung structure proceeded even after nicotine was eliminated from the animals. The data from this study do, however, implicate that nicotine exposure during the phase of alveolarisation probably induce changes on the gene level, which probably result in a faster ageing of the lung. This is supported by the fact that *klotho* gene expression is

essential for maintaining pulmonary integrity during postnatal life. Mutations of this gene during pregnancy and early lung development might give rise to lung lesions resembling pulmonary emphysema as the animals age (Suga et al. 2000). This might be due to affected lysyl oxidase activity and thus defective cross-linking of connective tissue fibres (Fiske and Kuhn, 1970; Suga at al, 2000). It is therefore plausible that maternal nicotine exposure result in the change in the genes responsible for maintaining the integrity of the lungs in adulthood and in this way induce gradual structural deterioration as the animals age.

It is interesting to note that while copper supplementation prevented the adverse effects of maternal nicotine exposure on the alveolar structure of the lungs of nicotine-exposed rat pups, it had no effect on the increased cellularity of the alveolar walls of these rats. If the increased cellularity of the alveolar walls is indeed due to suppression of apoptosis by nicotine, it implies that copper had no effect on this action of nicotine. The protective effect of copper is thus at a different level, probably by affecting connective tissue metabolism.

In conclusion, maternal nicotine exposure from the end of the saccular phase of alveolar development and through the alveolar phase of lung development up until weaning on postnatal day 21 resulted in lower alveolar number and internal surface area. Copper supplementation during lactation slowed down the rate of microscopic emphysema development, but is not preventing it entirely. These structural impairments may impair lung function in later life and is not associated with low birth weight, lack of nutrition, or length of gestation.

6.4 References

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CHAPTER 7

Effect of Maternal Nicotine Exposure on the Elastic Tissue Structure of the Lungs of the Offspring

7.1 Introduction

Connective tissue forms an important component of the lung and comprises mainly collagen and elastin fibres, proteoglycans and glycosaminoglycans. It also includes microfibril-associated glycoprotein (MAGP), (Brown-Augsburger et al. 1996) and non-collagenous glycoproteins, such as the fibulin family (McLaughlin et al. 2006). These connective tissue components give architectural stability to the lung and interference with any of them may compromise the ability of the lung to perform efficient gaseous exchange.

Elastic fibres are complex structures that contain at least 2 morphologically distinguishable components, namely amorphous elastin and microfibrils (Shifren and Mecham, 2006). Damage to the elastic component of the connective tissue framework of the lung, results in COPD. Apart from proteases that can cause destruction of the connective tissue framework of the lung, other factors have been implicated in tissue destruction leading to the development of COPD. These include oxidative stress and apoptosis of lung structural cells (MacNee, 2005; Owen, 2005). Alveolar macrophages of smokers, for example, release more reactive oxygen species than do cells from non-smokers (Shifren and Mecham, 2006). The antioxidant capacity of the plasma of smokers is also lower than that of non-smokers (Shifren and Mecham, 2006), and thereby renders the cells of the

lung more susceptible to oxidant damage. The effects of oxidants on elastic fibre homeostasis are most likely manifested through their ability to modulate the activity of proteinases, proteinase inhibitors, cross-linking enzymes and other extracellular matrix components involved in elastic tissue homeostasis, as opposed to a direct effect on elastin (Shifren and Mecham, 2006). This is illustrated by the fact that cigarette smoke blocks the cross-linking of elastin *in vitro* (Laurent et al. 1983). Studies by Chen et al (2005) showed that cells in a cigarette smoke-condensate have decreased levels of lysyl oxidase (LOX) protein accompanied by decreased LOX activity when compared to cells in a smoke-condensate free medium. This decrease in LOX protein in cigarette smoke-condensate-treated cells, is thought to be the result of oxidant induced down-regulation of LOX steady-state mRNA through inhibition of transcription initiation and increased instability of LOX mRNA transcripts (Gao et al. 2005). This implies that the oxidants in tobacco smoke interfere with elastic tissue homeostasis by reducing the half-life of elastic tissue while at the same time the replacement of damaged elastic tissue is not increased.

It has been reported that nicotine, an important component of tobacco smoke, induces oxidative stress *in vivo* and *in vitro* (Marwick et al. 2002; Rahman and MacNee, 1999; Asami et al. 1997; Aoshiba and Nagai, 2003). A study by Goksel et al (2005) showed that chronic nicotine administration to male Wistar albino rats caused oxidant damage in various organs by increasing lipid peroxidation and decreasing the activity of the endogenous antioxidants. It was indeed shown that experimental lung toxicity induced by the administration of nicotine to Wistar

rats, resulted in enhanced lipid peroxidation and the production of DNA-adducts (Ashakumary and Vijayammal, 1992). This can partially be attributed to the lowered ascorbic acid levels and thus the antioxidant effect of ascorbic acid. In a recent study (Kalpana and Menon, 2004) it was indeed shown that nicotine not only increases the oxidant load of the body, but also decreases its antioxidant levels, thereby decreasing the capacity to protect itself against oxidant damage. This supports earlier research which clearly showed that nicotine reduces the ascorbic acid content of the lungs of rats (Maritz, 1993; Maritz and van Wyk, 1997).

It was furthermore shown that nicotine inhibits cell growth and proliferation as well as protein synthesis in human periodontal ligament fibroblasts (Chang et al. 2002). This implies that the lungs of smokers could be more likely to develop damage of the connective tissue framework of their lungs than non-smokers. It furthermore implies that nicotine replacement therapies during pregnancy and even in the adult, may render the lungs more susceptible to damage.

In this project I showed that maternal nicotine exposure during all phases of lung development, as well as from the onset of the alveolar phase of lung development, resulted in the development of microscopic emphysema in the lungs of the offspring. Since emphysema develops as a consequence of elastic tissue damage (Shifren and Mecham, 2006), it is plausible that the microscopic emphysema seen in this project was due to damage to the elastic tissue framework of the lungs of the offspring.

