The Effect of Maternal Nicotine, Vitamin C and Nicotine + Vitamin C during gestation and lactation on neonatal lung growth and development

By

Samuel Siyabonga Rayise

Thesis presented in fulfilment of the requirements for the degree of Magister Scientiae at the Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape.

Supervisor: Professor G.S. Maritz
ABSTRACT

Maternal smoking is known to cause serious health risks to the unborn child. Recent studies implicate nicotine as the causative factor. Maternal nicotine exposure during pregnancy and lactation interferes with foetal and neonatal lung growth and development, rendering the lung more susceptible to damage and diseases. Therefore, the aim of this study was to investigate: 1) the effect of maternal exposure to nicotine (1mg/kg BW/day) during all phases of lung development: 2) and vitamin C supplementation (0.5mg/kg BW/day) to prevent the adverse effects of maternal nicotine exposure on lung development in the offspring. This is based on studies in our laboratories which suggested that nicotine reduces the blood and tissue vitamin C content of the mother, thereby rendering the neonate more susceptible to oxidation damage. The chief motivation of this study was to establish whether an anti-oxidant, such as vitamin C, can be administered to smoking pregnant and lactating mothers in order to combat the deleterious effects of nicotine on the lung development of their offspring. 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, compromising the ability of the lung to act as an organ of gaseous exchange. There was a decrease in the surface area available for gas exchange. The change occurred after the lung reached maturation and resembled microscopic emphysema. Vitamin C supplementation was unable to fully protect the neonatal lung against the adverse effects of maternal nicotine exposure; it however partially protected the neonatal lung against structural deterioration. Supplementation with vitamin C definitely offers possibilities as
a prophylactic to combat the detrimental influence of maternal nicotine-exposure on foetal and postnatal lung development.
DECLARATION

I declare that “An investigation into The Effect of Maternal Nicotine, Vitamin C and Nicotine + Vitamin C during gestation and lactation on neonatal lung growth and 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.

Samuel Siyabonga Rayise

Signed:
DEDICATION

This thesis is dedicated to my Father and Mother for their encouragement, support and making my dreams a reality. Big thanks especially to my supervisor, Prof. G.S. Maritz, for mentoring me. Most importantly, I am grateful to our Dear Lord for being my anchor through all of my studies.
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<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>one way analysis of variance</td>
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<tr>
<td>AP</td>
<td>activator protein</td>
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<tr>
<td>AK</td>
<td>adenylate kinase</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>BLMVECs</td>
<td>bovine lung microvascular endothelial cells</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>copper</td>
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<tr>
<td>DNA</td>
<td>2'-deoxy-5'-ribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>DPX mountant for histology</td>
</tr>
<tr>
<td>DHA</td>
<td>dehydroascorbate</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FAS</td>
<td>apoptosis stimulating fragment</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>G&amp;L</td>
<td>gestation and lactation</td>
</tr>
<tr>
<td>GCS</td>
<td>glutamyl cysteine synthetase</td>
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<tr>
<td>γ-GCS</td>
<td>γ-glutamyl cysteine synthetase</td>
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GSH  glutathione
H &E  haematoxylin and eosin
$H_2O_2$  hydrogen peroxide
HDF  human diploid fibroblasts
IL  interleukin
kDa  kilodalton
kg  kilogram
L  length of traverses
LPO  lipid peroxidation
LOX  lysyl oxidase
LDL  low – density lipoproteins
Lm  mean linear intercept
Lv  lung volume
m RNA  messenger ribonucleic acid
m  metre
M  molar concentration
MAGP  microfibril-associated glycoprotein
MAP  mitogen activating protein
mg  milligram
ml  millilitre
mm  millimetre
MMP  macrophage metalloprotein
N  number of fields
Na                   number of alveoli per unit area
nAChR            nicotinic acetylcholine receptor
NNK               4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRT                 nicotine replacement therapy
PMN                polymorphonuclear
PDGF               platelet derived growth factor
PFK                 phosphofructokinase
pH                 measure of the acidity of a solution
PThrP                parathyroid hormone related protein
Raf                Raf protein kinase
ROS                 reactive oxygen species
Sa                   internal surface area
SP                  surfactant protein
SDA                semidehydroascorbate
TBARS            thiobabituric acid reactive species
TGF-β           transforming growth factor beta
TNF                tumor necrosis factor
Va                 volume density of airspaces
Valv              volume proportion of alveoli (mean alveolar volume)
VEGF              vascular endothelial growth factor
Vt                  volume density of parenchymal tissue
WBC                white blood cells
α                  alpha
β  beta
γ  gamma
µ  micro
%  percent
\dot{\text{O}_2}^-  oxygen free radical
° C  degrees Celsius
5-HT  5-hydroxytryptamine
nAChR  nicotinic acetylcholine receptor
CHAPTER 1

Review of Literature

1.1. Introduction

Reports linking tobacco smoking with impaired health are numerous. Studies showed that cigarette smokers have the greatest risk of developing cancer of the oral cavity and throat. A number of respiratory diseases including emphysema and chronic bronchitis have been linked to the smoking habit (Huber, 1989). An alarming number of pregnant women in South Africa smoke despite being aware of the harmful consequences of smoking (Guthrie et al, 2001). 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 putting their unborn and young children at risk to a wide range of respiratory and other health problems. It is plausible that the action of cigarette smoke on the surfactant-system contribute to the increase incidence of respiratory diseases (fig. 1.1), including premature death.

Tobacco smoking during pregnancy is associated with adverse outcomes on the health of the foetus and infant. These include reduced birth weight and decreased gestational age (Davies and Albertiny, 1976). Colley et al (1973) link respiratory tract impairment in childhood to that in early adult life. Their study shows that childhood chest disease has a
more profound effect on the increased frequency of respiratory disability in early adult life than social class or air pollution.

Fig.1.1. Examples of some potential sites where smoke exposure may alter surfactant metabolism.
1: Particulates may have a direct detergent-like effect on the surface monolayer. 2: Lipid peroxidation induced by smoke exposure. 3: Transport of surfactant-specific phospholipids and altered secretion of lamellar bodies. 4 and 5: Reduced levels of SP-A and SP-D may affect hypophase processing directly or indirectly or alter SP-D regulation of DPPC synthesis. 6: Nicotine may alter receptor expression in foetal lung (Scott, 2004).
1.2. Nicotine

Nicotine has been implicated as the causative agent in some tobacco-related lung diseases. It is associated with alterations in tissue remodelling and an increased matrix deposition. Nicotine is able to cross the biological membranes including the blood brain barrier. The rate of nicotine absorption through biological membranes thus increases as the pH of the aqueous solution increases, whereas nicotine absorption decreases as the pH decreases. At an alveolar pH of 7.4, nicotine is able to pass easily across the cell membrane onto the circulation (Yildiz, 2004). Absorption through the alveolar wall is also dependent on the nicotine concentration in the smoke. It has been shown that the plasma nicotine levels in non-inhaling smokers is around 2.5-8.0 ng/ml, whereas the plasma nicotine levels in inhaling smokers reach 30-40 ng/ml nicotine (Yildiz, 2004). These observations demonstrated that absorption of nicotine through the buccal mucosa is poor and absorption through the lung is rapid.

About 70% of circulating nicotine is metabolized to cotinine, and it involves hydroxylation of nicotine by microsomal enzymes and conversion to the corresponding aldehyde. Pathways of nicotine metabolism can be divided into phase I and II metabolism of nicotine. The phase I metabolism involves the microsomal oxidation of nicotine and falls into four groups (figure 1.2, 1.3, 1.4) (Yildiz, 2004).
1. C – oxidation
2. Formation of nornicotine
3. N – oxidation
4. N – methylation

The phase II metabolism involves N – and – O glucuronidation of nicotine.

Apart from many oxidants in cigarette smoke (Scott, 2004), nicotine has been known to result in oxidative stress by inducing the generation of reactive oxygen species (ROS) in tissues. These ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation (LPO) (fig. 1.1). Lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharides, as well as protein cross-linking and fragmentation (MacNee, 2005). The mechanisms of ROS generation by nicotine are not clear. However, it has been reported that nicotine is chemotactic for polymorphonuclear (PMN) leucocytes and enhances the responsiveness of PMN leucocytes to activated complement C5a, thus generating ROS. Further, nicotine disrupts the mitochondrial respiratory chain leading to the increased generation of superoxide anions and hydrogen peroxide. Oxidative stress produces not only direct injurious effects in the lungs but also activates molecular mechanisms that initiate lung inflammation (MacNee, 2005).
Fig. 1.2. C-oxidation of nicotine (Yildiz, 2004).

Fig. 1.3. C-oxidation and methylation of nicotine (Yildiz, 2004).
Fig. 1.4. N- and O-glucuronidation of nicotine (Yildiz, 2004).
For nicotine to directly affect lung development, nicotinic cholinergic receptors must be present in foetal lungs. Nicotine has been found to up-regulate the alpha-7-nAChR expression and chronic nicotine exposure permanently inhibits the function of many of these nAChR. The alpha-7-receptors are very sensitive to inactivation by nicotine. Thus, prenatal nicotine exposure most likely leads to increased levels of inactivated α7-receptors in the developing lung (Sekhon et al., 1999). This would alter normal cholinergic transmission both by blocking the normal functioning of receptors and by allowing unopposed signalling by other nicotinic receptor subtypes that are more resistant to inactivation by chronic nicotine. It is possible that nicotine directly stimulate alpha-7-nAChR-bearing fibroblasts to lay down an excessive amount of connective tissue (Sekhon et al., 1999).

1.3. Lung Development

1.3.1. 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 saccular stages (Farrell, 1982).

1.3.1.1. Embryonic Phase

Human lung develops from the laryngo-tracheal groove and start to separate the lung bud from the prospective oesophagus (Farrel, 1982). The left and right branches, which give
rise to the left and right lungs, are also developed of day 13 of gestation in rats (Rothschild *et al*, 1996).

### 1.3.1.2. Pseudoglandular Phase

This phase, when the pre acinar branching pattern of airways and blood vessels is established, occurs at week 17 of gestation. 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).

### 1.3.1.3. Canalicular Phase

This phase takes place at approximately days 19 to 20 of gestation in rats (Farrel, 1982). 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. The production of surfactant follows the differentiation of type-1 and type-2 alveolar cells.

### 1.3.1.4. Saccular Phase

Saccular phase in rats begins on day 21 of gestation (Thurlbeck, 1978). During this period of lung development, differentiation of the alveolar epithelium and the production of surfactant continue. The potential airspaces are filled with foetal pulmonary fluid, which is expelled at the onset of labour and during vaginal delivery.
1.3.2. Postnatal Lung Development

Postnatal development of the rat lung occurs in three phases, referred to as the phases of lung expansion, tissue proliferation and equilibrated growth, respectively (Burri et al., 1974).

1.3.2.1 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 ten days of life and thereafter is related to lung volume (Burri et al., 1974).

1.3.2.2. 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. There is formation of the secondary septa 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, 1983).
1.3.2.3. Equilibration Phase

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, 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).

Each of these phases contribute to the development of the lung into a structural and metabolically mature and fully function organ that will meet the oxygen demands of the body. It is therefore, conceivable that interference with the “program” that control lung growth and development, may also have an adverse impact on lung growth and development and thus on lung structural, integrity and function as well as respiratory health. Various studies have indeed shown that maternal smoking result in a higher incidence of respiratory diseases in the offspring as well as negatively impacting on lung function (Davies and Albertiny, 1976; Bardy et al., 1993). Nicotine, an important component of tobacco smoke (Benowitz and Jacob, 1984) is considered to be the causative factor in some of the adverse effects of nicotine attributed to nicotine (Heusch and Maneckjee, 1998).
1.4. Nicotine and Lung Development

Many studies have also implicated nicotine in human lung cancer. Nicotine is able to activate the kinase ERK2, which results in increased expression of the Bcl-2 oncoprotein and suppression of apoptosis (Roman, 2004). Glucose is an essential source of energy in lung tissue and is necessary for the functional development of the lung (Bourbon and Jost, 1982). Maternal nicotine exposure during gestation and lactation results in sustained suppression of glycogenolysis and glycolysis in lung tissue of the rat fetus and neonate (Maritz, 1987). The lower glycogenolytic activity is due to a lower phosphorylase activity in the lungs of the nicotine exposed offspring (Maritz, 1986). The decrease in the glycolytic activity, and thus the flux of glucose through this pathway can be attributed to an inhibition of phosphofructokinase (Kordom et al, 2002). Both the persistent reduced glycolytic activity and high levels of cAMP are associated with premature onset of cell senescence (Kondoh et al, 2007). It is, therefore, plausible that the exposure of the offspring to nicotine during gestation and lactation and thus during all the phases of lung development, may induce premature senescence of lung cells of the offspring (Maritz, 2008).

Among the matrix molecules found to be highly expressed in tobacco-related lung disease is fibronectin. Cultured fibroblasts revealed that nicotine induces fibronectin gene transcription by acting on alpha-7-nAChRs, by eliciting an increase in intracellular cAMP, and by inducing the phosphorylation and nuclear translocation of CREB, a key transcription factor capable of initiating fibronectin gene expression (Roman et al, 2004). It’s also possible that, nicotine can induce the exaggerated deposition of fibronectin in the
lungs of animals exposed to tobacco. Newly deposited fibronectin, containing matrices are not inert, instead they affect cellular processes capable of modulating lung inflammatory and tissue repair responses. Fibronectin modulate many cellular functions such as cell adhesion, proliferation and apoptosis (Roman et al, 2004).

