The effect of maternal nicotine exposure on cell proliferation on the lungs of the offspring

By

Keitumetse Mothibeli
2769501

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Supervisor: Professor G.S. Maritz

KEYWORDS

Key words: tobacco; smoking; maternal; nicotine; cell proliferation; tomato juice; antioxidant; lung development; alveoli ; fetal programming.

ABSTRACT

Tobacco consumption and exposure to tobacco smoke is one of the biggest contributing factors to a growing epidemic of non-communicable diseases (NCDs), primarily cancers, diabetes, cardiovascular and chronic lung diseases which account for 63% of all deaths worldwide (WHO, 2011). An increased concern is in pregnant women who smoke. They not only expose themselves to nicotine, but also their unborn child. Cigarette smoking during pregnancy is associated with many developmental and growth complications. There are critical periods within the "program" that directs normal growth and development, during which the fetus is vulnerable to the effects of external factors. During these critical periods of development the program can be changed to increase the susceptibility of the fetal organs to disease and increased risk of adverse health consequences in adulthood. Health care professionals have tried to reduce the consumption of tobacco smoke by prescribing nicotine replacement therapy (NRT) to pregnant females as an alternative to smoking, without considering the effects of nicotine on the developing embryo and the health risk that might arise after birth. It is known that nicotine induces oxidant formation with resulting oxidative effects. This induces an overload of oxidants in the fetus and a decrease in the antioxidant capacity thereof. This may interfere with normal lung development.

Studies conducted in the past have shown that maternal nicotine exposure during **gestation and lactation** interfere with parenchyma development in the lungs of the offspring and also changes the "program" that controls the maintenance of lung structure in the long term. It was suggested that this could be due to the oxidant/antioxidant ratio imbalance created by nicotine exposure.

The present study addresses two questions. Firstly; does maternal nicotine exposure during **gestation**, affect lung development and function in the offspring postnatally? Secondly, will supplementing the diets of the rats with tomato juice, rich in antioxidants such as lycopene and vitamin C during gestation, prevent the adverse effects of maternal nicotine exposure on the developing lung of the offspring.

Wistar rats were used in the study. After mating, the rats were divided into 4 groups. One group received nicotine (1mg/kg body weight/day) only; a second group received tomato juice supplementation only, while the third group received both nicotine and tomato juice. The control rats were exposed to the same environmental conditions as the experimental groups. Morphological and morphometric techniques were used to evaluate the changes in the lung structure of the offspring at postnatal days 14, 21, 42 and 84.

The study showed that maternal nicotine exposure during gestation resulted in an accelerated increase in the body weight (BW) and lung volume (Lv) of nicotine exposed animals as they aged. The number of proliferating cells in the alveolar wall of these animals decreased with age, and maternal nicotine exposure during gestation resulted in a faster decrease in cell proliferation. A consequence of this is a faster deterioration of lung structure of the offspring later in life which is associated with a decrease in lung function. This change in the proliferating cells of the alveolar wall of nicotine exposed offspring was prevented by supplementing the nicotine exposed mothers diet with tomato juice. This means supplementing the diet of the mother with tomato juice prevented the adverse effects of the nicotine on the proliferating cells in the alveoli of the offspring.

DECLARATION

I declare that "*The effect of maternal nicotine exposure on cell proliferation on the lungs of the offspring"* is my own work, that it has not been submitted for any degree or examination in any other university and that all resources I have or quoted have been indicated and acknowledged by complete references.

Keitumetse Mothibeli November 2013

Signed: 

DEDICATION

This thesis is dedicated to my family and friends for their unceasing prayers and continuous encouragement. Thank you for your unwavering support. A very special thanks goes to my supervisor, Prof. G.S. Maritz, for mentoring me, always encouraging me, and most of all for believing in me. Most importantly I am grateful to God, El Shaddai for being my pillar of strength through all the challenges and lessons.

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AC	abdominal circumference
AMP	adenosine monophosphate
ATP	adenosine triphosphate
α	alpha
β	beta
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BPD	biparietal diameter
BW	body weight
CC	chest circumference
CC/Lv	chest circumference/ lung volume
C	control
COPD	chronic obstructive pulmonary disease
CRL	crown rump length
CRL/CC	crown rump length to chest circumference
Cst	static lung compliance
˚C	degrees Celsius
DNA	deoxyribonucleic acid
Dnp	days postnatally
ECM	extra cellular matrix molecules
EGF	epidermal growth factor
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Fig.	figure

Foxf1	forkhead box protein F1
Foxa2	forkhead box protein A
GSH	glutathione
g	gram
HC	head circumference
H_2O_2	hydrogen peroxide
H&E	haematoxylin and eosin
HNF	hepatocyto nuclear factor
HIER	heat induced epitope retrieval
hrs	hours
IGFBP	insulin-like growth factor-binding protein
IGF	insulin-like growth factor
kg	kilogram
kDa	kilodalton
kJ	kilojouls
Lm	alveolar linear intercepts
Lv	lung volume
Lv/BW	lung volume/body weight
mRNA	messenger ribonucleic acid
M	meters
µL	microliter
µm	micrometer
MTC	mid-thigh circumference
mg	milligram
ml	milliliter

mm	millimeter
min	minutes
N	number of fields counted
N	nicotine
N+T	nicotine + tomato juice
NA	not available
ND	not detectable
nAChR	nicotinic acetylcholine receptors
ng	nanogram
ng/ml	nanogram/millilitres
nmol/l	nanomole/litre
NBF	neutral buffered formalin
NCD	Non Communicable Diseases
No.	Number
NO	nitric oxide
NRT	Nicotine Replacement Therapy
O_2^-	oxygen free radicle
OH^-	hydroxide
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PBS	phosphate buffered saline
RTLF	respiratory tract lining fluid
ROS	reactive oxygen species

RNA	Ribonucleic acid
SD	standard deviation
Shh	Sonic hedgehog
SOD	superoxide dismutase
Spry2	Sprouty homolog
T+N	tomato juice and nicotine
TGFα	transforming growth factor alpha
TGF β	transforming growth factor beta
T	tomato
UL	upper tolerable intake level
VEGF	vascular endothelial growth factor
Va	Volume density
Vt	Parenchymal tissue
WHO	World Health Organization
Ypn	Years postnatally

CHAPTER 1

Introduction

The Fetal origin of adult disease theory was first introduced by Barker (1995). It is a theory used to understand that non-communicable diseases originate through nutritional deprivation of the fetus during "critical periods" of development that forces the fetus to adopt permanent adaptational changes in the physiology, structure and function of organs. It proposes that alterations in the environment within which the fetus develops, such as fetal nutrition and foreign substances, may change the structure, physiology and metabolism of the individual in such a manner that it predisposes the offspring to cardiovascular, metabolic and endocrine disease in adult life (Godfrey & Barker, 2000). Changes in the environment within which the fetus and neonate develops may therefore, have a profound effect on the future health of the individual.

Tobacco smoke contains more than 4000 different chemicals. Approximately 60 of these are tumor initiating or carcinogenic. Approximately 6 million people die from tobacco use and exposure to tobacco smoke worldwide (WHO, 2011). The World Health Organization (WHO) forecasts that 8 million people a year will die of smoking-related illness by the year 2030, making it the single biggest cause of death worldwide, with the largest increase to be amongst the women. The problem of tobacco smoking arises more with pregnant women, as it causes additional health problems. Pregnant women do not only expose themselves to the effects of tobacco smoke but also expose the unborn baby to the serious effects of tobacco smoke. Tobacco use during pregnancy is associated with a wide range of complications such as pre-term labour, reduced placental blood flow and complications to the fetus (Salihu et al.

2003; Wisborg et al. 2001). It has also been linked to increased incidences of abortions and sudden infant death syndrome (Di Franza & Lew, 1995). Studies have shown an increase in the use of NRT as it is believed to be a safer smoking cessation aid than the direct action of tobacco smoking. Yet, NRT depends on nicotine that is found in tobacco smoke and can have toxic effects on various organ systems in the fetus (Bruin et al. 2010; Ginzel et al. 2007). Nicotine is a habit forming substance that is able to cross the placenta from the maternal circulation (Matta et al. 2007) to the fetal circulation. It binds to nicotinic acetylcholine receptors (nAChR) on target cells in the airway, blood vessels, and alveolar cell wall in the fetal lung (Sekhon et al. 1999).