Copper supplementation prevents the adverse effects of maternal nicotine exposure. This study further shows that if maternal copper supplementation starts early enough, it will also prevent the late onset of microscopic emphysema in the lungs of the offspring. This implies that copper reduces the adverse effects of maternal nicotine exposure on the elastic tissue framework of the lungs of the offspring. The aim of this chapter was to determine the effects of maternal nicotine exposure and supplementation with copper on the elastic tissue component of the connective tissue framework of the lungs of the offspring.

7.2 Results

Measurements of the thickness (Fig. 7.1) of the elastic fibres showed that maternal nicotine exposure during gestation and lactation had no effect on the average thickness of the fibres in the alveolar walls. However, light microscopy pictures of thick sections (20 µm) of the lungs of the controls (Fig. 7.2 A) showed that the elastic fibre framework was intact. However, the elastic tissue framework of the lungs of the nicotine-exposed offspring (Fig. 7.2 B) showed severe damage which resemble the microscopic emphysema-like lesions seen in sections of whole lung (Chapter 5, Fig. 5.6). The elastic tissue framework of the lungs of the offspring that received a combination of nicotine and copper (Fig. 7.2 C) also showed signs of emphysema but was not as severe as that of nicotine-exposed animals. The elastic fibres in the alveolar walls of the control lungs were of even thickness and had a smooth appearance (Fig. 7.3 A). On the other hand, elastic fibres of the nicotine-exposed animals displayed areas of elastic fibre “condensation” (Fig. 7.3 B).

Thin sections (4 μm) of the lung tissue of the nicotine-exposed animals showed (Fig. 7.5) the elastic tissue fibres of the animals exposed to nicotine during gestation and lactation or during the phase of rapid alveolarisation only. The elastic fibres of these lungs showed localized thinning and breaks in the strands (Fig.7.5. B and C). In addition, it also showed localized condensation (Fig.7.5 A and B) of the elastic fibres such as in the thick sections (Fig. 7.3 B). The breaks in the elastic tissue fibres were associated with breaks in the alveolar walls (Fig. 7.5 B and C).

The elastic fibres of the lungs of the control 42-day old rats as well as of the rats exposed to nicotine and copper during gestation and lactation or only from the onset of the phase of rapid alveolarisation showed continuous elastic fibres in the alveolar walls. No elastic fibre condensation was evident in neither the control lung nor the lung tissue of the animals that were exposed to both nicotine and copper (Fig. 7.6 A and B).

Pores of Kohn with smooth openings occured in the lungs of the control animals (Fig. 7.6 A). The elastic tissue around the openings of the pores of Kohn were normal, whereas the elastic tissue at the sites of alveolar wall breaks were thinned and the openings in the walls were uneven (Fig. 7.4 A and B).

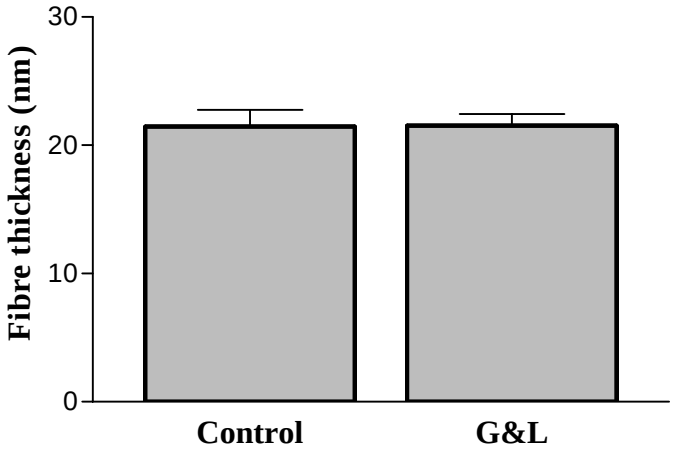

Fig. 7.1 The influence of maternal nicotine exposure during gestation and lactation on the elastic fibre thickness of the offspring's lungs.