The effect of nicotine on neonatal lung structure is reflected in a reduced radial alveolar count. This reduction in the radial alveolar count of lungs of nicotine-exposed rat pups is an indication of failure of alveolar development within the lung acini. It is indeed illustrated that maternal nicotine exposure during pregnancy and lactation interferes with elastogenesis in neonatal rat lung (Maritz and van Wyk, 1997). Since elastogenesis is essential for alveolar formation (Looshi and Potter, 1959), it is conceivable that nicotine will have an adverse effect on the connective tissue framework of the lung and thus lung function. Furthermore, maternal nicotine exposure also causes damage to the supporting connective tissue skeleton of neonatal rat lung (Dolley, 1995).

The mechanical properties of the lung are largely determined by the connective tissue networks laid down during development. The macromolecules most important for lung mechanics and structural integrity are collagen, elastin, and proteoglycans. Elastin is also an important architectural component that influences lung development, predominantly during the alveolar stage. The effects of oxidants on elastic fiber homeostasis are most likely manifested through their ability to modulate the activity of proteinases, proteinase inhibitors, cross-linking enzymes, as opposed to a direct effect on elastin. Interference with the balance between protein synthesis and breakdown will cause damage to the
connective tissue framework and overtime result in emphysema (Maritz and van Wyk, 2007).

Recent identification of nicotinic acetylcholine receptors (nAChR) in pulmonary fibroblasts suggests that in utero nicotine exposure may alter collagen expression. Because the alpha-7-nAChR receptor is primarily a ligand-gated calcium channel, activation of the alpha-7-receptor leads to increased intracellular calcium and the inhibition of the alpha-7-receptor leads to decreased intracellular calcium. Linking intracellular calcium to collagen gene expression, it has been shown that increased intracellular calcium decreases collagen gene biosynthesis in human fibroblasts (Sekhon et al, 1999). This supports the hypothesis that prenatal nicotine exposure leads to inactivated alpha-7-receptors, decreased fibroblast intracellular calcium, and therefore increased collagen. It is important to point out that there are also likely to be effects on collagen degradation. Therefore, it is likely that the increased collagen levels in the nicotine-exposed fetal lungs results from both increased collagen synthesis and from decreased degradation (Sekhon et al, 1999).

About 90% of collagen found in the lung is collagen type I and II. It is suggested that whereas collagen I predominantly plays a key role in providing tensile strength, collagen II mainly provides compliance. So if prenatal nicotine induces alterations in collagen type I and III, the will be changes in lung function (Lang et al, 1994).
Normal lung development and injury/repair utilize common mesenchymo–epithelial signalling pathways to maintain homeostasis. Epithelially–derived parathyroid hormone related protein (PTHrP) induces the differentiation of mesodermal alveolar interstitial fibroblast to lipid, containing interstitial lipofibroblast (LIF) via a PTHrP receptor–mediated, cAMP–dependent PKA pathway. The lipid–containing LIFs produce factors that induce the growth and differentiation of the adjoining type II cells, culminating in alveolar homeostasis. Factors that disrupt this cellular homeostatic mechanism by causing the transdifferentiation of lipofibroblast (LIFs) to myofibroblast (MYFs) lead to abnormal lung development and function. Nicotine induces pulmonary alveolar lipofibroblast–to–myofibroblast transdifferentiation. Alveolar interstitial lipo–to–myofibroblast transdifferentiation results in fail of alveolarization in the developing lung, which leads to an arrest in pulmonary growth and development. In the developing lung, myofibroblast are fewer in number and are predominantly located at the periphery of the alveolar septa where they very likely participate in the formation of new septa. However, in chronic lung diseases, myofibroblast not only increase in number but also are located in the center of alveolar septum in great abundance (Kehan et al, 2005).

1.5. Vitamin C (Ascorbic Acid)

Vitamin C is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species (fig.1.5). Humans do not synthesize vitamin C because they do not have the enzyme gulonolactone oxidase, which is essential for synthesis of the ascorbic acid. Vitamin C is an electron donor and therefore a reducing agent, it donates two electrons from a double bond between the second and third carbons of the 6-carbon
molecule. The reason why vitamin C is called an antioxidant is because by donating its electrons, it prevents other compounds from being oxidized. However, by the very nature of this reaction, vitamin C itself is oxidized in the process (Padayatty et al, 2003). The species formed after the loss of one electron is a free radical semidehydroascorbic acid or ascorbyl radical. Ascorbyl radical is relatively stable with a half-life of $10^{-5}$ seconds and is fairly unreactive (Padayatty et al, 2003). So it is possible that harmful free radicals can interact with ascorbate and the reactive free radical is reduced and the ascorbyl radical is formed in its place. Upon the loss of a second electron in ascorbyl radical, the compound formed is dehydroascorbic acid. Once formed, ascorbyl radical and dehydroascorbic acid can be reduced back to ascorbic acid by at least three enzyme pathways, as well as by reducing compound in biological systems such as glutathione. In humans, there is only partial reduction back to ascorbic acid, therefore all the ascorbic acid that is oxidized is not recovered (fig. 1.6). If dehydroascorbic acid is not reduced back to ascorbic acid, it is hydrolyzed irreversibly to 2, 3-diketogulonic acid. This compound is formed by irreversible rupture of the lactone ring structure that is a part of ascorbic acid, ascorbyl radical and dehydroascorbic acid. 2, 3–diketogulonic acid is further metabolized into xylose, xylonite and oxalate. The formation of oxalate has clinical significance because hyperoxaluria (over excretion of oxalate) can result in oxalate kidney stones in some people (Padayatty et al, 2003).
Fig.1.5. Vitamin C synthesis pathway and pentose pathway in animals. The reactions are catalyzed by the following enzymes: 1, UDP-glucose pyrophosphorylase; 2, UDP-glucose dehydrogenase; 3, nucleotide pyrophosphatase; 4, UDP-glucuronosyltransferase; 5, UDP-gluconuridase; 6, phosphatase; 7, β-gluconuridase; 8, glucuronate reductase; 9, gulonolactonase; 10, L-gulonolactone oxidase; 11, L-gulonate 3-dehydrogenase; 12, decarboxylase; 13, L-xylulose reductase; 14, xylitol dehydrogenase; 15, D-xylulokinase. Three possible mechanisms for glucuronate formation (a, b and c) are shown (Linster and Van Schaftingen, 2007).
Vitamin C acts as an electron donor for eight different enzymes. Three participate in collagen hydroxylation; these reactions add hydroxyl groups to the amino acid praline or
lysine in the collagen molecule. Two other vitamin C dependent enzymes are necessary for synthesis of carnitine, where carnitine is essential for the transport of fatty acids into mitochondria for ATP generation. Other three vitamin C dependent enzymes have the following function, one participate in the biosynthesis of nor epinephrine from dopamine, one adds amides groups to peptide hormones, greatly increasing their stability, one modulates tyrosine metabolism (figure 1.6) (Linster and Van Schaftingen, 2007).

The extracellular lining of the alveolus, which comes in close contact with cigarette smoke, is enriched with ascorbic acid. It is estimated that the ascorbate concentration of alveolar lining fluid of rat is 6.3 mmol/L. This considerably higher than the 0.1 mmol/L ascorbate concentration in normal rat serum. Ascorbic acid is more abundant in the fluid lining the alveolar than its oxidized metabolites, dehydroascorbate (McGowan et al., 1984). This implies that ascorbic acid acts as a first line of defence against inhaled oxidants. It furthermore means that a lowering in the ascorbic acid content in this fluid lining, such as during inhalation of cigarette smoke, will reduce the capacity of the lungs to protect itself (Bui et al, 1992).

1.5.1. Ascorbic Acid and Collagen formation

Proline monooxygenase is an enzyme that plays a role in the maturation of collagen. It catalyzes the conversion of specific proline residues to hydroxyproline. The enzyme does not hydroxylate free proline, though it can act on small peptides containing proline (McGowan et al, 1984). Molecular oxygen is the source of the oxygen atom in the hydroxyl group of hydroxyproline. In common with many other oxygen-using enzymes,
proline monooxygenase contains an iron atom. Hence, the enzyme is an iron metalloenzyme. The iron must be in the reduced (ferrous iron, Fe$^{2+}$), rather than in the oxidized state (ferric iron, Fe$^{3+}$) to support catalytic activity. A general property of ferrous iron is that it is not particularly stable. It can spontaneously oxidize to the ferric state. Ascorbate plays a vital role in maintaining the enzyme’s iron in the reduced state. The conversion of ferrous to ferric iron in the enzyme is not coupled to each event of hydroxylation, in other words, the conversion is not an obligatory component of the hydroxylation reaction. The oxidation of iron appears to occur after every ten to twenty hydroxylation events. Then the iron must be re-reduced for enzyme catalysis to continue. Ascorbic acid reduces the iron again and, in turn, is converted to semidehydroascorbate (Brody, 1994).

Vitamin C deficiency results in impairment in the hydroxylation of collagen. Properly hydroxylated collagen molecules self-associate to form a triple-helix structure within the cell. Collagen that is not hydroxylated and does not form the triple helix is not readily secreted from the cell and its secretion is impaired (McGowan et al, 1984).

1.5.2. Vitamin C as an Antioxidant in human biology

The species which receive electrons and are reduced by vitamin C can be divided into several classes:

1. Compounds with unpaired electrons (radicals) such as oxygen related radicals (superoxide, hydroxyl radical and peroxyl radicals).
2. Compounds that are reactive but are not radicals, including hypochlorous acid, nitrous acid related compounds.

3. Compounds that are formed by reactions with either of the first two classes and then react with vitamin C, e.g. alpha tocopheroxyl radicals.

4. Transition metal-mediated reactions involving iron and copper for example reduction especially of iron by ascorbate can lead to formation of other radicals (Padayatty et al, 2003).

Oxidants can react with three general classes of biomolecules.

1. Lipids.
2. Proteins.
3. DNA.

Lipids, membrane lipids and lipids in circulating lipoproteins such as low density lipoproteins (LDL) can interact with reactive oxygen species resulting in lipid peroxidation. Once lipid peroxidation form, they can react with oxygen to form highly reactive peroxyl radicals, and continued formation of lipid hydroperoxidation can result, a process termed radical propagation. Ascorbate can reduce the initiating reactive oxygen species so that initial or continued lipid peroxidation is inhibited (Brody, 1994).

Proteins also undergo oxidation by several mechanisms, a peptide chain can be cleaved by oxidants, or specific amino acids can be oxidized. The two amino acids most prone to oxidative attack are probably cysteine and methionine. Oxidation processes can affect DNA indirectly through protein oxidation and lipid oxidation or directly by oxidation of DNA. Guanine is the DNA base most susceptible to oxidative attack. DNA can also be
damaged by reactive nitrogen species, derived from nitrosamines e.g. nitric oxide radicals. Once nitrosamines give rise to reactive nitrogen species, prevention of mutagenic activity by ascorbate is less effective in prevention of DNA damage (Padayatty et al., 2003). Damage to DNA may result in changes in the “program” that control lung growth, development and aging, and induce fetal onset of adult respiratory diseases (Maritz, 2008).

1.5.3. Vitamin C concentration in humans as a functional dose

At plasma vitamin C concentration less than 4 µM, symptoms of scurvy may occur. Oral doses of 30 mg daily yield a steady-state plasma concentration of approximately 7 µM for men and 12 µM for women (Padayatty et al., 2003). Tight control of vitamin C concentration is mediated by tissue transport, absorption and excretion. The higher the dose, the less absorption occurs. Vitamin C reabsorption and excretion by the kidney play a key part in tight control of plasma and tissue vitamin C concentrations in healthy humans, and this control is lost in patients with end stage renal disease. In the kidney, vitamin C is filtered through the glomerulus’s and reabsorbed in the proximal tubule by vitamin C transporters SVCT1. When the transporters absorption mechanism approaches maximal velocity, additional vitamin C cannot be absorbed and is lost in urine. Its also important to note that ascorbate entry into mammalian cells is energy-dependent, being effected by two distinct Na\(^+\) dependent co-transporters, SVCT1 and SVCT2, which show distinct distributions. But dehydroascorbate (DHA) is transported by glucose transporters, particularly GLUT1, GLUT3 and GLUT4, and is therefore not energetically driven (Linster and Van Schaftingen, 2007).
Glutathione and vitamin C are thought to be the most abundant reducing agents in cells. Furthermore, glutathione is implicated in vitamin C recycling from DHA. It would make therefore sense for glutathione to exert a control on vitamin C synthesis. Several experiments performed with glutathione-depletion agents indicate that glutathione depletion favours vitamin C synthesis. Administration to adult mice of buthionine sulfoximine, an inhibitor of glutathione synthesis, led to a two-fold increase in the amount of vitamin C in the liver within 4 hours.

Vitamin C, in addition to its function as an antioxidant, acts as a prooxidant and generates ascorbate free radical and reactive oxygen species in biological system in the presence of transition metal ions (Fe$^{2+}$). The endothelium, a semipermeable barrier that plays a pivotal role in the maintenance of vascular integrity and function, is a prime target for the high levels of circulating prooxidants vitamin C (Kalpana et al, 2007), presumably leading to altered integrity of the endothelium (fig. 1.7). It has been reported that vitamin C, upon infusion into blood at a dose of 10mg/min for 120 minutes, improved the impairment of endothelial function in smokers.