Nicotine found in tobacco is one of the compounds that cause point mutations in the DNA molecule and, therefore, changing the program that controls development and growth of the cells leading to disease in adulthood (Hecht, 1999). Nicotine can damage the fetal lung, heart and central nervous system (Argentin & Cicchetti, 2004). It also passes to the baby through breast milk (Dahlstrom et al. 1990). Consequently, nicotine replacement therapy NRT is highly recommended by many health professionals as safe to assist with the quitting of smoking (Zwar et al. 2006), the effects that may come with nicotine exposure via NRT on the unborn baby are often overlooked.

The effects of nicotine on the offspring will be addressed, because nicotine induces changes in the *in utero* and external environment of the fetus and neonate respectively. During early phases of lung development, the fetal lung experiences rapid cell proliferation (Burri, 1997; Kaplan, 2000). During these phases of rapid cell proliferation, the cells are susceptible to changes in the environment (Barker, 2004). It is therefore important that the mother's diet, contain all the nutrients required for normal growth and development of the neonate.

Secondly, the exposure to foreign substances must be limited. Intake of high levels of oxidants, for example, may result in an imbalance in the oxidant/antioxidant environment into which the fetus develops and in this way interfere with normal development (Fardy & Silverman, 1995). Smoking contributes to the changes in the *in utero* environment (Hanrahan et al. 1992) within which the fetus develops. These changes may result in irreversible changes in the organ structure, function and metabolism during the critical time window that determines normal growth and development of the organs (Barker, 2001; Curhan et al.1996). Studies by Maritz & Thomas (1994) and Maritz & Windvogel (2003) showed that the lungs of rats that were exposed to nicotine via the placenta and the mother's milk were unable to maintain the structural and functional integrity of the lungs in the long term. Maternal nicotine exposure induces oxidative stress (Halima et al. 2010; Helen et al. 2000), and lowers the antioxidant capacity of the lungs of the offspring (Maritz & Wyk, 1997; Windvogel et al. 2008). Studies by Sekhon et al. 1999 have also demonstrated that maternal nicotine predominantly result in the decrease in the volume density of the airspaces of the alveolar region of the lungs of the offspring and in this way increased alveolar size.() This is followed by a decrease in the internal surface area available for gas exchange (Maritz & Dennis, 1998; Maritz & Windvogel, 2003; Sekhon et al. 2001). It has been suggested that these adverse effects of nicotine are due to its genotoxic effects (Argentin & Cicchetti, 2004), its oxidant properties (Hussain et al. 2001), and its capacity to induce oxidant formation in tissue (Sener et al. 2005; Suzuki & Ohshima, 2002). It is therefore conceivable that supplementing the mother's diet with antioxidant rich tomato juice will prevent the adverse effects of maternal nicotine exposure during gestation and therefore maintain of the lung parenchyma of the offspring in the long term.

This study serves to raise awareness on the effects of maternal smoking and NRT during pregnancy on the developing lung. The aim of this study is to determine whether supplementation of the diet of pregnant rats with lycopene rich tomato juice will protect the lungs of the offspring during gestation against the adverse effects of maternal nicotine exposure on the structure of the lungs of the offspring in the long term.

CHAPTER 2

Literature Review

The pulmonary system ensures optimal gas exchange between the atmospheric air and the circulating blood. Therefore, the lung tissue must undergo a series of complex processes of cell proliferation, and branching morphogenesis steps working interchangeably in the developing lung to ensure the development of a functional lung (Stenmark & Abman, 2005).

The development of the airways and vasculature is carefully and precisely controlled through molecular and physical factors. Some of the molecular factors include transcriptional regulators, growth factors, morphogens, and extra cellular matrix molecules (ECM), all of which must be carefully controlled at the right time and place to produce a properly mature and functional lung. The lung is structurally complex, and its growth and development depends on the integration of a multitude of signaling pathways that guide its development during embryogenesis. The master regulator of one of these signaling pathways is Sonic hedgehog (Shh) morphogen. The Shh signaling plays a crucial role in lung organogenesis by guiding the activity of downstream Gli transcription factor. Gli2 and Gli3 are thought to be the primary transcriptional mediators of Shh signaling. Rutter (2008) showed that Gli2 is the primary mediator of Shh signaling influencing embryonic lung growth and proliferation through cyclic regulation.

Much of what is known about lung development has arisen from studies of rodents. The major difference between the mouse and human lung arises from the asymmetric layout to accommodate the heart, with three lobes on the right and two lobes derived from the left primary bronchus, whereas mice have five secondary bronchi, they have only one lobe off the left primary bronchus and four derived from the right (Fig. 2.2). Rats and mice are born with

saccular lungs and they have to function at a more primitive stage than the human lung (Rutter, 2008; Schittny & Burri, 2007). Lung development of humans is almost complete at birth with just part of alveolarization and microvacular maturation occurring up to 2 years postnatally. Other scientist may argue that alveolarization continues well after birth, possibly being completed by +-3 years of age. The total number of alveoli in the fully develop human lung ranges from 300-600 million (Thurlbeck, 1982; Hislop, 2002; Hyde et al. 2004; Ochs et al. 2004).

2.1 Lung development

To understand the influence of various factors on the development of the lung into a mature organ capable of supplying the body with sufficient quantities of oxygen and to remove carbon dioxide from the blood, it is important to discuss the normal development of the lung.

The intrauterine stages of lung development can be divided into five phases, namely, embryonic, pseudoglandular, canalicular, saccular and alveolar phase (Pringle, 1986) (Fig. 2.1, table 2.1). The first phase is the specification of the lung primordium, followed by morphogenesis and cellular differentiation along the trachea. This phase is followed by branching morphogenesis and development of the lung parenchyma and finally, alveologenesis and differentiation of distal epithelial cell types into alveolar type I and type II cells (Minoo, 2000).

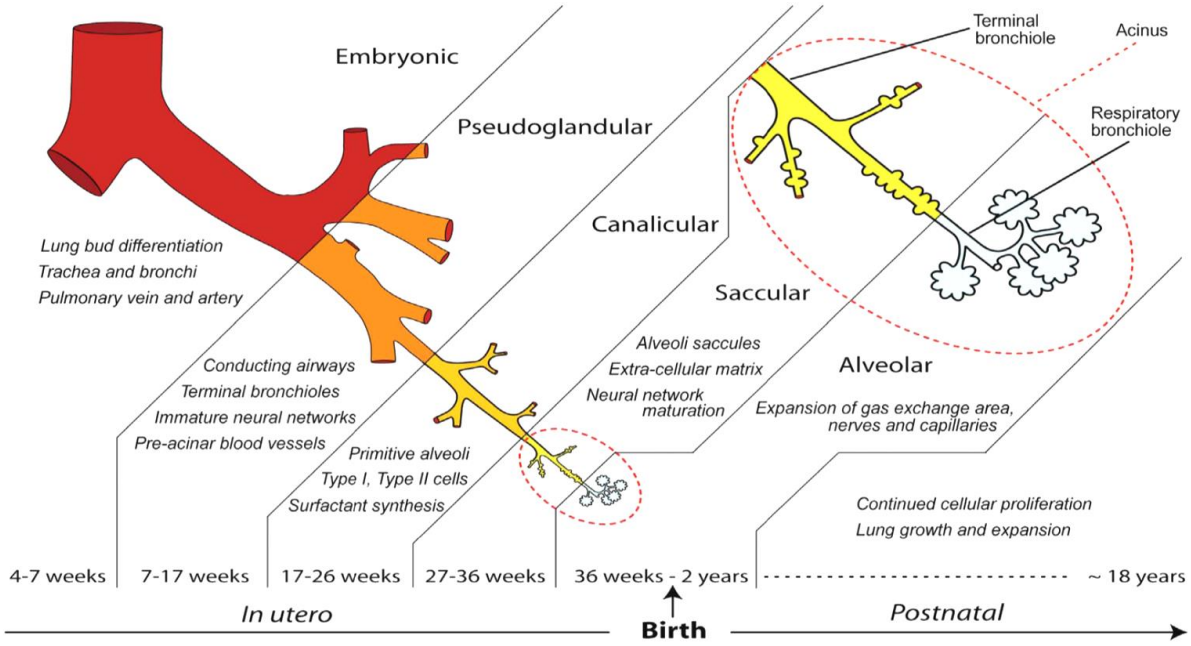


Figure 2.1. Stages of lung development in humans: diagrammatic representations of the timeline and developmental organization of trachea, primary bronchi, intrapulmonary bronchi, and acinus in the mammalian respiratory system. Reprinted from (Kajekar, 2007).