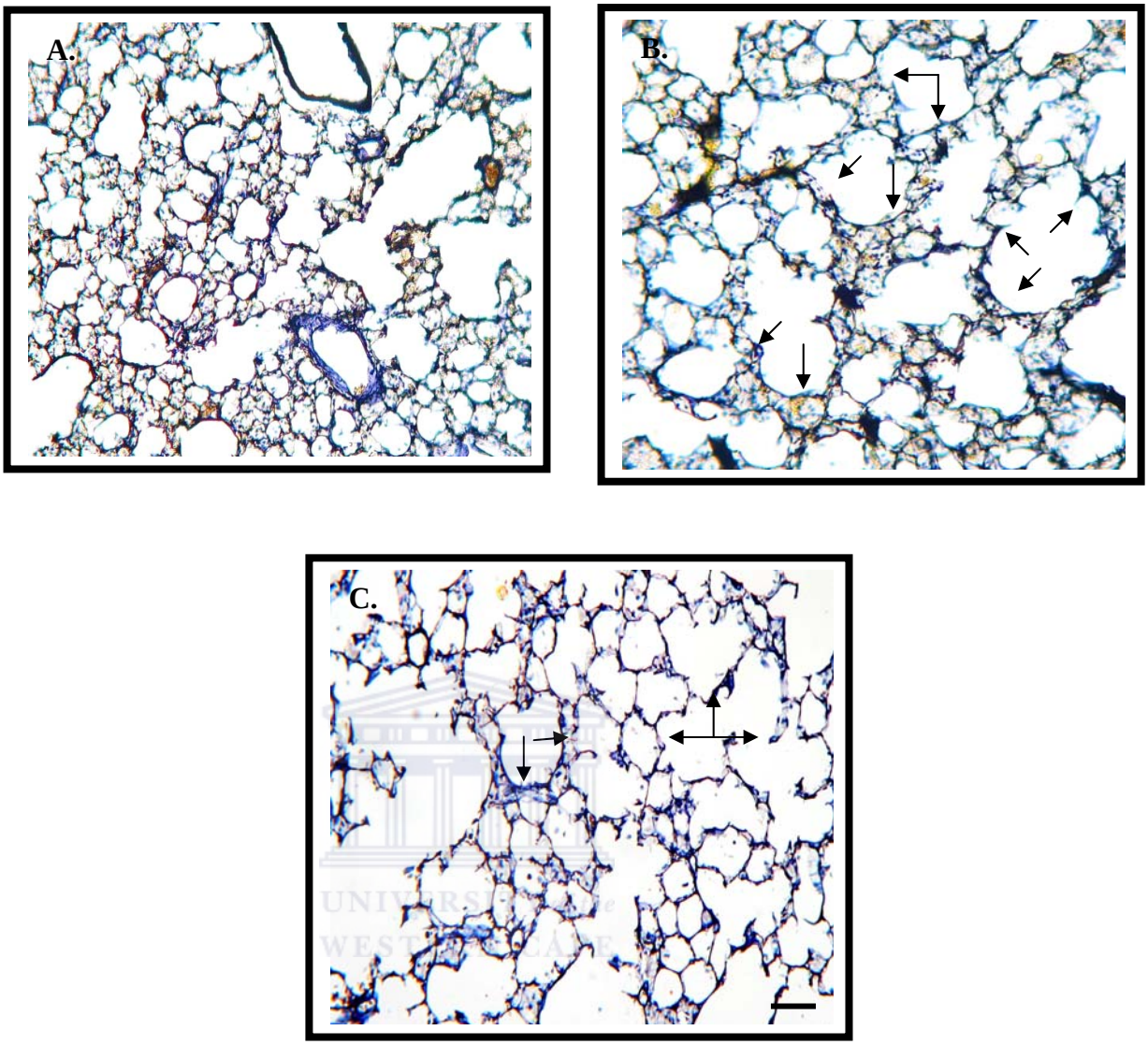

Fig.7.2. Illustration of elastic tissue framework in intact (A) control, (B) nicotine and (C) copper and nicotine-exposed rats. The framework of nicotine-exposed offspring (B) shows severe damage which resembles microscopic emphysema-like lesions (arrows) seen in sections of whole lung. The elastic tissue framework of the offspring that received nicotine and copper (C) also show signs of microscopic emphysema (arrows). (Bar = 16 µm)

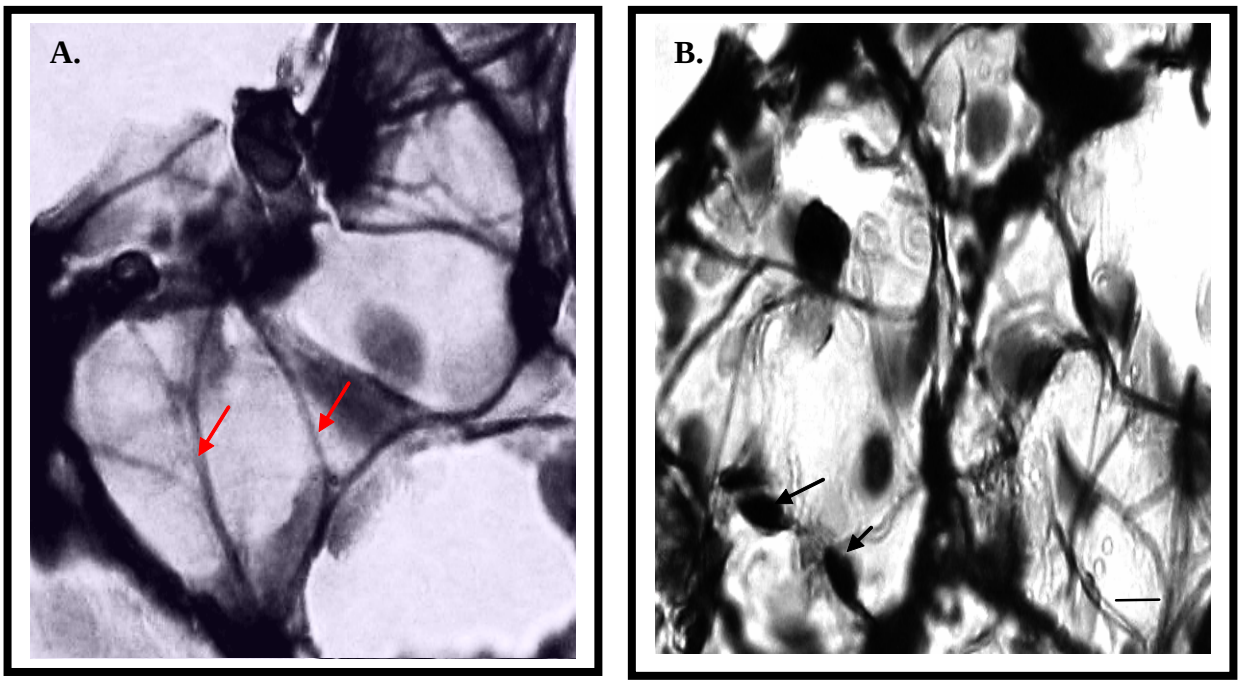

Fig.7.3. Illustration of elastic fibres in the alveolar walls of (A) control, and (B) of 42-day old rats exposed to nicotine via the placenta and mother's milk. Red arrows = Smooth elastic fibres. Black arrows = aggregates of elastic tissue. (Bar = 50 µm)