Endothelial dysfunction is well established as one of the primary events in the pathogenesis of atherosclerosis. Monocyte-endothelial interactions have been implicated in the initiation of endothelial dysfunction seen in many chronic cigarette smoking. (Fennessy, 2003)
Fig.1.7. Scheme of the proposed mechanism of vitamin C-induced PLD activation through MAPK regulation in the lung microvascular ECs. (Varadharaj et al, 2005).

Endothelin-1 (ET-1), an endothelial–derived peptide, is a potent vasoconstrictor and growth promoter that has a short plasma half-life, but a prolonged onset and duration of action. Impaired-flow-mediated dilatation (FMD) has been demonstrated by ultrasonic insonation in the brachial arteries of young otherwise healthy cigarette smokers. In FMD of the brachial artery, endothelial NO synthase (eNOS) is activated by the forces of increased sheer stress and stretch with the resultant release of NO, which induces relaxation of smooth muscle cells in the vessel wall. Vitamin C improves, but does not restore the normal dilatory response of the brachial artery to increase flow (Fennessy et al, 2003).
At pharmacological doses, vitamin C exposed to bovine lung microvascular endothelial cells (BLMVECs) shows formation of intracellular ascorbate free radicals ROS is dose and time dependent, leading in to alterations and loss of redox-dependent cell viability (Varadharaj et al., 2005). Exposure of BLMVECs to vitamin C increased the formation of extracellular and intracellular H$_2$O$_2$ in a dose dependent manner. Vitamin C, at pharmacological doses, induced activation of PLD in BLMVECs through oxidative stress and upstream activation of p38 MAPK and ERK1/ERK2. At a concentration of 5.7 and 10 mM for 120 minutes, the BLMVECs exhibited marked alterations in their cell morphology with considerable gaps between cells and formation of filopodia and lamellipodia. Vitamin C was able to cause a significant depletion of GSH (50% depletion) even at 1 mM concentration. At 3.5 and 10 mM concentration of vitamin C, further depletion of intracellular GSH to 64%, 72% and 80% in BLMVECs at 120 minutes, it is clear that vitamin C at pharmacological doses induces oxidative stress and loss of redox-dependent cell viability in BLMVECs (Varadharaj et al., 2005).

1.6. Lung Maintenance

Homeostatic alveolar maintenance requires a proper extracellular matrix maintenance, which means that inhibition of extracellular matrix deposition may lead to experimental emphysema. Furthermore, abnormalities of elastin fiber deposition lead to developmental lung arrest and an overall simplification of alveolar structure with airspace enlargement (Tuder et al., 2006). It has been proposed that alveolar destruction in emphysema involves
feedback interactive loops among apoptosis, oxidative stress, and excessive lung extracellular matrix proteolysis (fig. 1.8), (Tudor et al, 2006).

Fig. 1.8. Conceptual framework of positive interaction among apoptosis, protease/antiprotease imbalance, and oxidative stress in emphysema (Tuder, 2006).

In alpha-1-antitrypsin (A1AT)–deficient patients, emphysema occurs in a more global pattern, affecting all lobes, and involving microscopically most of the primary lobule that is, both the centrilobular and the peripheral components of the alveolar units connected to the respiratory bronchiole. The nature of the widespread alveolar destruction in alpha-1-antitrypsin (A1AT)-deficient patients remains unclear. With the increasing understanding that emphysematous lung destruction involves excessive proteolysis, apoptosis, and
oxidative stress, the association of A1AT deficiency with panlobular destruction may be explained if all three elements of this pathologic triad synergistically and simultaneously operate in the lung destruction in A1AT-deficient patients (fig. 1.9) (Tuder, 2006).

Fig.1.9. Pathogenesis of alphas-1-antitrypsin (A1AT) deficiency. A1AT deficiency (ZZ variant) may lead to a more global alveolar destruction since it causes (1) oxidative stress via its proinflammatory properties when polymerized, (2) loss of the antiprotease shield, and (3) loss of anti–active caspase-3 activity (Tuder, 2006).

Carbohydrates play an important role in the structural and functional development and maturation of foetal and neonatal lung tissue. The whole process of lung development
(cell growth and formation of connective tissue framework) depends on ATP energy derived from carbohydrate metabolism (Maritz and Najaar, 1994).

The environment imposes continuous biological selection, with consequences related to aging, as macromolecular damage accumulates and progressively overwhelms organ defences. There are molecular sensors that respond acutely to environmental stresses, such as those imposed by hypoxia or nutrient deprivation. RTP-801 or REDD1 (Regulated in development and DNA damage responses) a repressor of the mammalian target of rapamycin (mTOR), was initially discovered as a hypoxia-inducible factor (HIF)-1-α-inducible protein, whose expression is modulated by oxidative stress and capable of inducing apoptosis when over expressed in lungs of mice (Tuder et al, 2006). It has been recently observed that mice exposed to cigarette smoke show acute lung up-regulation of RTP-801. Interestingly, TSC-2 down-regulates VEGF both dependently and independently of its inhibitory effects on mTOR. The inhibition of mTOR decreases S6-kinase-phosphorylation, causing its inactivation, and stabilizes 4eBP1 (an inhibitor of the ribosomal machinery), both leading to decreased protein translation (fig. 1.10), (Shoshani et al, 2002).
Fig. 1.10. RTP-801 and growth or inhibitory cell signaling in cells exposed to nutrients or environmental stresses, respectively. RTP-801 activates tubersclerosis complex hamartin and tuberin proteins (TSC1 and TSC2, respectively), which via inhibition of Rheb kinase lead to mTOR inhibition and suppression of protein translation. mTOR is usually activated under conditions favoring cell growth during abundance of nutrients or due to insulin signaling. The activation of RTP-801 is targeted for cell protection during stress; however, given the nature of the environmental stress, overexpression of RTP801 may cause apoptosis, oxidative stress, or excessive inflammation. AKT = protein kinase B; P-AKT = phosphorylated AKT; PI3K = phosphotidylinositol 3-kinase; RHEB-GTP = RHEB-GTPase (Shoshani et al, 2002).
Fibroblast play an important role in the maintenance of alveolar structure as a change in fibroblast phenotype could be involved in the pathogenesis of emphysema. Lung fibroblasts from patients with emphysema show a reduced proliferation rate and altered growth factor response. Senescent cells (e.g. fibroblast) not only loose their ability to divide and respond to mitogenic stimuli but also display alterations in morphology and metabolic profile. As fibroblasts provide part of the lungs structural support and matrix that is essential for its integrity, so a senescent phenotype could affect tissue microbalance and structural maintenance of the lung (Muller, 2006).

1.7. Cellular and Lung Senescence

It is found that cigarette smoke is able to stop the proliferation of lung fibroblast and up-regulate two pathways linked to cell senescence, namely a biological process associated with cell longevity and an inability to replicate (Tsuji et al, 2006). Cellular senescence is defined as complete and irreversible loss of replicative capacity accruing in primary somatic cells (fig. 1.11). Major characteristics of a senescent cell are the following:

1. A distinct flat and enlarged cell morphology.
2. Resistance to apoptosis.
3. Altered production of inflammatory and growth mediators.
4. An increase in senescence associated \( \beta \)-galactosidase activity

(Nyunoya et al, 2006).
One major anatomical change found in the aging lung is smaller airways size, principally due to alterations in the supporting connective tissue. Other anatomical changes associated with aging include an increase in the diameter of alveolar sacs. These morphologic changes are considered to be due to changes in the relative proportion of decreased elastic tissue and increased collagen that occur with aging (fig. 1.12) (Chan and Welsh, 1998).

Fig.1.11. Simple schematic comparing (a) the cell senescence hypothesis of ageing (right hand flow) with (b) the dysdifferentiation hypothesis of ageing (left hand flow). The cell senescence hypothesis postulates that in the normal course of life there is cell loss. That loss is balanced by cell division which is actively monitored. One or more “replicometers” act to trigger permanent cell cycle exit (senescence) in individual cells. Cell cycle exit is associated with a broad alteration
in gene expression leading to an altered phenotype that affects the microenvironment in which the cell resides and ultimately the entire tissue. In the dysdifferentiation model chronic oxidative stress leads to a regearing of gene expression generating an altered cellular phenotype which contributes to tissue ageing. The two models have many essential similarities (Bird et al, 2003).

Fig. 1.12. Schematic representation of lung volume changes associated with aging. Note that with senescence, there is a decrease in the inspiratory reserve volume (IRV9), the expiratory reserve volume (ERV9), and the vital capacity (VC9). There is a corresponding increase in residual volume (RV9) and functional residual capacity (FRC9) such that the TLC remains about the same TV 5 tidal volume (Chan and Welsh, 1998).
Major physiological changes that occur in the aging respiratory system may reflect several mechanistic/structural changes including the following:

1. Diminished lung elasticity.
2. Increased stiffness of the chest wall.

The aging alveolus actually becomes shallower, containing less air. On average, aged alveoli are surrounded by larger pores, which also contribute to the diminished surface area. The is a generalized reduction in the number and thickness of elastic fibers with advancing age, particularly in the alveolar ducts, consistent with a loss of elastin (Nyunoya et al., 2006).

The disappearance of lung tissue during the development of emphysema may involve the progressive loss of capillary endothelial and alveolar epithelial cell through the process of programmed cell death (apoptosis). One known survival factor for endothelial cells is VEGF (vascular endothelial growth factor), initially characterized as a factor that increases endothelial permeability and induces endothelial cell growth. Withdrawal of VEGF leads to endothelial cell apoptosis in vitro and in vivo. It is postulated that VEGF signalling may be required for the maintenance of adult lung alveolar structures (Kasahara and Tuder et al., 2000).
CHAPTER 2

Statement of problem

The aim of the current investigation was to study the effect of maternal nicotine exposure and supplementation with vitamin C on the development of the airways of the rat lung throughout the entire period of gestation 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.

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

1. The phase(s) of lung development in which nicotine induces changes in lung structure and the possible consequences thereof to lung function.

2. The long-term consequences of maternal nicotine exposure on lung development of the offspring.

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

In this study, the offspring were exposed to nicotine, vitamin C and a combination of nicotine and vitamin C via the placenta or mother’s milk. Since nicotine has oxidant properties it might interfere with lung development (Sekhon et al, 1999). It was
previously shown that maternal nicotine exposure reduces the vitamin C content of the lungs of the offspring (Maritz and van Wyk 1997) and thus the anti-oxidant capacity of the nicotine exposed lungs. Therefore, vitamin C was given to the pregnant mothers to improve the anti-oxidant capacity of the nicotine exposed lungs and therefore, its ability to protect itself against the oxidant effect of nicotine. In this study morphometric and morphologic methods will be used to study the impact of maternal exposure to nicotine only, or vitamin C only, or a combination of nicotine and vitamin C on lung development, and maintenance of lung integrity of the offspring in the long term. The morphometric methods involve quantification of parameters of lung growth and maintenance such as alveolar numbers ($N_a$), internal surface area (ISA), lung volume ($L_v$), alveolar volume ($V_{alv}$) and others. For histological evaluation lung tissue samples of the lungs of the offspring was stained with haematoxylin and eosin for assessing the status of the lung parenchyma and to visualize changes such as microscopic emphysema. Staining for connective tissue was done to visualize the connective tissue for assessment of integrity. This may help to explain the site of action of nicotine.
CHAPTER 3

Materials and Methods

3.1. Experimental animals

The animals used in this study were virgin white rats from the Wistar strain and were bred in the animal laboratory of the Department of Medical Bioscience Science at the University of the Western Cape. Only animals free from visible signs of diseases and ill-health were used in the study. Animals were fed a stock diet of Epol rat cubes, and they had fresh drinking water. The rats were kept in a controlled environment to eliminate factors such as noise and unnecessary handling. The ambient temperature was maintained at 25 ± 1°C and a 12-hour light cycle was maintained.

The animals were mated for a week after which the sires were removed. The females were randomly divided into four experimental groups. The body weight of each female was recorded daily for the next seven days. A significant mass increase over this time indicated that mating was successful. The pregnant rats were placed in individual straw lined cages for the duration of the study.

From gestational day seven to day 21 post partum, the rats were given treatment as designated by the experimental group. Nicotine treated animals received 1 mg/kg body mass/day subcutaneously, whereas vitamin C treated rats received 0.5 mg/kg body mass/day. The control animals received a placebo of water to the dose of 1 mg/kg body
mass/day. Finally, the last experiment group was subjected to the following procedure: an injection of 0.5 mg vitamin C/kg body mass/day followed by injection of 1 mg nicotine/kg body mass/day. The dose of 1 mg/kg body mass/day subcutaneously lies within the range of intake of 0.16 to 1.8 mg/kg body mass/day of habitual smokers (Maritz and Woolward, 1992).

The animals were injected daily between 9h00 and 10h00 using 1.0 ml plastic disposable syringes and needles. Care was taken during handling and injection of the animals not to inflict pain and to prevent discomfort of the animals.