2.1.1 Embryonic phase

The human lung originates as a ventral endodermal pouch from the primitive foregut during the 4th or 5th week of embryonic life, and ends between the 7th (Rutter, 2008) or 8th week of gestation (Boyden, 1977). Lung development begins with the formation of the sulcus laryngotrachealis, which is the formation of a groove in the ventral lower pharynx. A bud forms at different times of development between humans, rats and mice (table 2.1). At this stage it is referred to as a true lung primordium (Kitaoka et al. 1996). After the respiratory primordial pouch is formed, it produces the two bronchial rudiments. The endodermal bud elongates, growing caudally and establishing the asymmetry of the main brochi, where it will divide into the primary left and right lung buds which develop into the airways of a mature lung.

Stage	*Gestational age*			*Main events*
	Human (weeks)	*Rats (days)*	*Mouse(days)*	
Embryonic	3.5 -7	11-13	9.5-14.2	Start of Organogenesis. Formation of lung Bud, tranchea, left and right brochus.
Pseudoglandular	5-17	13-18.5	14.2 -16.6	Establishment of a bronchial tree, preacinar bronchi formed
Canalicular	16-26	18.5-20	16.6-17.4	Formation of the prospective pulmonary acinus, increase of
Saccular	24-38	21-4dpn	17.4-5dpn	Capillary bed. Formation of saccules, alveolar ducts and sacs.
Alveolar	36-2 ypn	4-14dpn	4-14dpn	Formation of alveoli thinning of interalveolar septa
Micovascular maturation	Birth -3ypn	14-21dpn	14-21dpn	Fusion of capillary bed to Single layered network.

Ypn: Years postnatally Dnp: days postnatally

Table 2.1. Phases of lung development between human and mouse (Rutter, 2008; Schittny & Burri, 2007).

By the end of the 7-8th weeks of gestation in humans the developing embryo already has two recognizable left segments (upper and lower lung segments) and three upper right segments (middle and lower right lung segments).

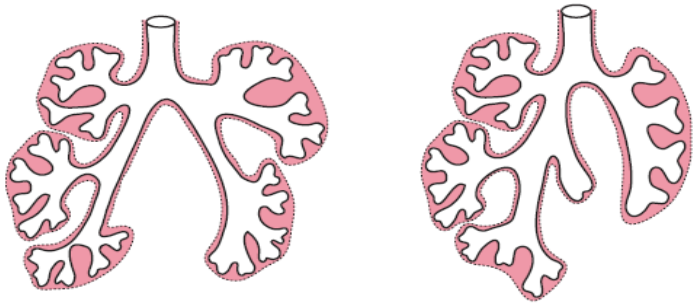
The embryonic phase (table 2.1) of rats start on day 11 and of mice on day 9.5 after mating from two endordermal buds sprouting from the ventral foregut. A trachea (containing two primary lung buds) and esophagus will then form at the same location from a single foregut tube. The two primary mouse lung bronchi will then grow out further into the splanchnic mesenchyme, the right primary bronchi will create four secondary bronchi and the left will

not branch therefore will create one lung lobe, shown in the late pseudoglandular phase (Fig. 2.2). All the bronchi will undergo further branching forming a mature airway tree (Rutter, 2008). The mechanisms and control of this stage is unclear.

2.1.2 Pseudoglandular phase

During the pseudoglandular phase (Fig. 2.3) in humans, bronchial development is complete and the lung has a glandular appearance. The pseudoglandular phase is from the 5th to the 17th week of gestation in humans, and from gestational days 13-18.5 in rats and 14.2 to 16.6 in mice (table 2.1). Airways are lined by columnar epithelium and separated by a poorly differentiated mesenchyme. The mesenchyme plays an important role in the development of the lung into a fully functional organ. The Mesenchyme directs the growth and cytoarchitecture of the lung and regulates the growth and differentiation of the lung epithelium at the cellular-molecular level via soluble growth and differentiation factors that are hormonally regulated. During this stage the rate of cell proliferation is at its highest. Large quantities of glycogen occur in respiratory epithelial cells.

During this branching stage the terminal buds contain a population of multipotent epithelial progenitors (Okubo et al. 2005). As the tubes extend, descendants of these cells give rise to the progenitors of the major cell types of the conducting airways (Perl et al. 2002). The appearance of morphologically differentiated epithelial cells begins proximally and proceeds distally (Perl et al. 1999). By the end of this phase, acinar outlines begin to appear as tubes and they continue to further grow and branch (Boyden, 1977; Crapo et al. 1980).



Human lung Mouse lung

Figure 2.2. Late pseudoglandular stage of the human and mouse lung (Rutter, 2008).

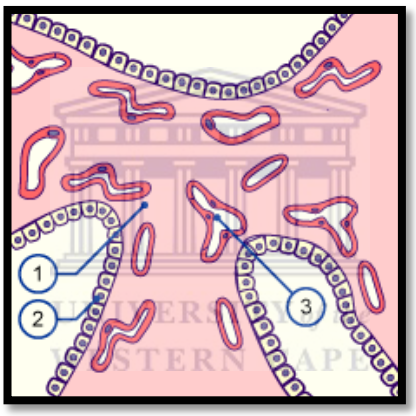


Figure 2.3. Lung tissue in the pseudoglandular phase: Lung has glandular appearance; Airways are lined with columnar epithelium and separated by poorly differentiated mesenchyme. 1) Lung mesenchyma; 2) Type II pneumocytes; 3) capillaries (Voigt et al. 1999).

2.1.3 Canalicular phase

At the 16th week of gestation in human life, and approximately 18 days in rats and 16 days in mice (table 2.1), the fetal pulmonary system enters the canalicular phase (Rutter, 2008). This stage is characterized by the proliferation of the mesenchyme and the development of a rich blood supply within the mesenchyme, and also by a flattening of the epithelium that lines the

airways. There is a rapid formation of capillaries. Along the acinus there is an invasion of capillaries that surround the acini and form a foundation for the later exchange of gases. Flattened type I pneumocytes differentiate from type II pneumocytes, together with the proliferation of the capillaries into mesenchyme (Fig. 2.4). Surfactant begins to appear. This is an important step towards the fetus being able to survive outside the uterus after pregnancy (Kitaoka et al. 1996). At the end of canalicular phase amniotic fluid is produced by the epithelium of the lungs.

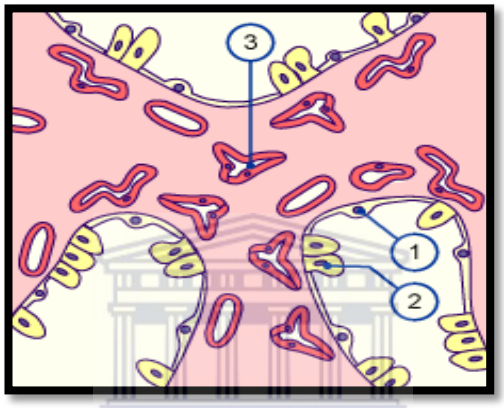


Figure 2.4. Lung tissue in the canalicular phase: Characterized by proliferation of the mesenchyme, development of rich blood supply, rapid formation of capillaries. 1) Type I pneumocytes; 2) Type II pneumocytes; 3) capillaries (Voigt et al. 1999).

2.1.4 Saccular phase

The saccular phase (Fig. 2.5) is between 24 and 38 weeks of gestation in humans and 21-4 dpn in rats (Rutter, 2008; Schittny & Burri, 2007). During this phase in humans, lung development and differentiation, together with the production of surfactant, continues. There is an increased development of capillaries and the alveolar septa develop. During this phase the surface area of the lung is increased due to saccular/alveolar formation. The interstitial spaces at this point are rich with fetal pulmonary fluid and the proportion of collagen and elastic fibers is still small. . There is a close correlation between the appearance of surfactant

in the lung extracts and in the amniotic fluid. Loss of glycogen from the type II pneumocytes is associated with the formation of surfactant.