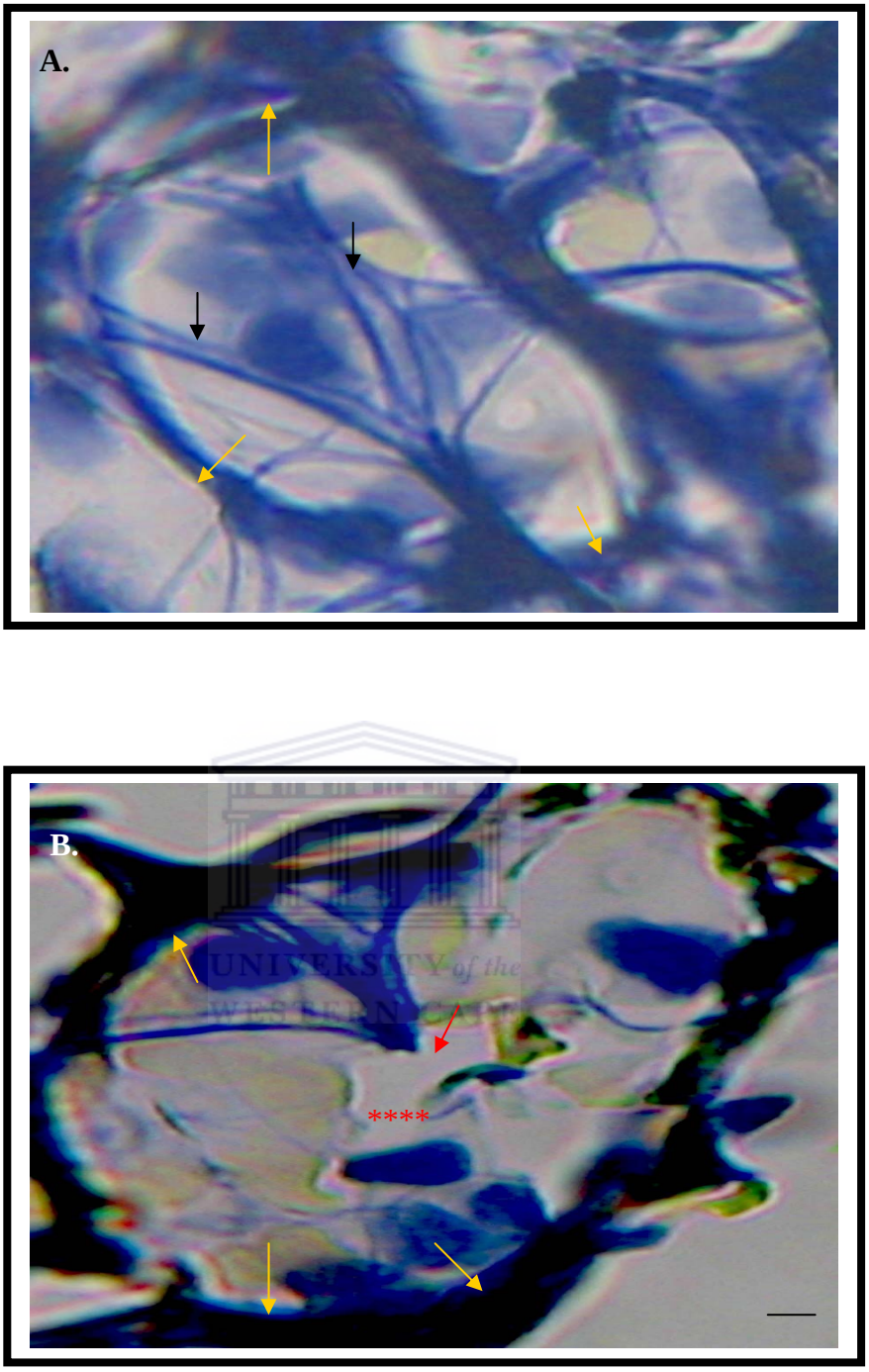

Fig.7.4. An alveolus illustrating intact (A) and breaks (B) in the connective tissue framework that supports the alveolar wall. Note the break (red asterisks) in the alveolar wall. Yellow arrows indicate elastic tissue of the alveolar mouth. (Bar=50µm)