### 3.2. Excision of lung tissue

Lung tissue for each of the experimental groups was obtained from the pups at week two, three and six. In each of the age groups a total of between 5 and 13 lung samples were obtained. Body weight was determined by weighing pups on a top loader laboratory balance (Denver instrument). Animals were then sacrificed by intraperitoneal injection of an overdose of 0.5 ml pentobarbital. The trachea was ligated and lungs were infused with 10 % formaldehyde. Lungs were fixed at a pressure of 25 mm Hg and 40 mm Hg in situ for 5 minutes, after which it was removed en bloc, and the lung were fixed in 10 % buffered formalin until processing.
3.3. Intratracheal instillation

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 fixative 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.

3.4. Measurement of Lung Volume

The lung volume was measured by the fluid displacement method. 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.
3.5. Sampling design

Fig.3.1. Illustration of a cascade sampling design in the lung, and the principles of systematic uniform random sampling have to be applied. (A) At the macroscopic level, the lung is cut completely into horizontal slices of thickness $t$, starting at a random position between 0 and $t$ (arrow). (B) These slices can be used for the estimation of total lung volume by the Cavalieri principle as well as for sampling of tissue blocks for light microscopy. Total lung volume $[V_{(lung)}]$ is the product of $t$ and the total cut area of the apical side of all slices (shown in red). (D) At a low light microscopic magnification, the volume fraction of parenchyma within lung $[V_{v(par/lung)}]$ is estimated by point counting.
3.6. Embedding and processing

After proper fixation in 10% buffered formaldehyde, the left lobe of the lung of each sample was placed in a plastic tissue processing cassette. Tissue was processed in a tissue processor, using fresh reagents for each processing cycle. The processing procedure involved is outlined in table 3.6.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 70% ethanol</td>
<td>1 hr</td>
</tr>
<tr>
<td>2 80% ethanol</td>
<td>1 hr</td>
</tr>
<tr>
<td>3 90% ethanol</td>
<td>1 hr</td>
</tr>
<tr>
<td>4 absolute ethanol</td>
<td>1 hr</td>
</tr>
<tr>
<td>5 absolute ethanol</td>
<td>1 hr</td>
</tr>
<tr>
<td>6 xylene I</td>
<td>1 ½ hr</td>
</tr>
<tr>
<td>7 xylene II</td>
<td>1 ½ hr</td>
</tr>
<tr>
<td>8 wax bath I</td>
<td>2 hrs</td>
</tr>
<tr>
<td>9 wax bath II</td>
<td>2 hrs</td>
</tr>
</tbody>
</table>

Table 3.6. Lung tissue processing procedure.

An embedding system was used for this process, prior to completing the embedding process, care was taken to standardise the orientation of all the lobes: the lateral aspect of each lobe was positioned such that it faced downward in the mould, with the superior aspect pointing to one of the short sides of the oblong mould. A very small volume of wax was then run slowly into the mould, the mould was placed on the refrigerated plate of the embedding system just long enough to permit the wax to start solidifying – thus securing the tissue in the required orientation. The mould was then removed from the refrigerated plate and the cassette was positioned such that the superior aspect of the lobe
was nearest to the pointed end of the cassette (fig. 3.2). The mould plus cassette was then filled with sufficient wax to permit proper adhesion to the cassette after the waxed solidified.

Fig. 3.2. Line diagram illustrating the standardised orientation of the processed lobe during the embedding process.
3.7. Microtomy

The tissue blocks were trimmed and sections of 5 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 5 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.

3.8. Microscope slide preparation

Sections of 5 µm were made by means of a precision rotary microtome (model: RMT-30). In order to ensure that the sampling was indeed from different levels in the lobe, and to eliminate the possibility that a section of the same alveolus appear in samples from two consecutive levels. So the 5 µm sections were obtained from the embedded tissue as follows:

1. Nearest to the lateral aspect of the lobe – level designated A.
2. In the paramedian plane of the lobe – level designated B.
3. Nearest to the medial aspect of the lobe – level designated C. (fig. 3.3)
Fig. 3.3. Line diagram of embedding lobe in profile, illustrating the protocol followed to obtain tissue sections from the three designated levels, namely A, B and C. Sectioning was executed from the superior to the inferior end of the lobe.

After cutting, the sections were floated on a water bath from where it was picked up on clean, marked microscope slides. Sections were fixed onto the glass slide in an incubator at 37°C for an hour, after which they were then stored in microscope slide boxes until staining could be executed.
3.9. Mayer’s haematoxylin and eosin staining technique

Haematin, the oxidation product of haematoxylin, is the natural dye that will cause staining of the tissue; the oxidation process is aided by sodium iodate. The poor affinity of haematoxylin for tissue is remedied by adding a mordant (aluminium salt) to the preparation. The nuclei of the tissue are stained a red colour, which is blued in the Scott’s Tap water to a dark blue shade. The cytoplasm is stained a reddish pink with eosin, the counterstain.

3.9.1. Reagents:

1. Xylene: Use as supplied by the manufacture.
2. Ethanol: Use in the following concentrations: absolute (as supplied by the manufacturer); 90%; 80% and 70%. The dilutions are done using distilled water.
3. Eosin: Dissolve 3.0 grams of eosin and 2.0 grams of phloxine in 1 litre distilled water.
4. Mayer’s alum haematoxylin: Combine 1.0 gram haematoxylin and 50.0 grams of potassium alum and dissolve in a small volume of water. Add 0.2 grams sodium Iodate, make up to a volume of 1 litre and leave to dissolve overnight at room temperature. Then add 50.0 grams chloral hydrate and 1.0 grams of citric acid and boil for 5 minutes. When cool. It is ready for use.
5. Scott’s Tap water: Dissolve 2.0 grams sodium bicarbonate and 20.0 grams magnesium sulphate in 1 litre distilled water.
6. 1% acid alcohol: Combine 1.5 litres of 96% ethanol with 0.48 litres of distilled water and 0.02 litres of concentrated hydrochloric acid. Mix well to form a homogeneous solution.

7. DPX: Use as supplied by the manufacturer.

3.9.2. Procedure:

1. Sections were fixed onto glass slides for 30 minutes in a hot air oven at 80°C.

2. Glass slides with sections were transferred to xylene and gently agitated for 1 minute and repeated on second xylene bath to remove any remaining traces of wax.

3. Transferred to absolute alcohol to initiate the hydration process

4. Continued the hydration process by repeating the procedure described in (3) in 90%, 80% and 70% ethanol.

5. Completed the hydration process and washed well in running tap water.

6. Stained in Haematoxylin for 15 minutes. Gently agitating the slides.

7. Washed in running tap water.

8. Sections were differentiated in 1% acid alcohol, and rinsed under tap water.

9. Sections were blued in Scott’s tap water for 30 seconds, and then rinsed under tap water.

10. Stained in Eosin for 1 minute. Washed in running tap water to rinse excess staining.

11. Sections were dehydrated for 30 seconds each in 70%; 80%; 90% and absolute alcohol.

12. Slides were cleared in two successive xylene baths, 30 seconds each.
13. Slides were mounted with DPX.

3.10. Verhoeff’s Method for Staining of Elastic Fibres

3.10.1. Materials

3.10.1.1. Staining Solution

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

3.10.1.2. Lugol’s Iodine:
Iodine 1g
Potassium iodide 2g
Distilled water 100ml

3.10.1.3. Van Gieson:
Saturated aqueous picric acid 100ml
1% acid fuschin 10ml

3.10.2. Procedure

1. The sections were washed with 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 distilled water.

7. The slides were counterstained with Van Gieson for three minutes.

8. They were then rinsed in distilled water.

9. The slides were dehydrated in alcohol, cleared in xylene and mounted with DPX mountant.

3.11. Van Gieson’s Method for Staining of Collagen Fibres

3.11.1. Preparation of solution:

1. Any of alum haematoxylins may be used; or better, iron haematoxylin.

2. Van Gieson’s picro-acid-fuchsin: 1% aqueous acid fuchsin, 5-15 ml, saturated aqueous picric acid (about 1.22%), 100ml.

3.11.2. Staining schedule:

1. Dewaxed, hydrated and applied nuclear stain.

2. Washed the slides with distilled water.

3. Stained for 3-5 minutes in Van Gieson’s solution (Solution B)

4. Washed quickly in distilled water.

5. Dehydrated the slides through ascending alcohols.

6. Cleared and mounted.
3.12. Morphometric Techniques

Various morphometric techniques can be used to assess abnormal lung development. Six of these techniques namely: lung volume (\(V_L\)), mean linear intercept (\(L_m\)), internal surface area (ISA), volume density of tissue (\(V_t\)) and air (\(V_a\)), lung alveolar volume (\(V_{alv}\)) and alveolar number (\(N_a\)) were used in this study to elucidate the effect that nicotine, ascorbic acid and nicotine plus ascorbic acid have on postnatal lung development in comparison with that of control animals. Used individually, the techniques quantify the effects that nicotine, ascorbic acid and nicotine plus ascorbic acid have on the lung. Used as a combination, it gives a global view of lung development and the extent of pulmonary damage due to nicotine exposure, as well as the possible preventative role of ascorbic acid where ascorbic acid plus nicotine was administered. The effect if ascorbic acid supplementation will also be assessed. Haematoxylin and eosin stained slides free of cutting artefacts were used for these assessment. Two microscope slides of each animal sample were used. At least six fields per microscope slide were counted.

3.12.1. Protocol followed to investigate the effect of treatment and age on lung development and damage:

From the sample population, two microscope slide preparation per age group per treatment were randomly selected for inclusion in this sample population. Each microscope slide preparation represented the lung tissue of a different animal from a different litter. In addition, a suitable microscope slide preparation of the lung tissue of an additional five animals per age group per treatment was used. The total sample population used was therefore seven animals per age group, per treatment (\(n = 7\)). With respect to the radial alveolar counts, mean linear intercept and internal surface area, an
average of six fields were assessed per microscope slide preparation. Hence the total number of fields assessed per age group, per treatment was 42 (n = 42).

3.12.2. Total number of alveoli per lung ($N_a$)

Tissue samples were taken randomly from the upper, middle and lower lung lobes. At least six tissue samples were taken per lung for processing for morphology and morphometry. Total number of alveolar was calculated as follows:

$$N_a = \frac{L_v \cdot V_a}{V_{alv}}$$

Where:

$L_v$ = lung volume (ml)

$V_a$ = alveolar air volume density ($\%$)

$V_{alv}$ = mean alveolar volume (ml)

3.12.3. Volume Density ($V_a$ and $V_t$)

The alveolar air volume density ($V_a$) and alveolar tissue volume density ($V_t$) was determined by using the point counting technique at 100 x magnifications. 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 10 x eyepiece and a 10 x objective were used to obtain a total magnification of 100 x. Two blocks were taken from the upper lobe, one from the middle lobe and two from the lower lobe. Non-parenchyma included tissue which had a diameter of more than 1.1mm.
The following alveoli were included:

1. Those that were found within the graticule.
2. Those that touched the lower and right borders of the graticule.

The alveoli that were excluded from the count included:

1. Those found outside the square on the upper and the left side of the graticule.
2. 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.

### 3.12.4. Mean Alveolar Volume

Mean alveolar volume ($V_{alv}$) was measured according to the following formula:

$$V_{alv} = L_m^3 \times \pi/3$$

Where:

$L_m = \text{Mean linear intercept.}$

Mean alveolar volume gives an indication of the size of the alveolus and hence the volume of it can contain. This means that it can be expected that alveoli with larger volumes has a reduced total surface area for gaseous exchange. It also implies the possibility of lesser alveolar surface area for gaseous exchange and because of the reduced number of alveoli.

### 3.12.5. Mean Linear Intercept (L$_m$)

Mean linear intercept, is the average distance between the walls of an alveolus. It is an index of alveolar wall size. The mean linear intercept will thus increase with an increase
in alveolar size such as in alveolar wall destruction. This indicates that surface area and
mean liner intercept, are inversely related. Thus, an increase in \( L_m \), as evidenced by
destruction of alveolar walls is indicative of a decreased surface area available for
gaseous exchange.

\[
L_m = N \times \frac{L}{m}
\]

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)
- \( m \) = the sum of all the intercepts.

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

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

1. Touched but did not intercept the left side of the vertical line.
2. Touched but did not intercept the upper side of the horizontal line.
3. 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:

1. Touched without crossing the right border of the vertical arm.
2. Touched without crossing the lower border of the horizontal arm.
3.12.6. Internal Surface Area (S_a)

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

\[ S_a = 4 \times \frac{L_v}{L_m} \]

Where:

\( L_v \) = lung volume.

\( L_m \) = mean linear intercept.

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 Keuls 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.
CHAPTER 4

Results

4.1. The influence of Maternal Nicotine, Vitamin C and Nicotine + Vitamin C on Body weight and Lung volume of the offspring.