Fetal breathing movements are essential for lung development (Wiggelsworth & Desai, 1979) and are detected in humans by the 11th week of gestation (Patrick et al. 1980). Lung expansions, together with rhythmic contraction of the diaphragm during fetal breathing, become important contributors to lung growth (Fig. 2.6). Mitogenic growth factors have been shown to be released during these rhythmic stretches (Smith et al. 1994).

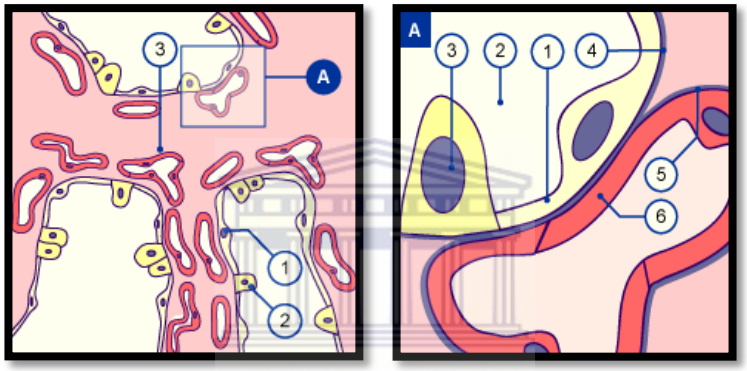


Figure 2.5. Lung tissue in the saccular phase. 1) Type I pneumocytes; 2) Saccular space; 3) type II pneumocyte; 4) Basal membrane of the air passage; 5) Basal membrane of the capillaries; 6) Endothelium of the capillaries (Voigt et al. 1999).

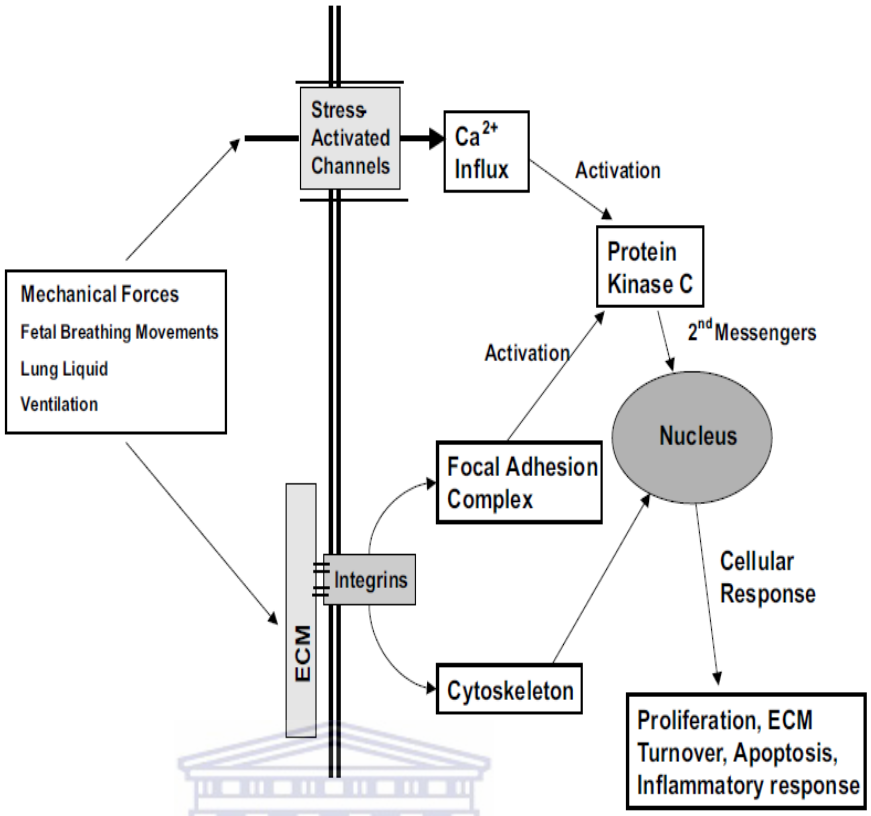


Figure 2.6. The effects of fetal breathing on lung growth and lung development (Copland and Post, 2004).

2.1.5 Alveolar phase

The alveolar phase in humans normally begins in the last few weeks of pregnancy and overlaps microvascular maturation. At this phase, alveoli are formed through a septation process that improves gaseous exchange (Burri, 1984). A third of the alveoli are normally fully developed in humans. Sacculi become more complex in structure and a large number of small protrusions form along the primary septa. These become larger and subdivide the sacculi into smaller subunits of alveoli (Kitaoka et al. 1996). In humans this phase takes place from week 36 of gestation to two years postnatally (Rutter, 2008). From birth to 3 years of age a second phase of alveolarization occurs, characterized by microvascular remodeling and a change from a dual to a single capillary bed (Miller & Marty, 2010; Rutter, 2008).

The alveolar phase in rats occurs during postnatal development and has three phases; lung expansion, tissue proliferation and equilibrated growth. Unlike humans, at birth, rats have lungs with no alveoli or alveolar ducts and it would require about three weeks after birth up to weaning at postnatal day 21 for the lung to fully develop into a mature and functional lung (Bolle et al. 2006). In humans lung development continues postnatally and is most rapid during the first 1-2 years as alveolar numbers increase from 50 million alveoli at birth (Dunnill, 1962; Langston et al. 1984) to 300-600 million in a developed lung (Thurlbeck, 1982; Oochs et al. 2004; Hyde et al. 2004).

Lung expansion is induced by breathing in air at birth. It has been shown that prenatal nicotine exposure might have a major impact on breathing regulation and patterning during the 1st week after birth (Sandberg et al. 2004). Although no direct evidence is available to support this, it is plausible that, together with the disynaptic growth of the airway, maternal nicotine exposure may adversely impact on lung growth and development.

2.2 Cell Proliferation

Cell proliferation is the increase in cell number as a result of cell division and growth. Cell proliferation is controlled by growth factors that bind to receptors on the cell surface. These receptors connect to signaling molecules that convey the message from the receptor to the nucleus. Here the transcription factors bind to DNA, turning on or off the production of proteins that cause cells to continue dividing (MacQuarrie et al. 2011). These factors all play a role in lung morphogenesis during growth and development (Maeda et al. 2007; Costa et al. 2001).

Although the primary focus of this study is to establish whether the exposure of the developing lung during gestation to nicotine via the placenta, will affect lung integrity in the longer term, I will briefly review the underlying factors that affect lung formation and development. This is important because during lung growth and development the lungs are very plastic and sensitive to changes in its environment. This is of particular importance since nicotine accumulates in fetal blood, maternal milk, amniotic fluid, fetal tissues (Dempsey et al. 2002, Luck et al. 1985; Szuts et al. 1978), and is genotoxic (Kleinsasser et al. 2005, Argentin & Cicchetti, 2004). It induces oxidant formation which interferes with DNA integrity (Skinner et al. 2010; Jablonka & Lamb, 2005).

2.2.1 The role of PCNA

Proliferating cell nuclear antigen (PCNA) was originally characterized as a DNA sliding clamp family PCNA is the processivity factor of Polδ and thus is the functional homologue of other processivity factors, the β subunit of the Escherichia coli DNA polymerase III holoenzyme (Pol III) and the product of gene-45 of bacteriophage-T4 (Kelman & O'Donnell, 1995; Wyman & Botchan, 1995). Since DNA polymerase epsilon is involved in resynthesis of excised damaged DNA strands during DNA repair, PCNA is important for both DNA synthesis and DNA repair (Shivji et al. 1992). PCNA plays an important role in nucleic acid metabolism, DNA replication and it is involved in DNA excision repair. It has also been suggested to be involved in chromatin assembly (Singhal et al. 1995). In a period of inactivity, dormancy and senescent the cells PCNA levels are low and with mitogenic stimulation there is a progressive increase in the level of PCNA shortly before DNA synthesis (Celis et al. 1988).