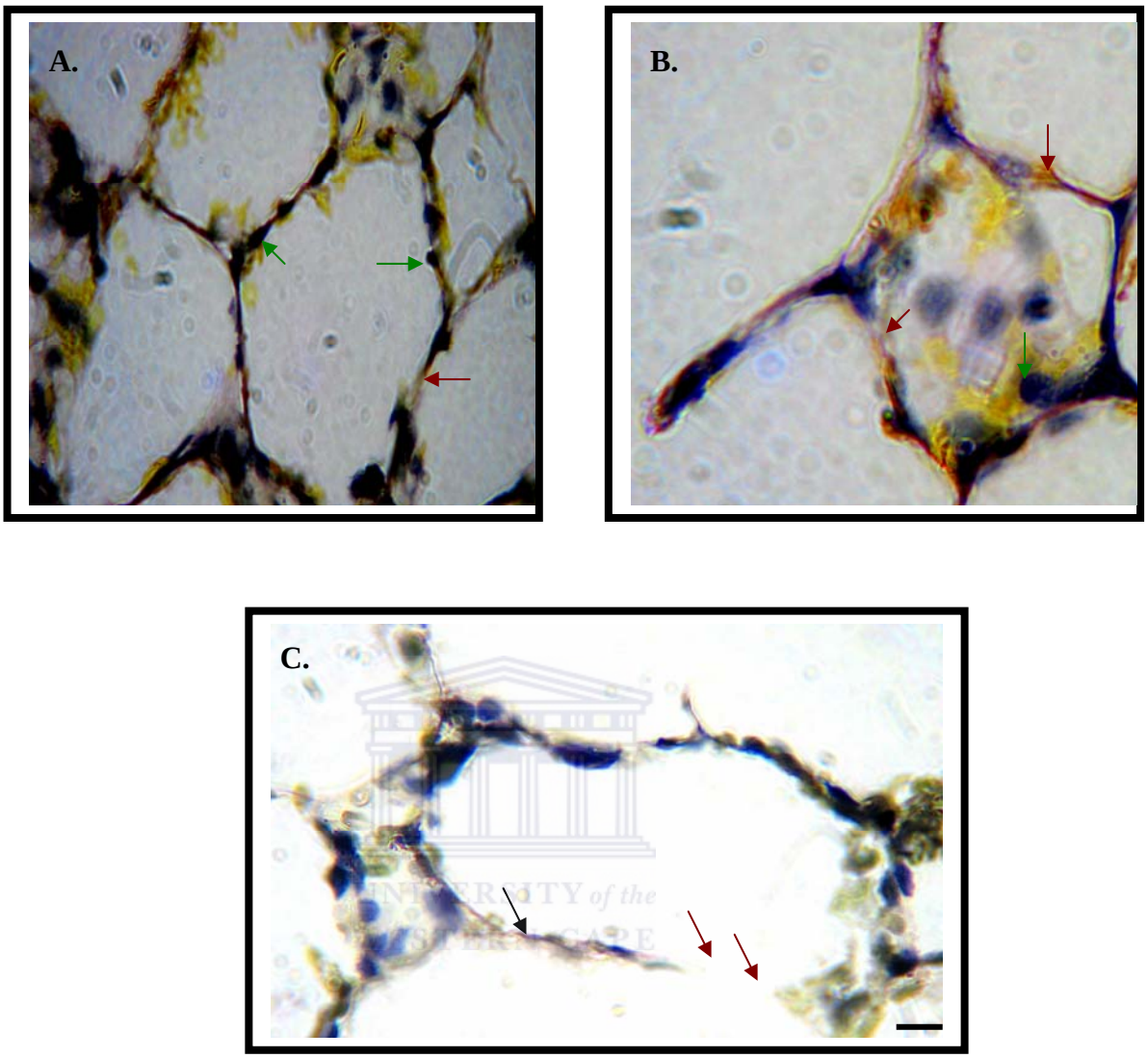

Fig.7.5. Light micrographs of thin (4 µm) sections of the lung tissue of 42-day old rats exposed to nicotine via the placenta and/or mother's milk. The elastic tissue (stained black) in the alveolar walls show localized aggregation of elastic fibres (green arrows) as well as breaks in the elastic fibres (red arrows) (A and B). Places the elastic fibres thinned (black arrow) with a break of the alveolar wall (double red arrows) (C). (Collagen = red fibres) (Bar = 50 µm)

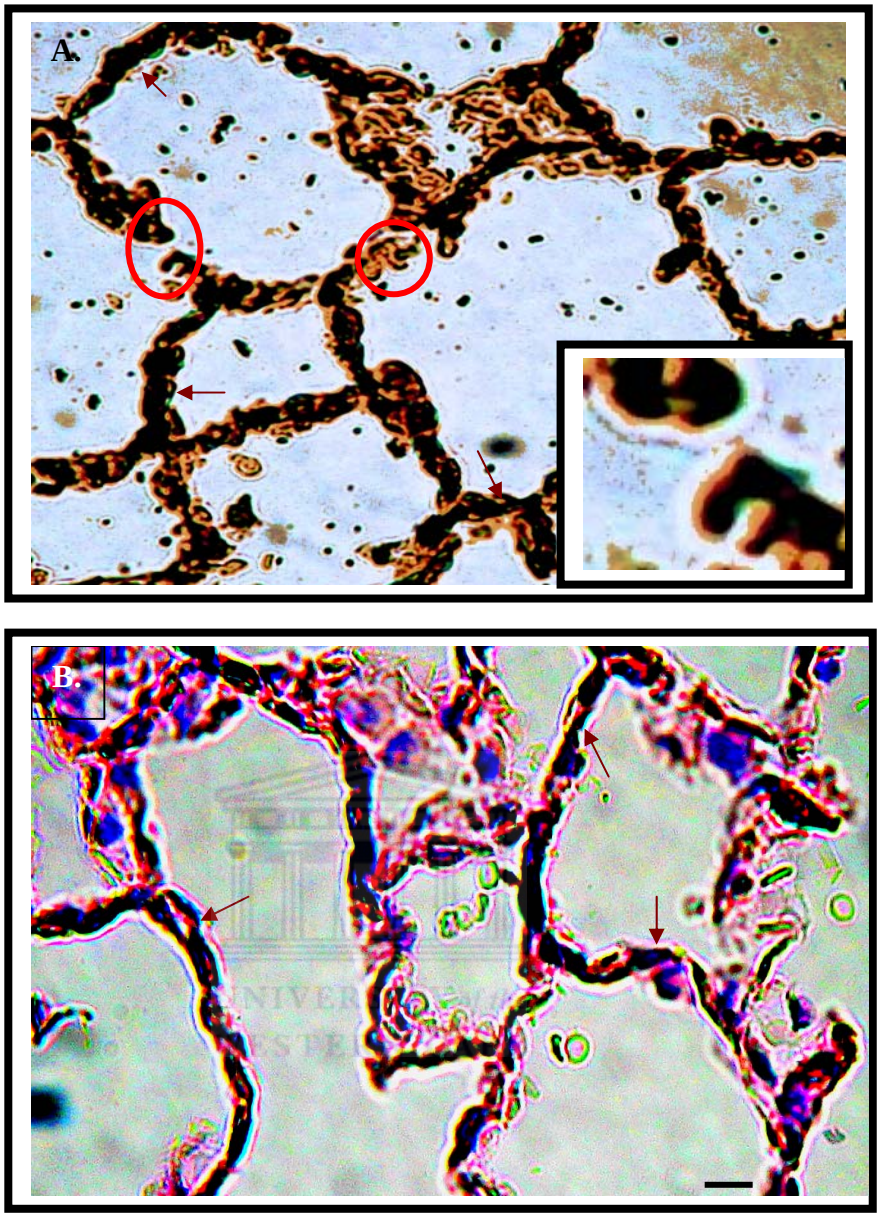

Fig.7.6. Light micrographs of elastic tissue in the lungs of 42-day old control animals (A) and animals exposed to both nicotine and copper (B). No aggregates or breaks of elastic fibres (red arrows) are evident. Smooth rounded openings of the pores of Kohn (A) (red circles) are present. Insert: Pore of Kohn illustrating normal elastic tissue and smooth opening. (Bar = 50 µm)

7.3 Discussion

The previous chapters showed that maternal nicotine exposure induced the late onset of changes in the parenchymal architecture of the lungs of the offspring, where these changes resembled premature ageing of the lungs. This included the enlargement of alveoli, decreases in alveolar numbers and of the internal surface area for gaseous exchange. This renders the lungs more susceptible to damage to the parenchyma and to the development of COPD (Dyer and Stockley, 1999). In this study it was indeed shown that microscopic emphysema occured in the lungs of the mature offspring of mothers that were exposed to nicotine during all the phases of lung development, as well as in the lungs of the rats exposed to nicotine via the mother's milk from the onset of the phase of rapid alveolar formation.