4.1.1. Body weight (BW)

The body weight (table 4.1.1. and fig 4.1(a)) of the control animals increased significantly (P <0.001) from 28.04 ± 2.76g on postnatal day 14 to 152.28 ± 23.63g on postnatal day 42. In animals exposed to nicotine during both gestation and lactation, the body weight also increased significantly from 19.03 ± 0.63g to 133.68 ± 5.42g (P <0.001). The same significance (P <0.001) was observed in the nicotine + vitamin C exposed animals. The increase was from 24.117 ± 1.60g to 148.08 ± 18.12g. The vitamin C exposed animals had an extremely significant increase, from 20.27 ± 1.10g postnatal day 14 to 143.39 ± 13.25g postnatal day 42. The body weight of the control animals was at postnatal day 14 (28.04 ± 2.76g) higher than that of the nicotine (19.03 ± 0.63g) and vitamin C (20.27 ± 1.10g) exposed animals (P <0.05). The body weight of the control and the nicotine + vitamin C exposed animals were the same (P >0.05). Also no differences in body weight were observed between the control and experimental animals on postnatal day 21 and 42 (P >0.05).
### Body Weight (g±SE)

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>28.04 ± 2.76</td>
<td>19.03 ± 0.36*</td>
<td>24.12 ± 1.60</td>
<td>20.27 ± 1.10*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>37.07 ± 1.97</td>
<td>31.69 ± 3.03</td>
<td>38.04 ± 4.81</td>
<td>31.81 ± 2.41</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>152.28 ± 23.6</td>
<td>133.69 ± 5.42</td>
<td>148.08 ± 18.1</td>
<td>143.4 ± 13.23</td>
<td>P &gt;0.05</td>
</tr>
</tbody>
</table>

P

| D14 vs. D42 | (P <0.001) | (P <0.001) | (P <0.001) | (P <0.001) |

#### Table 4.1.1 Comparison of body weight for each age group of each treatment. Significant difference from control:*P <0.05

### Lung volume (ml±SE)

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1.58 ± 0.08</td>
<td>1.36 ± 0.24</td>
<td>1.69 ± 0.09</td>
<td>1.31 ± 0.04</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>1.92 ± 0.12</td>
<td>1.83 ± 0.23</td>
<td>2.07 ± 0.16</td>
<td>1.92 ± 0.17</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>6.91 ± 0.65</td>
<td>5.30 ± 0.48</td>
<td>5.56 ± 0.25</td>
<td>5.99 ± 0.36</td>
<td>P &gt;0.05</td>
</tr>
</tbody>
</table>

P

| D14 vs. D42 | (P <0.001) | (P <0.001) | (P <0.05) | (P <0.05) |

#### Table 4.1.2. Comparison of lung volume for each age group of each treatment. Significant difference from control:*P <0.05

### Body weight/Lung volume (g/ml±SE)

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>17.83 ± 1.43</td>
<td>14.02 ± 0.71</td>
<td>14.27 ± 0.56</td>
<td>15.51 ± 0.71</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>19.41 ± 0.96</td>
<td>16.88 ± 1.41</td>
<td>18.2 ± 1.62</td>
<td>16.73 ± 0.96</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>25.37 ± 1.82</td>
<td>25.92 ± 2.72</td>
<td>26.49 ± 2.66</td>
<td>23.17 ± 1.56</td>
<td>P &gt;0.05</td>
</tr>
</tbody>
</table>

P

| D14 vs. D42 | (P <0.001) | (P <0.001) | (P <0.001) | (P <0.001) |

#### Table 4.1.3. Comparison of body weight/lung volume ratio for each age group of each treatment. Significant difference from control:*P <0.05
Fig. 4.1 (a, b and c). The effect of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on Body weight (BW), lung volume (Lv), and BW/Lv of the lungs of the offspring.
The data summarized in table 4.1.4 shows that the body weight of the control animals increased by 9.03 ± 0.79 g/week from postnatal day 14 to 21. However, it is interesting to note that between postnatal day 21 and 42 the body weight of the control animals increased by 46.4 ± 7.22 g/week. This increase in body weight per week after weaning is more than 40% faster than before weaning (P <0.001). For the nicotine exposed animals the increase in body weight between postnatal days 14 to 21 and 21 to 42 was 11.655 ± 2.4 and 34.33 ± 0.79 g/week respectively. This means that before weaning the increase in body weight of the control and nicotine exposed animals were no different. On the other hand, after weaning the increase in body weight of the nicotine exposed rat pups was 12.07 ± 2.5 g/week slower (P <0.05) than that of the control animals.

The rat pups exposed to vitamin C via the mothers’ blood and milk increased by 11.54 ± 1.31 g/week from postnatal day 14 to 21. Between postnatal days 21 to 42 the body weight increased by 37.19 ± 3.61/week. The nicotine + vitamin C exposed animals’ body weight increased by 13.92 ± 3.21 g/week from postnatal day 14 to 21; and by 36.68 ± 4.43 g/week between postnatal days 21 to 42. As for the control animals the increase in body weight between postnatal day 14 and 21, of the rats that were exposed to nicotine, or vitamin C, or nicotine + vitamin C during gestation and lactation, were slower than that between postnatal days 21 to 42. It is also interesting to note that the body weight of all the groups that received vitamin C, or a combination thereof, like the rats that were exposed to nicotine, increases slower (P <0.05) than the control between postnatal day 21 and 42.
Table 4.1.4 Body weight increase of the animals per week (g/week), between postnatal day 14 to 21 and postnatal day 21 to 42. Significant difference from control: *P <0.05

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Vitamin C</th>
<th>Nicotine + Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-21</td>
<td>9.03 ± 0.79</td>
<td>11.6 ± 2.4</td>
<td>11.54 ± 1.31</td>
<td>13.92 ± 3.21</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>21-42</td>
<td>46.4 ± 7.22</td>
<td>34.3 ± 0.79*</td>
<td>37.19 ± 3.61*</td>
<td>36.68 ± 4.43*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>P D14-21 vs. D21-42</td>
<td>(P &lt;0.001)</td>
<td>(P &lt;0.001)</td>
<td>(P &lt;0.001)</td>
<td>(P &lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

4.1.2. Lung volume

The lung volumes (table 4.1.2 and fig. 4.1(b)) of the control and nicotine exposed rat pups increased more than four and three-fold respectively from 1.58 ± 0.08 ml on postnatal day 14 to 6.91 ± 0.65 ml on postnatal day 42 (P <0.001) for the control rats and the nicotine exposed animals from 1.36 ± 0.24 ml to 5.30 ± 0.45 ml (P <0.001). The lung volume of the vitamin C exposed animals increased more than four-fold from 1.3 ± 0.04 ml to 5.9 ± 0.36 ml postnatal day 42, while that of the nicotine + vitamin C exposed animals increased more than three-fold from 1.69 ± 0.09 ml to 5.5 ± 0.25 ml postnatal day 42 (P<0.05). No significant difference in lung volume was observed between the control and experimental animals (P >0.05). The BW/Lv ratios were also not affected by maternal nicotine exposure (table 4.1.3 and fig. 4.1(c)).
Changes in lung volume in the animals showed that in control animals, the lung volume increased by $0.35 \pm 0.04$ ml/week between postnatal day 14 and 21, and postnatal day 21 and 42 by $1.66 \pm 0.18$ ml/week ($P <0.01$). In contrast to that of the control rats, the increase in the lung volumes of the rats that were exposed to nicotine via the placenta and mother’s milk between postnatal days 14 and 21 ($0.47 \pm 0.01$ ml/week) was not different ($P>0.05$) than the increase between postnatal days 21 and 42 ($0.47 \pm 0.08$ ml/week). The lung volume of the animals exposed to vitamin C only increased by $0.61 \pm 0.13$ ml/week from postnatal day 14 to 21, and between days 21 to 42 the lung volume increased by $1.36 \pm 0.06$ ml/week; and the lung volume of those rats exposed to both nicotine + vitamin C the lung volume increased by $0.38 \pm 0.07$ ml/week from postnatal day 14 to 21 and by $1.16 \pm 0.03$ ml/week between postnatal day 21 to 42 ($P <0.01$).

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Vitamin C</th>
<th>Nicotine + Vitamin C</th>
<th>$P$ control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-21</td>
<td>0.35 ± 0.04</td>
<td>0.47 ± 0.01</td>
<td>0.61 ± 0.13*</td>
<td>0.38 ± 0.07</td>
<td>$P &lt;0.05$</td>
</tr>
<tr>
<td>21-42</td>
<td>1.66 ± 0.18</td>
<td>0.47 ± 0.08*</td>
<td>1.36 ± 0.06</td>
<td>1.16 ± 0.03</td>
<td>$P &lt;0.05$</td>
</tr>
<tr>
<td>P D14-21 vs. D21-42</td>
<td>($P &lt;0.01$)</td>
<td>($P &gt;0.05$)</td>
<td>($P &lt;0.01$)</td>
<td>($P &lt;0.01$)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1.5. Lung volume increase of the animals per week (ml/week), between postnatal day 14 to 21 and postnatal day 21 to 42. Significant difference from control: *$P <0.05$
4.2 The influence of Maternal exposure to nicotine, vitamin C, or nicotine + vitamin C on the volume density of the airspaces and parenchymal tissue of the offspring.

4.2.1 Volume density of air ($V_a$) and parenchymal tissue density ($V_t$)

The $V_a$ and $V_t$ (table 4.2 and fig 4.7 a and b) of the control animals remain constant between postnatal days 14 ($V_a$: 76.13 ± 1.87 and $V_t$: 23.88 ± 1.87) and 42 ($V_a$: 77 ± 0.87 and $V_t$: 23 ± 0.87). On the other hand, the $V_a$ of the nicotine exposed animals (85.78 ± 0.61) of the day 14 rat pups is higher than that of the control (P <0.01), but decreases as the animal ages to 81.43 ± 0.33 on postnatal day 42. As a result of this decrease the $V_a$ on postnatal day 42 is not different (P >0.05) from that of the control animals of the same age. This implies that the parenchymal tissue density on day 14 of the nicotine exposed pups was lower (P<0.01) than that of the control rats of the same age but resembles that of the control on day 42. The $V_a$ and $V_t$ of the lungs of the rat pups exposed to vitamin C only or to both nicotine and vitamin C were not different from that of those rat pups that were exposed to nicotine only via the placenta and mother’s milk. There was also no significant difference (P >0.05) in the $V_a$ and $V_t$ of the rat pups exposed to vitamin C only or to both nicotine and vitamin C from that of the control animals.
The table below presents the results of a study regarding the influence of different factors on the metabolic rate of cows. The factors include the following: 0.05 > p ≥ 0.001; 0.001 > p ≥ 0.0001; p ≤ 0.0001.

**Table 4.2: The Influence of Metabolic Rate on the Metabolic Rate of Cows**

<table>
<thead>
<tr>
<th>p (0.0001)</th>
<th>p (0.001)</th>
<th>p (0.01)</th>
<th>p (0.1)</th>
<th>p (1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001 &gt; p</td>
<td>0.001 &gt; p</td>
<td>0.01 &gt; p</td>
<td>0.1 &gt; p</td>
<td>1.0</td>
</tr>
<tr>
<td>88</td>
<td>79</td>
<td>74</td>
<td>69</td>
<td>64</td>
</tr>
<tr>
<td>100</td>
<td>102</td>
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<td>108</td>
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<td>120</td>
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</tr>
<tr>
<td>130</td>
<td>132</td>
<td>134</td>
<td>136</td>
<td>138</td>
</tr>
</tbody>
</table>

Note: Values are expressed as metabolic rate (metabolic rate).
Fig. 4.2 (a and b). The effect of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on the tissue and alveolar air volume of the offspring.
4.3 Internal Surface area and Mean Liner Intercepts

4.3.1. Internal Surface area ($S_a$)

Table 4.3.1 and fig 4.3. indicates that the $S_a$ of the control animals increased from 150.12 ± 10.16 cm$^2$ on postnatal day 14 to 584.53 ± 40.24 cm$^2$ on postnatal 42 (P <0.001). Similar observations were made with the other groups. That is, the $S_a$ of the nicotine exposed animals increased from 139.62 ± 3.86 cm$^2$ on postnatal day 14 to 410.13 ± 30.78 cm$^2$ on postnatal day 42 (P <0.001); the $S_a$ of the nicotine + vitamin C exposed animals increased from 145.31 ± 10.42 cm$^2$ on postnatal day 14 to 490.71 ± 21.11 cm$^2$ on postnatal day 42 (P <0.001) and the $S_a$ of the vitamin C exposed animals increased from 135.98 ± 10.41 cm$^2$ on postnatal day 14 to 501.12 ± 13.26 cm$^2$ on postnatal day 42 (P <0.001).

The increase in the $S_a$ of the nicotine and vitamin C exposed rats was at 26.05 ± 4.02 and 22.56 ± 4.01 cm$^2$/week respectively, slower (P>0.05) than the 29.09 ± 4.51 and 30.88 ± 5.68 cm$^2$/week of the control rats and those that received both nicotine and vitamin C. This trend is maintained between postnatal days 21 and 42 where the increase in $S_a$ of the nicotine exposed rats was at 81.66 ± 6.65 and that of the vitamin C group at 112.02 ± 7.24 cm$^2$/week as oppose to the 140.77 ± 19.19 and 137.61 ± 18.33 cm$^2$/week of the vitamin C group. The increase in the $S_a$ of the nicotine group during this phase of lung growth was also slower (P<0.05) than that of the vitamin C group.
The increase in the $S_a$ (fig. 4.3) of the various groups follows the same trend as the increase in body weight of the rats (fig. 4.1.1).