2.2.2 Factors affecting Lung Morphogenesis

Lung morphogenesis is controlled by transcriptional factors, growth factors, extracellular matrix molecules, intergrins, and intercellular adhesion molecules. Together these factors influence the local gene network which direct endodermal patterning and lung branching morphogenesis, left-right asymmetry, vascularization and response to mechanical stress (Fig 2.8) (Copland & Post, 2004). Transcriptional factors play an important role in the control of gene expression during development (Minoo, 2000). Fibroblast growth factor (FGF) FGF-9 and FGF-10 are involved in controlling lung growth through epithelial-mesenchymal signaling mechanisms. FGf10 is critical in airway branching, cell proliferation, coordinating alveolar smooth muscle cell formation and vascular development (Bellusci et al. 1997; Mariani, 2007). FGF9 is involved in mesenchyma proliferation. Both these fibroblast growth factors are necessary to induce Sonic hedgehog (Shh) expression and Shh signaling. Shh with its transcription factor Gli control lung formation where Gli2 is linked to the expression of certain G_1/S Cyclin proteins which are key regulators of cell proliferation.

The first phase of lung development is the development of the lung primordium during the embryonic phase (Fig. 2.1). The lung primordium is formed within the expression of hepatocyto nuclear factor (HNF-3b and HNF-3a) (Minoo, 2000). Shh, FGF8, N-cadherin, activin β and activin receptor II A and HNF 4 influence left and right laterality in the lung (Boettger et al. 1999; Levin, 1997; Meyers & Martin, 1999).

The most crucial mediator of branching morphogenesis is the FGF pathway (Fig. 2.7). FGF10 in the mesechyme binds to FGFr2 which is responsible for budding and branching. FGFr2 found on the epithelium induces Sprouty2 (Spry2). Spry 2 inhibits FGF10. Shh also

downregulates FGF10. Inhibition of Shh and overexpression of Spry 2 causes impairment of branching (Bellusci et al. 1997). Additional factors responsible for branching morphogenesis are transcriptional factors HNF-3, HFH-4, GATA6, N-Myc and NKx2.1 that are expressed in the lung epithelium (Minoo, 2000; Zhou et al. 2007).

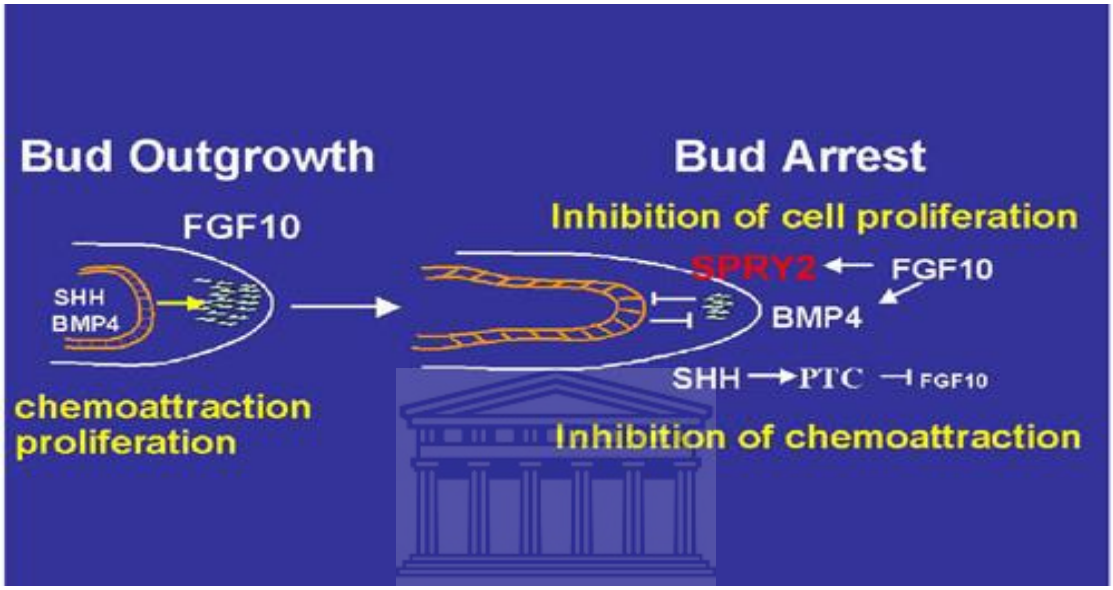
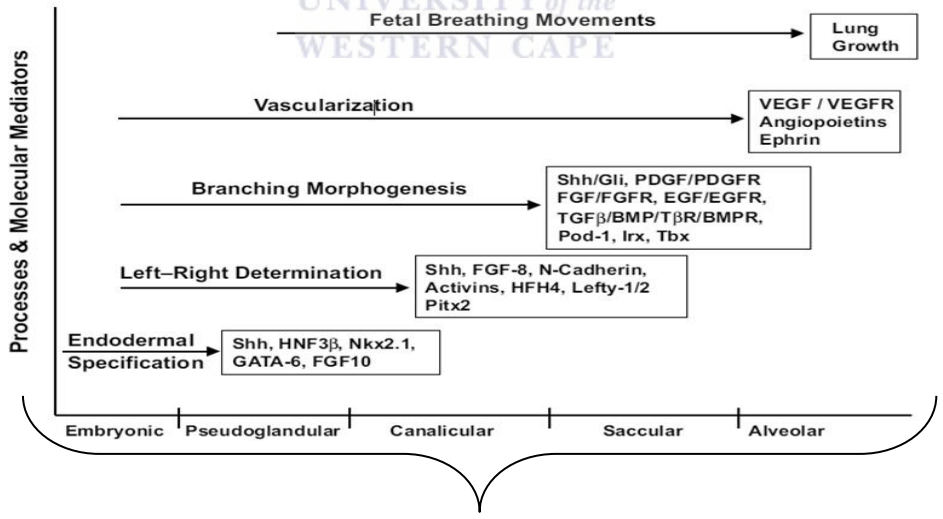


Figure 2.7. Interactions of FGF, Shh and Spry 2 during lung bud outgrowth and lung bud arrest. (Warburton et al. 2003).

Left hand figure: shows a bud that is beginning to extend. (FGF10) expression is shown as a clump of green mesenchymal cells that chemoattracts the epithelium (in orange), towards the pleura (in white). Shh is expressed at low levels, which facilitates the chemotactic activity of FGF10. BMP4 also plays key roles in bud extension.

Right hand figure: The bud has extended and is undergoing bud arrest. FGF10 has induced expression of Spry2 in the epithelium to high levels, which inhibits further chemotaxis in response to FGF10 signaling. BMP4 is also induced at higher levels and inhibits cell proliferation and bud extension. Through Patched (PTC) Shh acts to negatively regulate *FGF10* expression in the mesenchyme near the bud tip. Inhibiting cell proliferation and chemoattraction, resulting in bud arrest.

The growth of the alveoli and the airways are coordinated by the growth of the vasculature (Burri, 1984) (Fig. 2.8) , which is controlled by the growth factor such as vascular endothelial growth factor (VEGF) that induced proliferation, differentiation, survival of endothelial cells and crucial for the development and maintenance of the alveoli . In studies by Tsao et al. (2004) expression of angiogenic factors including VEGF and its receptors (VEGF-R1 and VEGF-R2) increased postnatally simultaneous with alveolirization. Lastly in lung development there is alveologenesis and differentiation of distal epithelial cell types, alveolar type I and type II. This phase of development is controlled by FGF and PDGF signalling pathways (Weinstein et al. 1998). The lineage history of type I and type II cells remains unknown; nonetheless studies done by Minoo (2000) have shown that alveolar type II cells are defined by the synthesis of surfactant protein genes whose transcription is dependent on NKx2.



Phases of Lung development

Figure 2.8. Regulatory factors of lung development (Copland and Post, 2004).