The connective tissue of the lung forms a 3-D network that serves as a framework or skeleton of the lung (Wilson and Bachofen, 1982). The lung is dependent on the connective tissue framework for its stability and for the maintenance of the gas-exchange function of the lung. Furthermore, the elastic tissue component of the connective tissue framework plays an important role in alveolar formation (Bourbon et al. 2005). It therefore implies that damage to the connective tissue framework of the lung will compromise its stability, as well as its function as a gas-exchanger. Furthermore, the integrity of the connective tissue framework is not only dependent on the integrity of the connective tissue fibres and the associated extracellular matrix, but also on the integrity of the fibroblasts that produce it (Absher, 1995).

The elastic tissue component of the connective tissue framework plays an important role in lung function in that it determines the compliance of the lung (Thibeault et al. 2006). Injury that is selective for elastin results in emphysema, examples being the injury produced by the injection of pancreatic elastase or papaine, enzymes relatively specific for elastin (Kuhn, 1997). In the model of elastase-induced injury, it has been suggested that despite the restoration of the elastin content of the lung to normal subsequent to the insult, emphysema results because the organization of the elastic fibres is disrupted and the network is discontinuous (Kuhn, 1997). In the present study it was shown that the average thickness of the elastic fibres of the control and nicotine-exposed lungs were the same. Although the average thickness was the same, the elastic tissue of the nicotine-exposed rats showed localized thinning, as well as localized aggregates of elastic fibres. In addition, breaks in elastic fibre strands occured. This implies that the connective tissue framework that supports the alveolar walls were compromised, which conceivably resulted in the microscopic emphysema that was observed in the lungs of the nicotine-exposed offspring (Chapter 5, Fig. 5.6 and Chapter 6, Fig. 6.7). This is supported by the damage to the elastic tissue component of the connective tissue framework of the lungs of the nicotine-exposed offspring.

The exact mechanism whereby maternal nicotine exposure induces the late onset of microscopic emphysema is not clear. It is known that growing elastic fibres are almost always surrounded by glycoprotein microfibrils, which have been thought to be involved in the orientation of the fibres (Cleary and Gibson, 1983 and Ross

and Klebanoff, 1971). This hypothesis was mainly based on experiments showing that, upon inhibition of lysyl oxidase by beta-aminoproprionitrile fumarate *in vivo*, aortic elastic tissue grew by lateral apposition of roundish aggregates which were always permeated by cytochemically recognized glycosaminoglycans (Baccarani et al. 1985; Pasquali-Ronchetti et al. 1984). It was suggested by Pasquali-Ronchetti et al (1984 and 1985) that trapping of glycosaminoglycans among elastin was induced by persistence of positive lysine amino groups on elastin offering binding sites for these negatively-charged matrix molecules. They also suggested that this creates binding sites for negatively-charged matrix molecules and that elastin/glycosaminoglycan association could be normal during elastin fibre synthesis. This might keep tropoelastin in solution in the extracellular space, at least up to the deamination of lysine epsilon amino groups by lysyl oxidase. Fornieri et al (1987) hypothesized that the electrostatic events during certain periods of intense elastic tissue synthesis (Keeley, 1979; Keeley and Johnson, 1984) would prevent newly synthesized tropoelastin molecules from random spontaneous aggregation far from growing elastic fibres. It was indeed shown by some researchers (Bressan et al. 1983 and 1986; Cox et al. 1974) that tropoelastin secreted by cells is highly hydrophobic, displaying a great tendency to aggregate *in vitro* with increasing temperature up to physiological levels. *In vivo* this characteristic would lead to the formation of many random elastin aggregates (Fornieri et al. 1987). Studies by the latter researchers showed that the degree of lysyl oxidase inhibition and thus the level of free epsilon amino groups on elastin, determine the elastin/glycosaminoglycan association and thus aggregate formation. They showed that abnormal elastin fibres and

elastin/glycosaminoglycan association only occurred when lysyl oxidase activity was markedly inhibited. It is therefore conceivable that the aggregates seen on the elastic fibres of lung tissue of rats that were exposed to nicotine via the placenta and mother's milk was due to inhibition of lysyl oxidase. It supports previous suggestions by Maritz and Windvogel (2003) that the changes in the elastic tissue framework of the lungs of the offspring was due to a decreased lysyl oxidase activity.

In a study by Kida and Thurlbeck (1980) it was shown that inhibition of lysyl oxidase activity during a critical period of postnatal lung development resulted in irreversible structural changes, including larger airspaces that predisposed the lung to injury later in life. Copper is essential for lysyl oxidase activity (Rucker et al. 1998). In another study it was shown that the copper content of the lungs of the rat offspring that were exposed to nicotine via the placenta and mother's blood, was significantly lower (Maritz et al. 2000) at the onset of the phase of rapid alveolarisation. This phase depends on a rapid deposition of elastic fibres (Emery and Mithal, 1960) and thus on optimal lysyl oxidase activity. The lower copper content and thus lysyl oxidase activity at this crucial phase of lung development may have an adverse effect on the development of the connective tissue framework of the lung parenchyma. The copper content of the lungs return to normal between postnatal days 7 and 14. It can thus be suggested that since the copper content of the lungs of the nicotine-exposed rat pups returned to normal before postnatal day 14, that lysyl oxidase activity also returned to normal. However, despite this the deterioration and aggregate formation in the long term

was not prevented. The reason for this is not known, but might be due to an imbalance in the control of extacellular matrix and connective tissue synthesis and breakdown. Part of the imbalance could be due to age-related changes in lysyl oxidase expression (Reiser et al. 1987; Ding and Gray, 2001) and activity (Fornieri et al. 1989). It was shown that mice lacking lysyl oxidase-like 1 activity did not deposit normal elastic fibres and resulted in enlarged air spaces in the lungs (Liu et al. 2004). It is therefore conceivable that the breaks in the connective tissue fibres of the nicotine-exposed rats was due to inefficient lysyl oxidase function due to premature ageing of the lung. If this is so, it implies that maternal nicotine exposure not only affect the formation of the elastic tissue component of the connective tissue framework, but also adversely affects the maintenance of the framework through premature ageing of fibroblasts and lysyl oxidase. This is supported by the observation that maternal copper supplementation during gestation and lactation prevented coacervation of elastic fibres.