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>150.12 ± 10.16</td>
<td>139.62 ± 6.86*</td>
<td>145.31 ± 10.24</td>
<td>135.98 ± 7.41*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>179.21 ± 18.65</td>
<td>165.67 ± 12.31</td>
<td>167.87 ± 16.53</td>
<td>171.86 ± 13.93</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>584.53 ± 40.24</td>
<td>410.13 ± 30.78*</td>
<td>490.71 ± 21.11</td>
<td>501.12 ± 26.26</td>
<td>P &lt;0.05</td>
</tr>
</tbody>
</table>

P D14 vs. D42 (P <0.001) (P <0.001) (P <0.001) (P <0.001)

Table 4.3.1. Comparison of internal surface area for each age group of each treatment. Significant difference from control: *P <0.05

Fig.4.3. The influence of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on the internal surface area ($S_a$) of the lungs of the offspring
The $S_a/L_v$ ratio of the nicotine exposed animals ($70.32 \pm 4.63$ cm$^2$/ml$^2$) was 24% less ($P < 0.001$) than that of the control animals ($92.74 \pm 3.35$ (cm$^2$/ml$^2$) at postnatal day 14. The animals exposed to vitamin C only had a $S_a/L_v$ ratio of $70.93 \pm 4.12$ cm$^2$/ml$^2$, which was 22% smaller ($P < 0.01$) than the control animals. At postnatal day 14 there was no significant difference between the animals that were both exposed to nicotine and vitamin C ($85.96 \pm 4.08$ cm$^2$/ml$^2$) to the control animals ($P > 0.05$). It is also noticed that at postnatal day 14 the animals exposed both to nicotine and vitamin C, the $S_a/L_v$ ratio was 17% higher ($P < 0.01$) than that of the nicotine exposed animals. At postnatal days 21 and 42 there was no difference ($P > 0.05$) between all of the experimental groups.

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Vitamin C</th>
<th>Nicotine + Vitamin C</th>
<th>$P$ control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-21</td>
<td>29.09 ± 4.51</td>
<td>26.05 ± 4.02*</td>
<td>22.56 ± 4.01*</td>
<td>30.88 ± 5.68</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>21-42</td>
<td>140.77 ± 19.19</td>
<td>81.66 ± 6.15*</td>
<td>112.02 ± 7.24*</td>
<td>137.61 ± 18.33</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>$P$ D14-21 vs. D21-42</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
</tr>
</tbody>
</table>

Table 4.3.2 Internal surface area changes of the lungs of the animals per week (cm$^2$/week), between postnatal day 14 to 21 and postnatal day 21 to 42. Significant difference from control: *$P$ < 0.05

4.3.2. Mean Linear Intercepts ($L_m$)

The mean linear intercepts of all the experimental animals are summarized in table 4.3.3 and fig. 4.3.2. No significant differences were observed in any of the groups. The $L_m$ of the control animals increased from 42.5 ± 1.82 µm on postnatal day 14 to 45.5 ± 0.96 µm
on postnatal 42 (P <0.05). On the other hand the mean linear intercepts of the nicotine exposed animals decreased from 56.33±0.67 µm on postnatal day 14 to 50 ± 1.65 µm on postnatal day 42 (P <0.05). The mean linear intercepts of the animals exposed to both nicotine and vitamin C also decreased from 48 ± 2.04 µm on postnatal day 14 to 45.3 ± 2.02 µm on postnatal day 42 (P <0.05). The mean linear intercepts of the vitamin C only exposed animals decreased from 52.25 ± 0.25 µm on postnatal day 14 to 50 ± 2.48 µm on postnatal day 42 (P >0.05). This means that the $L_m$ of those animals that were exposed to a combination of nicotine and vitamin C decreased by 5.9 % as oppose to the 4.5 % of animals that were exposed to vitamin C only and the 12.6 % of those exposed to only nicotine.

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>42.5 ± 1.82</td>
<td>56.33 ± 0.67*</td>
<td>48 ± 2.04*</td>
<td>52.3 ± 0.25*</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>21</td>
<td>43.75 ± 1.25</td>
<td>53.5 ± 0.96*</td>
<td>47 ± 1.08</td>
<td>53 ± 0.81*</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>42</td>
<td>45 ± 0.96</td>
<td>50 ± 1.65*</td>
<td>45.3 ± 2.02</td>
<td>50 ± 2.48*</td>
<td>P &lt;0.05</td>
</tr>
</tbody>
</table>

Table 4.3.3. Comparison of mean linear intercepts for each age group of each treatment.

Significant difference from control:*P <0.05
Fig. 4.3.2. The influence of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on the linear intercept of the lungs of the offspring.

The mean linear intercepts of the nicotine (56.33 ± 0.67 µm) and vitamin C only exposed rats (52.25 ± 0.25 µm) was 25% and 19% respectively higher (P < 0.001) than the control animals (42.5 ± 1.82 µm) at postnatal day 14 and 21. No significant difference is seen when comparing the control animals and the nicotine + vitamin C exposed animals (P > 0.05) at postnatal day 21 and 42.
4.4 Alveolar Volume and Alveolar Number

4.4.1. Alveolar Volume (V_{alv})

The data summarized in table 4.4.1 and illustrated in fig. 4.4.1 show that the V_{alv} of the control animals remain unchanged between days 14 and 42. On the other hand, those animals that were exposed to nicotine showed a decrease (P <0.05) in V_{alv} between postnatal days 14 to 42. From the data it is also clear that the V_{alv} of the control animals for all the age groups was smaller than that of the animals exposed to only nicotine, or only vitamin C. The V_{alv} of those that were exposed to a combination of nicotine + vitamin C was not different from that of the control animals. The alveolar volume of the nicotine exposed animals at postnatal day 14 was at $18.74 \pm 0.66 \times 10^4 \mu L$, 56.4% higher (P <0.001) than the $8.18 \pm 1.04 \times 10^4 \mu L$ of the control animals. From postnatal day 14 to postnatal day 42 the alveolar volume of the nicotine exposed animals gradually decreased to $14.23 \pm 1.38 \times 10^4 \mu L$, where the latter was 30.4% higher (P <0.001) than the $9.90 \pm 0.61 \times 10^4 \mu L$ of the control animals. The alveolar volume of the animals exposed to the combination of nicotine and vitamin C at postnatal day 14 and 21 was $11.77 \pm 1.48 \times 10^4 \mu L$ and $10.92 \pm 0.77 \times 10^4 \mu L$ respectively, 37 % and 32 % respectively lower (P <0.01) than that of the nicotine animals. Calculations show that the alveolar volume of the animals exposed to only vitamin C was $(14.94 \pm 0.22 \times 10^4 \mu L)$ 45.2% higher (P <0.01) than the $8.18 \pm 1.04 \times 10^4 \mu L$ of the control animals. At postnatal day 21 the alveolar volume of the vitamin C animals was at $15.62 \pm 0.72 \times 10^4 \mu L$, 43.5% higher (P <0.001) than the $8.83.3 \pm 0.76 \times 10^4 \mu L$ of the control animals. At postnatal day 42 the alveolar
volume of the animals exposed to nicotine only, vitamin C only, or to both nicotine + vitamin C was the same (P >0.05).

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>8.18±1.04</td>
<td>18.74 ± 0.66*</td>
<td>11.77 ±1.48</td>
<td>14.94 ± 0.2*</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>21</td>
<td>8.83±0.76</td>
<td>16.08 ± 0.85*</td>
<td>10.92 ± 0.77</td>
<td>15.62 ± 0.7*</td>
<td>P &lt;0.001</td>
</tr>
<tr>
<td>42</td>
<td>9.90±0.61</td>
<td>14.23 ± 1.38*</td>
<td>9.86 ± 1.20</td>
<td>13.38 ± 1.9*</td>
<td>P &lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.4.1. Comparison of mean alveolar volume for each age group of each treatment.

Significant difference from control:*P <0.05

Fig.4.4.1. The influence of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on the alveolar volume ($V_{alv}$) of the offspring.
4.4.2. Alveolar Number

Table 4.4.3 and fig. 4.4.2 shows that on postnatal day 14 the alveolar number of the lungs of the control animals was 2.54 ± 0.55 million, which is 62% higher (P <0.05) than the 0.97 ± 0.11 million of the nicotine exposed animals and 64% higher (P <0.05) than the 0.98 ± 0.11 million of the vitamin C exposed animals. The alveolar number of the animals that were exposed to a combination of nicotine and vitamin C was, however, the same as for the control animals at postnatal day 14. On day 21 after birth the alveolar number of the control rats and those exposed to both nicotine and vitamin C was not different, but that of the other two groups were lower (P <0.05). At postnatal day 42 the alveolar number of the lungs of the control animals was at 3.97 ± 1.09 million, 56.45% higher (P <0.01) than the 1.73 ± 2.07 million of the nicotine exposed animals. Even though the alveolar number of the nicotine exposed animals and rat pups exposed to vitamin C was the same at postnatal day 14 and 21, the alveolar number of rats exposed to both nicotine and vitamin was (2.16 ± 1.82 million) 34% higher (P <0.05) than that of the rats exposed to nicotine only. The alveolar number of the 42-day-old rats that were exposed to vitamin C only was the same (P >0.05) as that of the animals exposed to both nicotine and vitamin C.

Fig 4.4.3 and table 4.4.5 shows that the alveolar number/body weight ratio of the control, nicotine and vitamin C exposed animals were significantly different on postnatal days 14 and postnatal day 42 (P <0.05). Like the alveolar number/body weight ratio, the alveolar number/surface area ratio of the control, nicotine and vitamin C exposed animals were significantly different on postnatal day 14 and postnatal day 42 (P <0.05).
<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>2.54 ± 0.55</td>
<td>0.97 ± 0.11*</td>
<td>2.05 ± 0.21</td>
<td>0.98 ± 0.11*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>3.29 ± 0.55</td>
<td>1.91 ± 0.56*</td>
<td>3.27 ± 0.42</td>
<td>1.97 ± 0.40*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>3.97 ± 1.09</td>
<td>1.73 ± 2.07*</td>
<td>2.16 ± 1.82*</td>
<td>2.29 ± 2.76*</td>
<td>P &lt;0.01</td>
</tr>
<tr>
<td>P D14 vs. D42</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.3. Comparison of alveolar number for each age group of each treatment. Significant difference from control:*P <0.05

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1.67 ± 0.23</td>
<td>1.04 ± 0.09*</td>
<td>1.43 ± 0.09</td>
<td>0.98 ± 0.05*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>1.88 ± 0.17</td>
<td>1.29 ± 0.21</td>
<td>1.85 ± 0.1</td>
<td>1.32 ± 0.15</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>6.20 ± 0.83</td>
<td>4.18 ± 0.26*</td>
<td>5.31 ± 0.29</td>
<td>4.54 ± 0.39*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>P D14 vs. D42</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.4. Comparison of alveolar number/surface area ratio for each age group of each treatment. Significant difference from control:*P <0.05

<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Nicotine + vitamin C</th>
<th>Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.91 ± 0.2</td>
<td>0.51 ± 0.04*</td>
<td>0.89 ± 0.1</td>
<td>0.49 ± 0.4*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>0.89 ± 0.1</td>
<td>0.61 ± 0.04</td>
<td>0.89 ± 0.1</td>
<td>0.61 ± 0.03</td>
<td>P &gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>2.18 ± 0.3</td>
<td>1.30 ± 0.1*</td>
<td>1.83 ± 0.2</td>
<td>1.59 ± 0.2*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>P D14 vs. D42</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.5. Comparison of alveolar number/body weight ratio for each age group of each treatment. Significant difference from control:*P <0.05
Fig. 4.4.2 (a and b). The influence of maternal exposure to nicotine, vitamin C or a combination of nicotine and vitamin C on the alveolar number (N_a) and, alveolar number/ internal surface area (S_a) of the lungs of the offspring.
Fig. 4.4.3. The influence of maternal exposure to nicotine, vitamin C and a combination of nicotine and vitamin C on the alveolar number (N\(_a\))/BW ratio of the offspring.