2.2.3 Nicotine and Lung morphogenesis

Nicotine is said to decrease the release of TGF-ß1 a growth inhibitor of many cell types including mesenchymal cells (Minoo, 2000) and increase the release of bFGF and PDGF from endothelial cells (Cucina et al. 2000). Therefore the decrease in TGF-ß1 and an increase in bFGF due to nicotine exposure are suggestive of an increase in cell proliferation and mitogenic activity (Cucina et al. 2000). Nicotine can also impair alveolarization; the process in which it does this is not yet well understood. But it is believed that it is closely associated with altered angiogenesis in the lung (Jarzynka et al. 2006; Heeschen et al. 2001). It is therefore conceivable that interference with the synthesis, control, and release of these growth factors, may also interfere with normal lung growth and development. This may induce changes in lung development and render it more prone to disease later in life.

2.3 Sensitivity of cells to changes in the environment

2.3.1 Ability of fetal cells to protect themselves

The embryo contains genes that will determine the normal development of the individual from the embryonic phase though the fetal and neonatal phase to maturity. A "program" exists in the gene of the developing embryo which directs the growth and development of the individual. Different tissues of the body grow during periods of rapid cell division. These periods are called "critical periods" or "critical windows of development". During periods of normal growth and rapid cell proliferation organs are vulnerable to factors such as lack of nutrients, drugs and external factors, such as air pollution and food additives. The effect of these factors tends to differ based on the phase of development (Miller & Marty, 2010). It has been suggested that an individual's susceptibility to disease in later life may depend on the intrauterine environment during periods of rapid cell proliferation and differentiation (Barker,

2001). Studies conducted by McCance & Widdowson, (1974) showed that nutrition is important during periods of development, and that brief periods of undernutrition may reduce the number of cells in some organs, changing the "programme" that controls normal development.

The fetus main adaptation to a lack of nutrients and oxygen is to slow the rate of cell division. (Barker & Clark, 1997), especially in those tissues that are undergoing critical periods of development at the time. Disproportionate growth can occur because different tissues have critical periods of growth at different times. It is therefore possible that the infant is of normal weight and size at birth but with organs that are not fully developed. This may result in permanent changes in the "program" that determines the growth and the development of those organs that are effected. It is therefore important to note that the utero environment, genetic information from parents, and concentration of growth factors or hormones are important in determining the growth and development of the fetus to maturity and determine the state of health in adulthood. The lifestyle of the parents can therefore severely impact on the "program" that controls lung growth and development as well as maintenance of lung structural and functional integrity in the longer term. Smoking, for example, is associated with a higher incidence of respiratory diseases in the offspring (Oswald & Medvei. 1955; MMWR, 1989).

2.3.2 Fetal onset of adult disease.

Growth depends on the availability of the nutrients and oxygen supply. For example, the fetal experience including nutrition and other environmental factors during postnatal and early postnatal development influence developmental plasticity, therefore altering structure, physiological state and behavior in response to environmental conditions (Barker, 2004). For

instance a well-nourished mother will have offspring that are adapted to affluent conditions, whereas mothers who have a low level of nutrition will have offspring adapted to lean environments. However, if the environment into which the baby is born differs from the one it was adapted to *in utero*, it may result in an increase in susceptibility to disease such as heart disease, diabetes mellitus or hypertension (Barker 1998, 1994; Forsdahl, 1977). Animals develop a variety of characteristics during pregnancy that are well adapted to the environment in which they are likely to live. Therefore, it is correct to say that if the mother's forecast of her offspring future environment is incorrect the health of her offspring may suffer severely, and proper 'forecast' can facilitate survival.

Smoking and nicotine like nutrition, changes the environment within which the fetus develops, and depending on the time of exposure, induce different metabolic and structural adjustments in the offspring. This may determine the response of the offspring in terms of health in the long term.

2.4 The Lung and the natural anti-oxidant pool

The lung acts as a primary portal for the transportation and the entry of oxygen and various oxidants into the body. It is also important to understand that the metabolism of oxygen give rise to a number of reactive oxygen-derived free radicals (Ward, 1986) that require the antioxidant defence system of the lung for protection against oxidation injury. The respiratory epithelium is said to be lined by a respiratory tract lining fluid rich in antioxidants; this overlying layer of fluid has been identified to help against oxidative injury (Kelly et al. 1995, 1999). The antioxidant defence system of the epithelial lining fluid consists of compounds such as reduced glutathione (GSH), ascorbic acid (vitamin C) and uric acid, lipophilic antioxidants such as α-tocopherol (Vitamin E), retinol (vitamin A) and plasmalogens,

antioxidant enzymes such as superoxide dismutases, catalase, and the glutathione peroxidases and metal binding proteins, caeruloplasmin and transferrin (Kelly et al. 1999, Putman et al. 1997). The complex defences of antioxidants in the lungs provide protection of the distal lung structure from damaging oxidant effects. Inflammation in the lung may result in activation of macrophages and neutrophils and the release of free radicals due to respiratory burst. The imbalance between oxidant/antioxidant may contribute to lung injury, which may cause the leakage of capillaries (Messent et al. 1997), altered surfactant metabolism (Putman et al. 1997; Holm et al. 1988; Crim & Longmore, 1995) and diminished surfactant function (Gilliard et al. 1994).

The antioxidant enzyme system of the fetal lung, such as superoxide dismutase, catalase, and glutathione peroxidase, is initially not well developed. Studies on rat, rabbit, hamster, and guinea pig showed that rapid elevations in fetal lung antioxidant enzyme levels occur during the final 10% to 15% of gestation. The increase in the activity of the individual antioxidant enzymes prior to birth averaged approximately 150% to 200%. These findings suggest that late gestational changes in the principal pulmonary antioxidant defense system represents a normal "preparation for birth", required to assure successful functioning of the neonatal lung in the relatively oxygen-rich external environment (Frank & Sosenko, 1987). This implies that the fetus is for most part of gestation dependent on the antioxidant capacity of the mother for protection. This further means that any condition which creates an oxidant/antioxidant imbalance in the mother will render the fetus more susceptible to oxidant induced damage.

The mother's antioxidant capacity can be restored by removing the cause of the oxidant/antioxidant imbalance or by supplementing the mother's diet with antioxidant vitamins. However, it has been shown that supplementing the diet with vitamins can have

detrimental effects. For example the Food and Nutrition Board of the Institute of Medicine in the United States has set an upper tolerable intake level (UL) for vitamin E at 1,000 mg for any form of supplementary alpha-tocopherol per day. Vitamin E can act as an anticoagulant and may increase the risk of bleeding problems but the set UL levels has shown no evidence bleeding problems, based on the result of animal studies (Hennekens, 2007). It has also been shown that antioxidants often are more effective if they are used together with other phytonutrients (Rao & Argarwal, 1999). This means it is safer and more effective to consume fresh fruit and vegetables to maintain a normal oxidant/antioxidant balance.

Antioxidants are substances that neutralize oxygen free radicals, which are normally produced by certain chemicals, smoking and radiation. Antioxidants are naturally accruing in the body and are found in food and may be formulated in a form of a supplement. The most common antioxidants used are vitamin C (ascorbic acid), vitamin E (a-tocopherols) and lycopene (Sies, 1997).

Antioxidant/oxidant imbalances in bronchoalveolar lavage fluid are said to contribute to oxidative stress in respiratory disease (Schock et al. 2001). Antioxidants found in the epithelial lining fluid of the lung contain high concentrations of low molecular weight antioxidants such as ascorbic acid, uric acid, and glutathione. These antioxidants provide the first line of defense against exogenous and endogenous oxidants (Kelly et al. 1995). It is therefore conceivable that depletion of these first line of defense by high levels of oxidants, such as during smoking will render the lungs very susceptible to oxidant damage. By maintaining a normal antioxidant level, the lungs will be protected against oxidant damage.

2.4.1 Lycopene

Lycopene is a bioactive carotenoid present in many fruits and vegetables (Table 2.2). It is almost exclusively found in tomatoes and tomato–based products imparting the red colour. Tomatoes are a rich source lycopene which acts as an antioxidant providing protection against lipid, protein, and DNA damage (Agarwal et al. 2001).

It was reported by researchers at Juntendo University School of Medicine (Kasagi et al. 2006) that lycopene, which is a potent antioxidant, prevented smoke-induced emphysema in mice. It was suggested by them that tobacco smoke induced emphysema in the rats in their experiment by causing an oxidant overload. This is conceivable because tobacco smoke contains 10^{15} of oxidants per puff and hence puts oxidative stress on the lung (Church & Pryor, 1985).