Apart from changes in lysyl oxidase activity, maternal nicotine exposure may also affect the integrity of the elastic fibres in the lungs of the offspring by affecting the maintenance of existing fibres. Recently it was proposed that an increase in oxidants may result in damage to the connective tissue framework of the lung (Shifren and Mecham, 2006). This is plausible under certain circumstances since nicotine stimulates the formation of oxidants and specifically induces peroxidation of membrane lipids (Goksel et al. 2005). The lowering of lung ascorbic acid by nicotine (Maritz, 1993) makes this a possible mechanism while the lung is exposed to nicotine. However, lipid peroxidation will only be increased if nicotine

is present. Since microscopic emphysema was only detected 3 weeks after nicotine withdrawal, it is unlikely that nicotine will induce oxidant formation in the lungs of the offspring 3 weeks after nicotine withdrawal, since nicotine's half-life is 11.2 hours in newborns and about 90 minutes in adults (Dempsey et al. 2000, Luck and Nau, 1984). It is therefore unlikely that nicotine, after 21 days of withdrawal, will still be present in the lung at a level high enough to increase the oxidant levels of the lungs of the offspring to such an extent that it will cause breaks in the connective tissue framework of the lung.

In a study by Ranga et al (1979) it was shown that there is a loss of elastic fibres in the lung as the animal ages. They attribute it to a loss of pseudo-elastin. Previous studies also show that ageing is associated with emphysematous changes in rat lung (Saldiva et al. 1988). Diminished lung elastic recoil occured with age, suggesting that it is a true ageing phenomenon. Furthermore, it is associated with a marked increase the incidence of COPD in the lungs of humans (Dyer and Stockley, 1999). This can be attributed to ageing fibroblasts, since fibroblasts provide part of the lung's structural support and matrix that is essential for its integrity (Absher, 1995), and a senescent phenotype could affect tissue microbalance and structural maintenance of the lung. It was shown in this project that lungs of rats exposed to nicotine via the placenta and/or mother's milk aged sooner than that of the control rats. It is therefore plausible that exposure of the offspring to nicotine via the mother's blood and/or milk, resulted in a "reprogramming" of the lung fibroblasts to age faster than in those that were not exposed to nicotine. Since lung fibroblasts from patients with emphysema show a

reduced proliferation rate (Holz et al. 2004; Nobukuni et al. 2002), altered growth factor response (Noordhoek et al. 2003) and lower number of population doublings in long-term culture (Holz et al. 2004), it is plausible that maternal nicotine exposure enhanced the rate of fibroblast ageing in the lungs of the offspring. An increase in the number of senescent cells over time may result in retarded maintenance of the support structures of the lung and thus contribute to a gradual destruction thereof. If this is indeed so, it explains the appearance of the emphysematous lesions in these lungs despite the withdrawal of nicotine at weaning.

Based on the above, I hypothesize that maternal nicotine exposure as late as the onset of the phase of rapid alveolarisation changed the "program" that controls the maintenance of the connective tissue framework of the lungs of the offspring.

Since the response to nicotine exposure was the same for those animals that were exposed to nicotine during all the phases of lung development or only from the onset of the phase of alveolarisation, it is clear that smoking or nicotine replacement therapy by the mother, (neither of which are recommended) should not start before alveolar formation in the lungs of the offspring is completed.

In conclusion, it appears that the changes seen in the lung parenchyma of those animals that were exposed to nicotine during all the phases of lung development or only from the onset of the phase of rapid alveolarisation, was due to the adverse effect of nicotine on the lung connective tissue framework and thus its role in

supporting the lung parenchyma. The exact mechanism of action of nicotine is not yet known. Studies to determine the mechanism(s) of action of nicotine is ongoing.

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CHAPTER 8

Conclusion and Future Perspectives

8.1 Introduction

There are 2 groups of companies that benefit commercially from tobacco and its major alkaloid, nicotine. The first group are the tobacco companies targeting their potential clients in more and more subtle ways to downplay the adverse health consequences of smoking and to expand their market.

Although most people know that smoking is not healthy, many do not realize just how deadly cigarettes are. Each year smoking steals more than 5 million years of potential life from over 400 000 Americans who die from illnesses linked to smoking (Meister, 2006). According to the report, it is helpful to put the statistics in perspective to better understand the impact of cigarette smoking in comparison to that of 6 other major causes of death in the USA: alcohol abuse, drug abuse, AIDS, motor vehicle crashes, homicide, and suicide. All 6 of these causes combined kill only half as many people as cigarettes do.

It is therefore important that governments should strictly enforce anti-tobacco legislation and education on tobacco and realize that the economic, social, health and educational burden that results from smoking tobacco far outweigh the financial gain awarded to them by tobacco companies. Current strategies by the government of South Africa include an intention by the Department of Health

(DOH) to amend the bill against tobacco (DOH, 2006). These include stricter action against users and providers of tobacco products and include the following:

a. Children under the age of 18 years will not be allowed to purchase tobacco and will not be allowed in designated smoking areas.

b. An increase in the penalty for the selling of tobacco to children under the age of 18.

c. Penalties will be imposed on employers for failing to protect their employees from tobacco smoke.

d. Penalties will be imposed on those selling tobacco products in health institutions.

e. Owners of public places will face penalties for not enforcing the no smoking action in smoke free areas and

f. It will also be deemed an offence for any individual to smoke in a non-smoking area.