The alveolar number in the control animals increased by 0.75 ± 0.1 million /week between postnatal days 14 and 21 and between postnatal days 21 and 42 by 0.36 ± 0.18 million /week. The alveolar number of the nicotine exposed animals increased between postnatal day 14 to 21 and 21 to 42 by 0.94 ± 0.45 million and 0.51 ± 0.50 million /week respectively. During the same period of time the alveolar number of the experimental animals exposed to vitamin C only increased by 0.9 ± 0.29 million /week from postnatal day 14 to 21; and between days 21 to 42 the alveolar number increased by 0.69 ± 0.79 million /week. The nicotine + vitamin C exposed animals alveolar number increased by 1.23 ± 0.21 million /week from postnatal day 14 to 21; and by 0.76 ± 0.47 million /week between postnatal day 21 to 42.
<table>
<thead>
<tr>
<th>Age Days</th>
<th>Control</th>
<th>Nicotine</th>
<th>Vitamin C</th>
<th>Nicotine + Vitamin C</th>
<th>P control vs. experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-21</td>
<td>0.75 ± 0.1</td>
<td>0.94 ± 0.45</td>
<td>0.9 ± 0.29</td>
<td>1.23 ± 0.21*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>21-42</td>
<td>0.36 ± 0.18</td>
<td>0.51 ± 0.50</td>
<td>0.69 ± 0.79</td>
<td>0.76 ± 0.47*</td>
<td>P &lt;0.05</td>
</tr>
<tr>
<td>D14-21 vs. D21-42</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td>(P &lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4.6. Alveolar number increase of the animals per week (million/week), between postnatal day 14 to 21 and postnatal day 21 to 42. Significant difference from control: *P <0.05

4.5. Morphology Results

Figure 4.5 is light micrographs that illustrate structural differences between control animals (A); nicotine exposed animals (B) and combination of nicotine + vitamin C exposed animals (C) at postnatal day 42. Evaluations of the (B) and (C) light micrographs, the alveolar appear larger and in many areas the septae are missing. The enlarged air sacs that occur in light micrograph (B) resembles emphysematous lesions and the structural disturbances seen on light micrograph (C) seem to resemble senile emphysema. The control lung (light micrograph (A)) appears normal.
Fig. 4.5.1. Lung tissue of 42-day-old control rats (A) and rats exposed to nicotine (B), vitamin C (C), and a combination of nicotine and vitamin C (D) via the placenta and mother’s milk during gestation and lactation where ME indicate some of the enlarged alveoli characteristic of microscopic emphysema. (Bar= 0.5 µm)
Fig. 4.5.2. Elastic tissue of lungs of 42-day-old A) control rats, and rats exposed to B) nicotine, C) vitamin C, and D) a combination of nicotine and vitamin C via the placenta and mother’s milk during gestation and lactation. Red arrows = breaks in elastic tissue strands.
CHAPTER 5

Discussion of results

5.1. Maternal nicotine exposure and the development of the neonatal rat lung as a gas exchanger

Studies by Barker (1996) suggested that certain adult onset diseases may result from changes in the *in utero* environment prior to birth. These diseases have been associated with foetal growth reflected as small size for age. These diseases include high blood pressure, diabetes mellitus and others (Barker *et al.*, 1996). Where most of the previous studies dealt with nutrition and growth and the link with foetal onset of adult diseases, recent research showed that certain lifestyle factors, such as smoking and exposure to nicotine also affect lung development and growth (Maritz, 2008). It is suggested that these changes in lung growth and development induced by changes in the *in utero* environment may have long term consequences in terms of respiratory health (Li *et al.*, 2000). Changes that were observed include impaired glucose metabolism (Maritz, 1992) as well as structural changes (Maritz *et al.*, 2003). It is interesting to note that most of these changes occur after nicotine withdrawal (Maritz and Windvogel, 2005). Adequate nutrition is essential for foetal and neonatal development as it supplies energy and precursors, such as amino acids, to support growth. Inadequate nutrition supply will therefore result in stunted foetal and neonatal growth, and will result in newborns that are small for their age. It is suggested that poor foetal nutrition not only results in a smaller body size, but also results in foetal adaptations that ‘programme’ future propensity to
adult disease (Lucas et al. 1999). Smoking is associated with constriction of the blood vessels that transport nutrients to the developing foetus. It is, therefore, suggested that the lower body weights of babies born from smoking mothers is due to a lower nutrient supply (Davies and Albertiny, 1976). In the current study it was shown that maternal nicotine exposure during gestation and lactation resulted in lower body weight at postnatal day 14. However, due to catch-up growth between postnatal days 14 and 21, the body weight of the 21-day-old nicotine exposed rats were not different from that of the control rats of the same age. The lower body weight at postnatal day 14 and thus at birth can in all likelihood be attributed to an inadequate nutrient supply from the mother’s blood. Once the intake of nicotine decreases as the animals grow older and are more dependent on solid food than mother's milk, the growth rate increases. Although no direct evidence exists to support this, it is possible that nicotine or a product of nicotine metabolism also suppressed growth in a reversible manner. At least two authors (Chung-Ming, 2005 and Jordanov, 1990) have shown that nicotine accumulates in the developing lung and that its concentration in the amniotic fluid is higher than in the mother’s blood. This means that nicotine, with its oxidant effect, is present in the developing lung which may induce changes in the events that control lung growth, development and aging.

It is also very important to note that in the current study the rat pups began to eat solid food from postnatal day 12, which is towards the end of the phase of rapid alveolarisation. As the animals grew older they ate more solid food and the milk intake gradually decreased and thus also the intake of nicotine via the mothers milk up to postnatal day 21 when they were weaned. 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 after weaning. It is therefore unlikely that changes in lung structure and metabolism that occur after weaning can be attributed to a lack of nutrients or to the presence of nicotine in the tissue of the offspring. Taking these observations into account, it is conceivable that the changes in lung structure reported in this study, and that occur after weaning on postnatal day 21, is due to changes in the factors that controls lung development, maintenance of lung integrity and aging.

In order for the lung to operate effectively as an organ of gas exchange at birth, the lung must have developed a large internal surface area. This is achieved by the formation of millions of thin-walled alveoli, as well as a dense capillary network to ensure proper gas exchange between blood and alveolar air (Harding and Hooper, 1993). Any interference with lung growth may render the lungs less effective as a gas-exchanger and even more susceptible to respiratory disease in later life (Landau, 2006). It has also been well demonstrated that elastic tissue formation by the fibroblast in the alveolar septa plays an important role in alveolarisation (Emery, 1970). Since lung structure and function is closely associated, changes in lung structure will result in compromised respiratory function. The connective tissue framework of the lung plays an important role in maintaining lung structure and function. It also plays an important role in lung development and in particular alveolar formation (Burri, 2006). Elastic tissue in particular plays an important role in normal breathing by maintaining lung compliance (Thibeault, 1999). Damage to the connective tissue framework of the lung will thus result in compromised lung function. It is therefore important to ensure that lung development
in utero as well as after birth is maintained to ensure normal lung function and resistance to disease. This includes adequate nutrition and to refrain from a lifestyle, such as smoking or nicotine replacement, that might compromise the in utero environment where foetal development occurs.

The data from the current study demonstrated that the proportion of alveolar air in the nicotine-exposed offspring was higher at postnatal day 14 than that of the control group. This implies that the volume density of the parenchymal tissue of the 14-day-old nicotine exposed pups was lower than that of the control rats of the same age. However, by day 42 after birth no significant difference were seen in the air and tissue densities in the rat pups exposed to nicotine or to vitamin C only, or to a combination of nicotine and vitamin C. This means that after weaning and thus from the onset of nicotine withdrawal, the volume density of the lung parenchyma of the nicotine rats returned to a level that resembles that of the control animals. It is imperative to note that the volume density of the tissue of the 14-day-old rats that were exposed to both nicotine and vitamin C was the same as for the control animals. This indicates that maternal vitamin C supplementation during gestation and lactation prevented the effect of maternal nicotine exposure on the tissue densities of the lungs of the offspring.

The lung is predetermined to develop a gas exchanger area with enough reserve capacity which can provide an adequate supply of oxygen to the organism and thus to satisfy the energy demands of the body, even during strenuous exercise conditions. The surface area for gas exchange increases as the animal grows by the formation of new alveoli through
septation (Burri, 1974). Any interference with the process of alveolar development during foetal and neonatal phases of lung development may therefore reduce the area available for gas-exchange and thus the capacity of the lung to meet an increase in oxygen demand. It is important to note that the internal surface area and alveolar number of the 14 day-old nicotine-exposed rat pups were lower than that of the control animals despite the fact that the lung volume was not affected by maternal nicotine exposure. This means that the alveolar volume of the lungs of the rats that were exposed to nicotine via the placenta and mother’s milk must be larger than that of the other groups. The data in this study indeed show that the mean alveolar volume of the nicotine exposed rats of the same age was larger than that of the control animals.

The internal surface area of the nicotine-exposed animals was the same as that of the controls at postnatal day 21. This implies that maternal nicotine exposure had no influence on the formation of the secondary septa during the phase of lung development that follows the phase of rapid alveolarisation. On the other hand, at postnatal day 42, the alveolar number was smaller than that of the control animals of the same age. In addition, the alveolar volume was larger than that of the control rats. It is, therefore, not surprising that the internal surface area of the nicotine exposed rat pups was lower than that of the control animals of the same age. It is interesting to note that when the alveolar number is corrected for body weight, the alveolar number/body weight ratio of the nicotine exposed animals was lower than that of the control animals. This means that alveolar formation after weaning was slower than the increase in body weight as the animals mature. It is also plausible that it was due to a slow degradation of the lung parenchyma over time. As
a consequence the increase in alveolar number and thus internal surface area was not proportional to the increase in body weight. Following on that the alveolar surface area available for gas exchange of the lungs of the nicotine exposed rat pups was lower than that of the control animals and consequently also the reserve capacity. This is supported by the morphological data which show signs of emphysema at postnatal day 42. This is further supported by the lower alveolar number/internal surface area ratio of the nicotine exposed animals compared to that of the controls because the rate of alveolar number increase in the controls exceeded that of the nicotine exposed animals while at the same time the alveolar volume of the control animals remained smaller than that of the nicotine exposed animals.

In neonatal rat lung, fibroblast elastin expression peaks during the second postnatal week, that is during the phase of rapid alveolarization, and declines rapidly thereafter (Noguchi and Samaha, 1991). This signify that interference with lung fibroblast integrity at this stage of lung development may result in impaired lung growth and development, this is of significance especially since elastin is associated with the development of alveoli (Kehan, 2005) and thus with the development of the internal surface area for gas exchange to meet the demands of the body for oxygen during resting as well as exercise conditions. The importance of the fibroblasts in maintaining lung structure and function is also demonstrated by the fact that lung fibroblasts from patients with emphysema show a reduced proliferation rate and premature aging which is characterised by slow degeneration of the lung parenchyma (Holz, et al, 2004; Miller and Welker, 2006; Verbeken, et al, 1992). As a consequence the capacity of the lung as gas-exchanger
decreased. It is therefore plausible that the reduced lung internal surface area of the 42-day-old nicotine exposed offspring was due to an inability of the lung fibroblasts to maintain the structural integrity of the lungs, in all likelihood due to premature aging of the fibroblast (Miller and Welker, 2006). This is attributed to altered “programming” due to the changes in the in utero environment that the developing lungs were exposed to (Maritz and Windvogel, 2005). The reason for the altered “programming” is not clear, but it has been shown that oxidants and nicotine can induce point mutations in DNA (Kleinsasser, Sassen, Semmler et al, 2005). This is done by the direct effect of nicotine, by a slower flux of glucose through the glycolytic pathway and by an oxidant/anti-oxidant imbalance, which may all change the “program” that controls lung development and aging (Maritz et al, 2008). The latter can be attributed to the fact that nicotine induces peroxidation of membrane lipids (Kalpana and Menon, 2004).

Since the internal surface area of the current control and nicotine-exposed animals were the same on postnatal day 21, it implies that the effect of maternal nicotine exposure on alveolar formation and maturation was exerted mostly during the phase of equilibrated growth of the lung and after nicotine was removed from the lungs by the normal metabolic processes. It has been reported that between postnatal days 14 and 21, just after the phase of rapid alveolarisation, which ends on postnatal day 13, the number of fibroblast in the lung parenchyma decreases (Burri et al, 1974). This decrease in fibroblast occurs by means of programmed cell death or apoptosis, which peaks between postnatal days 17 and 19 (Bruce, 1999). The decrease in fibroblast numbers can be attributed to the normal development processes associated with the slower rate of alveolar
formation that followed the earlier rapid phase of alveolar formation. Fewer fibroblasts are thus required to meet the demands of the lung at this stage of growth and development.

Apoptosis of fibroblasts in the alveolar walls also plays a key role in thinning of the alveolar septa that occurs after the phase of rapid alveolarisation (Schinttny et al, 1998). The rate of apoptosis after alveolarisation increases owing to a decrease in the Bcl-2 and an increase in BAX in the fibroblast on postnatal day 16 (Yang et al, 1995). Nicotine activates mitogen-activated protein (MAP) kinase signalling pathway, specifically extracellular signalling-regulated kinase (ERK2), resulting in an increased expression of Bcl-2 protein and inhibition of apoptosis (Heusch and Maneckjee, 1998). Thus, while the animals were exposed to nicotine it could, via this signalling pathway, suppress apoptosis and consequently slow apoptosis and thus slower thinning of the alveolar walls. It is probable that the role of this signalling pathway in the control of apoptosis will be the same in the fibroblast of the lungs of the offspring after 21 days of nicotine withdrawal since the half life of nicotine is only about 90 minutes, unless the program that controls expression of these proteins were changed by nicotine during gestation and lactation. This is possible because nicotine can cause point mutations in DNA (kleinsasser et al, 2005). It is also likely that if the fibroblast of the nicotine exposed lungs age faster than in control lung, that it may enhance apoptosis in these cells. The morphometric and morphologic data from this study show that the lungs of the 42-day-old nicotine exposed animals developed emphysema-like lesions. Although no direct evidence is available to support this, it is plausible that nicotine inhibited the proliferation of the lung fibroblast
and fibroblast-mediated responses and therefore in this way contributed to the development of emphysema. It may also have increased the rate of apoptosis and in this way contributed to the induction of emphysema in this study. Studies to investigate this are being planned. This is plausible if nicotine changed the “program” that controls the signalling pathways that control apoptosis.