Food	Lycopene (mg/100 g)
Tomato Foods	
Tomatoes, raw	0.9–4.2
Tomato sauce	7.3–18.0
Tomato paste	5.4–55.5
Tomato juice	5.0–11.6
Tomato soup	8.0–10.9
Catsup	9.9–13.4
Others	
Watermelon, fresh	2.3–7.2
Papaya, fresh	2.0–5.3
Grapefruit, pink	0.2–3.4
Guava, raw	5.3–5.5
Vegetable juice	7.3–9.7

Table 2.2. Lycopene in fruits and vegetables (Pohar et al. 2003).

It is therefore plausible that supplementing the diets of the individual that is exposed to tobacco smoke with anti-oxidant rich fruit or vegetables may protect the body against the harmful effects of an overload of oxidants.

In vitro studies have shown that lycopene is an effective antioxidant, a radical scavenger, and an anticarcinogenic (Miller et al. 1996; Mortensen & Skibsteb, 1997; Di Mascio et al. 1989). Due to its potent antioxidant effects it has been thought to be responsible for protecting cells against oxidative damage and thereby decreasing the risk of chronic diseases (Rao & Agarwal, 1999). It has also been shown to modulate hormonal and immune systems as well as metabolic pathways (Fuhramn et al. 1997; Astorg et al. 1997). A study that was conducted at the University of Toronto showed that dietary lycopene supplementation using spaghetti sauce and tomato juice increased serum lycopene by 2 fold and protected serum lipids, proteins and lymphocyte DNA from oxidative damage. It was also proven that lycopene found in tomato paste also increased the serum levels of lycopene by 2, 5 fold but failed when provided in the form of fresh tomatoes (Gartner et al. 1997).This can be attributed to the processing of tomatoes which result in the increase of bioavailability of lycopene also; in fresh tomato about 95% of lycopene is in the trans and about 5% in the cis-form. Upon heating, the relative proportion of cis-isomers can increases to 30% of the total lycopene (Shi & Magure, 2000). In a study done on ferrets by Boileau et al. (2002), it was shown that the cis-isomers of lycopene are preferentially taken up indicating that they are more bioavailable than the trans form. In human plasma the percentange of cis- and trans- isomer is about 50-50 (Clinton, 1998) with different lycopene levels in every tissue (Table 2.3).

Mean Lycopene level and SD,[nmol/g wet weight]		
Tissue	**Human**	**Rat**
Testes	4.4–21.4	NA
Adrenal gland	1.9–21.6	NA
Liver	1.3–5.7	20.30(1.90)
Prostate gland	0.80	0.32(0.06)
Breast	0.78	NA
Pancreas	0.70	NA
Lung	**0.22–0.57**	**0.115(0.015)**
Kidney	0.15–0.62	NA
Colon	0.31	0.046(0.006)
Skin	0.42	NA
Ovary	0.30	NA
Stomach	0.20	NA
Brain	ND	0.017(0.006)

Table 2.3. Concentrations of Lycopene in Human Tissue (Agarwal & Rao, 2000; Stahl & Sies, 1992; Kaplan et al. 1990; Schmitz et al. 1991; Nierenberg & Nann, 1992) and Rat tissue (Jain et al. 1999).Note: NA=not available, ND=not detectable SD=standard deviation.

2.4.2 Lycopene and the lung

The lung is exposed to oxidative stress due to the relatively high oxygen levels that it is exposed to by virtue of its function as a gas-exchanger. It is therefore dependent on a continuous supply of antioxidants to protect itself against oxidative damage. Dietary antioxidants that are found in the lung epithelial lining acts as a first line of defense against this form of damage (Arab et al. 2002). In lung tissue lycopene and β-carotenoids can be measured (Table 2.3), and studies have shown that they provide a significant additional level of protection against oxidative and ozone induced damage. This was supported in clinical trials which showed that the lung accumulates lycopene, and that the supplementation with dietary carotenoids reduced DNA damage as measured by Comet Assay (Arab et al. 2002).

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CHAPTER 3

Nicotine

3.1 Introduction

Nicotine is obtained from the dried leaves of *Nicoticina tabacum* and *Nicotiana rustica*. It is rapidly absorbed from the mucosal surfaces, and it is a habit forming substance. The free alkaloid is readily absorbed from the skin and lungs. Although it is rapidly absorbed from the lungs, it is poorly absorbed from the mouth and nasopharynx (Gori et al. 1986). The internal surface area of the lung, which is about 143 m^2 (Gehr et al. 1978), is a major site of nicotine absorption by smokers (Berne & Levy, 1998; Rubinstein et al. 2012). Inhalation is required to give appreciable absorption of nicotine.

Nicotine is distilled from burning tobacco. An average cigarette contains about 800 mg of tobacco and 9-17 mg of nicotine, of which about 10% is normally absorbed by the smoker. This varies with the type of smoke, and the habit of the smoker (Rang et al. 1999). When smoked, approximately 1.5 mg of the nicotine absorbed by the lung produces a venous nicotine peak level of 20–30 ng/ml. Within about 10 minutes after smoking the nicotine levels drop by about half, and more slowly over the next one to two hours. The rapid decline is due to redistribution between the blood and other tissues. The slower decline is due to hepatic metabolism, mainly by oxidation to an inactive ketone metabolite called cotinine (Benowitz, 1988). Absorption across biological membranes also greatly depends on the pH of nicotine. Nicotine is a weak base with a p*K*a of 8.0 (Fowler, 1954). In its ionized state, found in acidic environments, nicotine does not easily cross biologic membranes. Therefore the basic environment must be favorable for nicotine.

Nicotine is metabolized in the liver by cytochrome P450 enzymes, mostly CYP2A6, and CYP2B6 (Rang et al. 2003; Messina et al. 1997). In non-pregnant women, 70-80% of nicotine is metabolized to cotinine (Benowitz & Jacob, 1994). High levels of cotinine are metabolized to 3'-*trans*-hydroxycotinine (Nakajima et al. 1996; Messina et al. 1997). Equally, nicotine and cotinine undergo *N*-glucuronidation, whereas 3'-hydroxycotinine undergo *O*-glucuronidation (Jacob & Benowitz, 1991; Benowitz & Jacob, 1994; Benowitz et al. 1994). Cotinine has little or no effects on cognitive performance and on cardiovascular effects in humans (Benowitz et al. 1983; Keenan et al. 1994). *Trans* 3-hydroxycotinine, the main metabolite of cotinine, also has no cardiovascular effects (Scherer et al. 1988).

3.2 Nicotine Metabolism during Pregnancy

Nicotine from tobacco or NRT readily crosses the placenta, concentrate in the fetal environment (fetal blood and amniotic fluid) and is absorbed via the fetal skin (Onuki et al. 2003). It is also detected in breast milk during lactation (Jordanov, 1990; Lambers & Clark, 1996).

Dempsey et al. (2002) found that the metabolic clearance of nicotine and cotinine during pregnancy is increased. This resulted in the decrease in the half-life of cotinine and nicotine. In non-pregnant adults the average half-life of cotinine is 17 hrs, whereas that of smoking pregnant woman is 9hrs (Benowitz & Jacob, 1994).

Since the enzymes involved in the metabolism of foreign substances are not yet well developed in the fetus during about 85 to 90% of pregnancy, the metabolism of nicotine in the fetal liver is slow resulting in a longer half-life (Frank & Sosenko, 1987; Walther et al. 1991).

On the contrary, clearance of nicotine and cotinine is increased during pregnancy (Dempsey & Benowitz, 2001). Lambers & Clark (1996) found that there was a higher concentration of nicotine in fetal tissue compared to maternal blood levels. Therefore, the cells of the developing lung and other fetal organs are exposed to higher concentrations of nicotine for a longer period of time than in the mother. This means that the cells of the developing lung are exposed to the genotoxic (Kleinsasser et al. 2005, Argentin & Cicchetti, 2004) and oxidant effects of the nicotine (Bruin et al. 2008) for longer periods of time. Since rapidly dividing cells are more vulnerable to the effects of foreign substances such as nicotine (Rehan et al. 2007), it is conceivable that nicotine exposure during gestation and/or early postnatal life via maternal milk may interfere with growth and development of the tissues.