It is hoped that the amendment to the bill will help to curb the dangers of smoking to some extent and make it more difficult for smokers to employ their smoking practices, but as is the case with most drugs, people will ultimately try to overcome the system to feed their addiction.

In the USA it has recently emerged that over the years the tobacco companies have actually increased the nicotine content of cigarettes (Brown, 2006). It was also shown that the blood nicotine content of smokers using “smokeless” cigarettes is higher than in the blood of those using conventional cigarettes

(Meister, 2006). New advertisements (see addendum) are also used to convince smokers and potential smokers that using cigarettes made of “natural” tobacco is safer. This downplays the health hazards of smoking and ignores the role of nicotine as an addictive agent and its role in having an adverse effect on health, especially of the offspring of smoker. Therefore, taking into consideration the ignorance of people, the difficulty to stop the habit together with misleading advertisement, it is not surprising that smoking of tobacco will not be stopped.

A second group of companies benefit from the knowledge that smoking is a proven health hazard and that cigarette smokers can significantly reduce the development of smoke related diseases by quitting the habit. This is difficult even with smoking cessation programs. Their inability to quit is due to the fact that they are addicted to nicotine. Consequently, many of the smoking cessation programs switched to the use of nicotine replacement therapies. These programs claim that *cigarettes kill, not nicotine*. According to the ACSH report (Meister, 2006), nicotine is not especially dangerous and that it does not cause cancer or emphysema. According to this report there is no evidence that it plays a direct role in the development of heart disease or stroke, although it does have some effects on the circulatory system.

Although there is compelling experimental and clinical evidence that nicotine harms the developing foetus in several ways (Maritz, 1987 and 1988; Chen and Kelly, 2005), this report does not mention findings of any research regarding the effect of maternal nicotine exposure on the health of the offspring in the short or

long term. In my study, like in several other studies (Maritz, 1987 and 1988; Gamieldien and Maritz, 2004; Sekhon et al. 1999), I clearly showed that maternal nicotine exposure even as late as from the onset of the phase of alveolar development, resulted in the late onset of microscopic emphysema in the offspring. These effects are not due to a reduced nutrient supply to the offspring during gestation and/or lactation because the body weights of the offspring were not affected. Growth was also proportional. This implies that nicotine changed the program that directs growth, development and maintenance of lung structural integrity in an irreversible manner.

Insufficient research has been done to validate the use of nicotine replacement therapy in pregnant women. The use of nicotine replacement therapy to assist pregnant women to stop smoking should therefore not be encouraged. This is supported by the lack of evidence that NRT aids smoking cessation in pregnancy. Furthermore, new evidence shows that offering a remedy for a risky behaviour, such as smoking, inadvertently promotes it by suggesting that the risk is manageable (Bolton et al. 2006).

8.2 Future Perspectives

The success rate of only 5% of the various smoking cessation campaigns are not very good, nor are the strategies introduced by government. This clearly shows that new strategies must be developed to prevent the harmful effect of smoking on the health of the smoker. Nicotine is used in various programs to assist smokers to quit the habit, but with very little knowledge regarding its effect on health in

general. More research should be done in order to elucidate the mechanisms of action of nicotine and the other possible substances involved in the aetiology of disease progression from lighting up a cigarette.

Since my project shows that maternal nicotine exposure results in premature ageing of the lungs of the offspring, and renders it more susceptible to disease, it is important to investigate the site and mechanism of action of nicotine. It is therefore sensible to further investigate the effect of maternal nicotine exposure on the lung connective tissue architecture, as well as the microenvironment where the connective tissue framework is formed and maintained. This will assist in determining the site and mechanism of action of nicotine. In addition the effect of products of nicotine metabolism, such as nor-nicotine, and NNK, must be studied. Once the information is available, strategies, such as nutrient supplementation, can be developed. Since most parents don't quit smoking when the mother is pregnant it makes sense to develop a strategy to prevent the harmful effects of maternal nicotine exposure on neonatal development.

This project showed a possible mechanism of action of nicotine in the lungs of the offspring. Follow up studies are required to determine the site and action of nicotine and on strategies to prevent the harmful effects of maternal nicotine exposure during gestation and lactation on the respiratory health of the offspring. In addition to policies to address nicotine intake during pregnancy and lactation and educational programs, strategies can be to develop dietary supplements to prevent the effect of nicotine on the lungs of the offspring.

Due to the fact that institutions such as the ACSH fail to recognize the impact of maternal nicotine exposure on health of the offspring, it is important that the findings of this and other studies must be published in relevant journals and presented at various congresses. It must be brought to the attention of policy-making bodies to address the consequences of maternal nicotine exposure on the respiratory health of the offspring. Education regarding the health hazards of smoking must be extended to the impact of maternal nicotine exposure on the health of the offspring. The availability of NRT must also be under strict control to prevent the harmful effects of nicotine on the developing foetus.

In summary, it is clear that the nicotine intake during pregnancy and lactation is having an adverse effect on lung development in the offspring. It is also clear that a site of action is the connective tissue framework of the developing lung. Although copper replacement showed some positive results, it is not preventing the premature ageing of the lungs of the offspring. It is also evident that NRT programs to aid individuals to stop smoking are not advisable because of the negative effect of nicotine on the development of the foetus.

In conclusion, it is essential to investigate the effect of nicotine exposure during gestation and lactation on lung development in depth to understand its mechanism of action and to develop strategies to counter its effects. Apart from the scientific component, strategies to prevent the effect of nicotine and by implication many of the effects of smoking, a multidisciplinary approach will be necessary to develop strategies. These will include the involvement of health and education officials.

8.3 References

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8.4 Addendum

(Sante Fe Natural Tobacco Company, 2006)