In the current study it is apparent that fibroblast function was still adequate up to at least postnatal day 21. Since the emphysema-like lesions only developed after nicotine withdrawal on postnatal day 21, it is conceivable that the fibroblast proliferation rate, as well as fibroblast-mediated response may be compromised at a younger age than the average. If this is so it may explain the early development of the emphysema-like lesions that was observed in the lungs of the 42-day-old nicotine exposed rats. It is also possible that maternal nicotine exposure during gestation and lactation, and thus the exposure of the developing lung to nicotine levels that is higher than that in the mother’s blood (Jordanov, 1990), induced changes in the “program” that control fibroblast metabolism and aging. If this is so, it offers a possible answer as to why maternal nicotine exposure rendered the lungs of the offspring more susceptible to damage and emphysema. This is supported by a number of studies that have shown some genes that have been candidates for determining the susceptibility to pulmonary emphysema (Finlay et al, 1997; Smith and Harrison, 1997). With regard to this, it has been reported that the disruption of the klotho gene resulted in the gradual development of pulmonary emphysema in mice. It is possible that maternal nicotine exposure changes these genes that are responsible for the control of lung development and the maintenance of lung integrity after birth as the lungs
mature and age (Finlay et al., 1997). Furthermore, research has shown that long-term nicotine exposure results in a predisposition for the induction of genetic instability (Guo et al., 2005). Gene instability requires two critical elements, namely an inappropriate cell cycle progression, and DNA damage. Long-term nicotine exposure, through the activation of Ras pathways and up-regulating cyclin D1, disrupts the G1 arrest. It also augments the production of ROS which may lead to DNA damage. This implies that exposure to nicotine via tobacco smoking or nicotine replacement therapy to quit smoking will make the lungs more prone to the development of respiratory diseases, and an increased propensity to develop emphysema later in life even if the individual never smoked (Guo et al., 2005).

From the above mentioned findings it can be concluded that not only smoking, but also 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 run. This implies that although no effects will be observed in the short term, nicotine replacement therapy, during pregnancy and lactation will have a long-term effect in the maintenance on lung integrity and respiratory health of the offspring.

5.2. Maternal vitamin C supplementation during maternal nicotine exposure

Apart from being an important anti-oxidant, vitamin C (ascorbic acid) is essential for collagen formation. Vitamin C is a co-factor for hydroxylases and mono-oxygenase enzymes involved in the synthesis of collagen (McGowan et al., 1984). Because subcutaneous administration of nicotine reduces the vitamin C content of adult rat lung
(Maritz and van Wyk 1997), it is possible that the intake of nicotine via tobacco smoking or via nicotine replacement therapy during gestation and lactation, will reduce the blood and tissue vitamin C content of the neonate, thereby rendering it more susceptible to oxidant damage. It may also reduce the vitamin C content of the mother’s blood and thus the potential of the mother to protect the offspring against the harmful effects of nicotine. Maternal vitamin C supplementation during gestation and lactation may overcome the effect of nicotine on the blood vitamin C content of the mother's blood. This will ensure that enough vitamin C is available for development of the foetus and neonate and for its protection against the harmful effects of nicotine. Vitamin C also protects the DNA of the cells from the damage caused by free reactive oxygen species (ROS). It prevents harmful genetic alterations within cells (Gaby and Singh, 1991). Studies by Maritz (1993) also illustrated that maternal vitamin C supplementation during pregnancy and lactation prevented the inhibition of glycolysis in neonatal lung induced by maternal nicotine exposure. This means that vitamin C supplementation maintained a level in the blood of the mother and that of the foetus sufficient to protect the developing lung.

Currently, as expected, maternal vitamin C supplementation and exposure to a combination of vitamin C and nicotine had no influence on the lung volumes of the offspring. The data from the present study also illustrated that the foetal lung was, contrary to expectations, adversely affected by maternal vitamin C supplementation, because vitamin C supplementation resulted in an increase in mean alveolar volumes in the offspring of the experimental animals. The mean alveolar volume of the animals that was born to the mothers who received vitamin C supplements during gestation and
lactation was the same as those that were exposed to nicotine only. This might be due to a pro-oxidant effect of vitamin C (Paolini et al, 1999). If it is so, it means that the amount of vitamin C used to supplement the daily intake of the mother was too high. On the other hand, although the mean alveolar volume of the animals exposed to both nicotine and vitamin C tend to be lower than that of the control animals, it proved not significant (P >0.05), but it was significantly lower (P <0.05) than that of the nicotine-exposed animals. No apparent reason for this is can be attested to. However, since nicotine reduce the vitamin C content of lung tissue, it is possible that the level of vitamin C was too low for it to act as pro-oxidant (Harris, 1996). This conceivably resulted in a vitamin C level in the tissue that was within the levels that will enable it to act as an anti-oxidant and so prevented the oxidant activity of nicotine and other oxidants. It is thus conceivable that the adverse effects of nicotine and of excessive vitamin C was neutralized resulting in normal alveolar volume. Consequently the surface area available for gas exchange of the animals exposed to a combination of nicotine and vitamin C was larger than that of the animals that were exposed to nicotine only.

Tobacco smoking activates neutrophils to generate reactive oxygen species (ROS) in the lung (Tate and Repine, 1984). These reactive species can then interact with the alpha-1-proteinase inhibitor and inactivate this protective enzyme (Pryor, 1986). Vitamin C has been shown to protect human alpha-1-proteinase inhibitor from direct deactivation by nicotine as well as deactivation by free radicals generated by activated neutrophils (Pryor, 1986). However, no white blood cells (WBC) are seen in lungs of vitamin C exposed rats or the rats that were exposed to nicotine during gestation and lactation. Therefore, the
adverse effects of nicotine or vitamin C on the developing lung were not due to influx of white blood cells into the lungs and the consequent release of oxidants into the lungs, but rather to the effect of nicotine and vitamin C at tissue level. The results from this study show that vitamin C supplementation can potentially protect the lungs of the offspring against the adverse effects of maternal nicotine exposure. However, since it can act as pro-oxidant it is necessary to prevent the intake of too high doses of the vitamin, especially when pregnant.

In conclusion, the partial protection by vitamin C can be attributed to the fact that vitamin C is water soluble while nicotine is water as well as lipid soluble (Yildiz, 2004). This may reduce the capacity of vitamin C to protect the lung against the oxidant effects of nicotine. It might be better to use a combination of vitamin C and fat soluble vitamins with anti-oxidant properties such as vitamin E or vitamin A or a combination thereof. This suggestion is supported by a study which showed that a combination of vitamins E and C maintain Bcl-2 in functional form by their membrane stabilizing action (Ramanathan et al, 2005). It also reduced oxidant induced apoptosis (Barrosso et al, 1997). Further studies to establish whether a combination of these vitamins will protect the lungs of the offspring from the adverse effects of maternal nicotine exposure is necessary.
5.3. Maternal nicotine exposure and premature aging of the lungs – Future research

An increase in alveolar volume and a decrease in the number of alveoli is a characteristic of senile emphysema. This is a condition that characterizes lung aging. In the present study it was shown that the lungs of the rats that were exposed to nicotine via the placenta and mother’s milk displayed signs that resemble those associated with premature aging and thus of senile emphysema. This means that maternal nicotine exposure during pregnancy and lactation, and thus during phases of rapid lung cell proliferation associated with lung growth and development, induced changes in the processes that control lung aging and maintenance of lung structure. As a consequence the lungs age prematurely. It is therefore likely that senescent cells will occur prematurely in the lungs of the nicotine exposed rats.

In addition, suppression of glycolysis induces premature aging of cells (Kondoh et al, 2007). Since maternal nicotine induce irreversible inhibition of glycolysis in the lungs of the offspring, it is conceivable that it will result in premature aging of the cells in the lung. Furthermore, experiments with human diploid fibroblasts (HDF) showed a drastic deregulation of carbohydrate metabolism in senescent cells. This is characterized by an imbalance of glycolytic enzyme activities and the failure to maintain ATP levels. This resulted in up-regulation of adenylate kinase and of the levels of AMP, which is known to act as a growth-suppressive signal that induces premature senescence (Zwerschke et al, 2003). This further supports the findings of the present study because maternal nicotine
exposure increases the AMP content of the lungs of the offspring (Maritz and Burger, 1993).

Senescent cells not only lose their ability to divide and to respond to mitogenic stimuli, but also display alterations in morphology and metabolic profile (Bird et al., 2003). This phenotype can be induced by oxidative stress (Balin et al., 2002). It is therefore plausible that factors that induce premature aging of lung fibroblasts will also affect not only growth and development of the lung, but will also adversely affect the maintenance of the lung structure and function since the fibroblasts provide part of the structural support and matrix that is important for its integrity (Absher, 1995).

Zwerschke et al. (2003) reported that about 90% of the consumed glucose in human diploid fibroblasts is converted to lactate. These cells displayed aerobic glycolysis, characterized by a high rate of lactate production from glucose in the presence of oxygen. It is interesting to note that more than 40% of the glucose consumed by lung tissue is also changed to lactate in the presence of adequate amounts of oxygen (Peterson et al., 1984, Kerr et al., 1979). The lactate is produced by the type I alveolar epithelial cells and in all likelihood the interstitial fibroblasts. Other cells such as the type II alveolar epithelial cells and alveolar macrophages have a very active Krebs cycle and respiratory chain which implies that most of the glucose consumed by these cells will be converted to CO₂ and H₂O and ATP (Kerr et al., 1979).
The fact that the lifespan of many species can be extended through caloric restriction, suggests a critical role for alterations of carbohydrate metabolism in the control of regulatory processes that influence cell proliferation and survival (Lane et al., 2001). Studies by Lin and Neubauer (2000) established a new concept according to which changes in carbohydrate metabolism, and in particular the regulation of glycolytic energy production, contribute to the control and regulation of cell proliferation and survival. This indicates that glucose flux through glycolysis is important for normal growth, tissue maintenance and aging.

Studies by Lee et al. (2001) showed that the AMP levels increased drastically in senescent human diploid fibroblasts. Lee et al. (2001) suggested that the increase is in all likelihood due to the up-regulation of adenylate kinase in these cells. Lee et al. (2001) also demonstrated that the expression of genes involved in energy generating pathways in duodenum of aging rats, specifically cytochrome-c oxidase, ATP synthase and sodium-potasium ATP-ase was down-regulated. Studies by Ethier et al. (1989) also indicated an increase in adenosine release from cultured human lung fibroblasts from aged donors, which is likely due to an enhanced breakdown of ATP together with a concomitant increase in the AMP levels in these fibroblasts. The higher levels of AMP serve as a strong anti-proliferative signal on the cells and aging of the fibroblasts. It is therefore conceivable that changes in metabolic control which will result in a down-regulation of glycolysis and of and increase in AMP will result in premature aging of the lung fibroblasts and other lung cells. It was indeed shown by Muller et al. (2006) that lung fibroblasts from patients with emphysema show markers of aging. It is therefore possible
that premature aging will induce development of emphysema earlier in animals that were exposed to nicotine via the placenta and mother’s milk as observed in this project.

Since vitamin C prevents inhibition of glycolysis (Maritz, 1993), it is likely that vitamin C supplementation will prevent premature aging caused by a slow flux of glucose through this pathway. Since vitamin C is not completely prevented the harmful effects of maternal nicotine supplementation suggest that other mechanisms are also involved in inducing structural changes in the lungs of the offspring. Although no direct data is available to substantiate it, it is plausible that it was due to direct effect of nicotine on the DNA if the lung cells.

Further studies are therefore important to establish 1) whether maternal nicotine exposure during pregnancy and lactation indeed induce premature aging in the lungs of the offspring, and 2) if so, whether it is due to an increased oxidant load in the lungs of the offspring of those exposed to nicotine via the placenta and mother’s milk.
CHAPTER 6

Conclusion

Several studies showed that maternal smoking interfere with lung growth and development in the offspring resulting in an increased incidence in respiratory diseases in the offspring (Huber, 1989). Nicotine is implicated as a causative factor and studies indeed illustrated that maternal nicotine exposure irreversible suppress glycolysis and glycogenolysis (Maritz, 1987). It was also shown that maternal nicotine exposure adversely affects structural development of the lungs of the offspring and thus rendering them more susceptible to disease (Dolley, 1995).

The objectives of this study were therefore to:

1. Determine the effect of maternal nicotine exposure during gestation and lactation on lung development from the end of the phase of rapid alveolar formation up to weaning on postnatal day 21, and

2. The larger term effect on lung structure after weaning up to postnatal day 42.

3. And whether vitamin C supplementation protects the lung against the effect of maternal nicotine exposure. Thus, lung integrity will be studied on rats during lactation and after lactation. It is important to note that the animals received nicotine and vitamin C only via the placenta and mother’s blood. After weaning the animals received no nicotine or vitamin C supplementation.
The most important findings are:

1. Maternal nicotine exposure during gestation and lactation change the “program” that control normal aging. This is characterized by a decrease in alveolar number and increase in alveolar volume with a resultant decrease in internal surface area as the lung age.

2. Maternal vitamin C supplementation during gestation and lactation also resulted in an increase in alveolar volume and a decrease in alveolar number. It is proposed that the changes seen are due to the pro-oxidant effect of excess vitamin C intake.

3. Simultaneous exposure of the mother to both nicotine and vitamin C prevented most of the adverse effects on lung growth and development in the offspring.

4. The data suggested that the adverse effect of maternal nicotine exposure is due to its oxidant properties.

Based on the above data it is suggested that:

A. 1. Nicotine replacement therapy not to be used during pregnancy and lactation to quit smoking.

2. Vitamin C supplementation be used with caution.

B. 1. Research be planned to establish whether maternal nicotine intake induce premature lung aging, and

2. Whether premature aging is due to oxidant overload in the lungs of the offspring.
References


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