3.3 Nicotine and the lung

Once nicotine enters the fetal circulation it interacts with nicotinic acetylcholine receptors (nAChRs) in the fetal lung. In a study conducted on fetal monkeys it was shown that maternal nicotine exposure up-regulates α7 nAChR expression in the fetal lung. This is associated with increases in collagen and elastin content of the lung. Maternal nicotine exposure also results in an increase in the number of alveolar type II cells in the alveolar walls of the offspring (Sekhon et al. 1999, 2002; Lieberman et al. 1992; Maritz & Thomas, 1995). This is associated with nicotine induced damage to the type I alveolar epithelial cells which is followed by type II cell proliferation and differentiation to replace the damaged type I cells and thus to maintain the alveolar walls (Berthiaume et al. 2006; Fehrenbach, 2001; Hodes, 1999; Rehan et al. 2009). Interaction of nicotine with nAChRs on the surface of rodent bronchial epithelium promotes cell proliferation (Cattaneo et al. 1997; Minna, 2003) and this contributed to dysanaptic lung growth (Wongtrakool et al. 2007). Fetal nicotine exposure has

largely been associated with reduced blood flow rate in infants of smoking mothers (Hanrahan et al. 1992), impairment in infant breathing (Stick et al. 1996; Wasowicz et al. 1994; Gilliland et al. 2002; Harding,. 1995), and breathing control (Ueda et al. 1999), retardation of lung growth (Collins et al. 1985), decreased elastic tissue staining (Maritz & Woolward, 1992), and induced neuro-endocrine cell hyperplasia in fetal lungs (Van Lommel, 2001; Joad et al. 1995), collectively these changes alter pulmonary structure and function. Nicotine also up-regulates surfactant protein gene expression, where pulmonary surfactant plays an important role in mounting of an immulogical defense, activating intrinsic cellular response (Creuwels, 1997). Nicotine also affects signaling pathways for example, increasing the activity of protein kinase C (Cattaneo et al. 1997; Minna, 2003), and phospholipase C causing the inhibition of apoptosis (Mai et al. 2003).

3.3.1 Nicotine and ROS

Nicotine induces the formation of free radicals and reactive oxygen species (Hecht, 1999) and at the same time reduces the antioxidant capacity of the lung (Dennis et al. 2005). This creates an oxidant/ antioxidant imbalance if not treated. Apart from the excessive amounts of oxidants in smoke (Church & Pryor, 1985), an increased number of inflammatory leukocytes and alveolar macrophages also contribute to the increased oxidant stress in the lungs of smokers (Kilburn & McKenzie, 1975). Smoking generates ROS that interact with mitochondria, and mitochondrial DNA (Droge, 2002) to produce DNA damage and an increase of p53 mutations which may increase the propensity for lung cancer (Liu et al. 2006). It is interesting to note that lung cancer from smokers shows a distinct, unique p53 mutation spectrum that is not observed in lung cancer from nonsmokers (Hernandez-Boussard & Hainaut, 1998). Data from the literature suggests that chronic nicotine exposure weakens

and compromises genomic integrity (Guo et al. 2005). This is partially achieved through ROS generation by nicotine (Guo et al. 2005). Alcohol consumption and tobacco are associated with p53 mutations in non-small cell lung carcinomas. However, alcohol is not a known mutagen and thus apparently enhances the mutagenic effects of cigarette smoke (Toyooka et al. 2003).

Reactive oxygen species (ROS) play an important role in physiological signal transduction and pathogenesis of disease. Normal cells in the body try to remove ROS by using scavenger enzymes in order to avoid the harmful effects of oxidative stress. An example is superoxide dismutase. This enzyme converts superoxide anions into hydrogen peroxide that can be detoxified by catalase and glutathione peroxidase (Kondoh et al. 2003). It is important to note that a balance must be maintained between antioxidant ability of the target cell and ROS, as it is a normal product of cellular metabolism and excessive amounts can cause deleterious effects.

3.3.2 Oxidative stress and the lungs

The respiratory tract lining fluid is the first line of defense against inhaled oxidants because it forms an interface between the epithelial cells and the environment (Kelly, 2003). There are three ways in which oxidants can cause damage to the epithelial cells.

1) A direct interaction of toxins from cigarette smoke, for example, free radicals that penetrates the respiratory tract lining fluid (RTLF) that acts as a protective shield against inhaled oxidants (Cross et al. 1994; Dye & Adler, 1994).

2) Damage of cells by toxic reactive products that are generated by an interaction between cigarette smoke and RTLFs (Dye & Adler, 1994).

3) Lastly, reactions occurring subsequent to activation of inflammatory-immune processes initiated by (1) and/or (2) (Cross et al. 1994, 1984; Dye & Adler, 1994).

Studies by Taylor et al. (1986); Chow (1993); and Cantin & Crystal (1985) have shown that there is an increase in oxidative stress in smokers, and in patients with COPD. Cigarette smoke contains more than 10^{15} free radicals per puff. It also contains a complex mixture of over 4700 chemical compounds (Church & Pryor, 1985). These free radicals cause damage to epithelial cells of the lower respiratory tract through oxidative injury of the membrane lipids, proteins, carbohydrates and DNA. The injury can impair cellular function, induce apoptosis and stimulate dysfunctional matrix remodeling (Hiura et al. 2000). Thus, in smokers where tobacco smoke is inhaled regularly the antioxidant capacity of the RTLF can be severely compromised; even the antioxidant capacity of the epithelial cells can be compromised. This may result in premature aging of cells and a gradual deterioration of the alveolar walls of the lung.

Maternal smoking is associated with an increased level of oxidative stress markers in the mother of the offspring (Noakes et al. 2007; Orhon et al. 2009). Unless these oxidants are neutralized by antioxidants, it will be transferred to the fetus where it may interfere with the cell integrity and thus metabolism. It may also program the lungs of the offspring to become more susceptible to disease in the longer term. Genomic stability may also be compromised. Studies conducted by Orhon et al. (2009) and Hussain et al. (2001) have indeed shown that nicotine exposure results in oxidative stress in fetal, neonatal and adult tissues. It has also resulted in the decrease in activity of the enzymatic antioxidants superoxide dismutase (SOD) and catalase that protect tissues against free radical injury. Studies demonstrated that the transgenic expression of SOD1 in mice protects against the development of cigarette smoke

and elastase-induced emphysema (Foronjoy et al. 2005). It is, therefore, conceivable that the increase effects of maternal nicotine exposure and ROS will not only affect mitochondrial DNA and oxidant/antioxidant balance, but would likely affect energy delivery to mechanisms that control normal development of the lung.

In vitro studies showed an increase in the amount of oxidants (O_2^- and H_2O_2) was associated with an increase in the number of alveolar leukocytes and macrophages in cigarette smokers as compared to non-smokers (Schaberg et al. 1992; Richards et al. 1989; Davis et al. 1988; Ludwig & Hoidal, 1982; Bridges et al. 1985; Van Antwerpen et al. 1995). Nicotine and the oxidants can cause point mutations in the DNA molecule (Jablonka & Lamb, 2005). It is therefore plausible that nicotine can cause a change in the program that controls lung growth, and maintenance of lung structure, in the long term.

3.3.3 Role of glucose metabolism in lung development and maintenance.

Glucose uptake and metabolism is an important and essential pathway for cell proliferation (Holley & Kiernan, 1974; Pardee, 1974) and maintenance of lung structure and is therefore necessary for the functional development of the lung (Bourbon & Jost, 1982; Maniscalco et al. 1978). Glucose is also the main source of α-glycerophosphate important for pulmonary surfactant synthesis in the lung of an adult whereas in fetal lungs the synthesis of surfactant is largely dependent on a loss of cellular glycogen from alveolar type II cells just before birth (Salisbury–Murphy et al. 1966). During the alveolar phase of development, lung tissue is more dependent on glycogen as an energy source and surfactant synthesis. Maternal nicotine exposure suppresses phosphorylase synthesis resulting in lower phosphorylase activity. Consequently the glycogenolytic activity is lower with nicotine exposed offspring. (Maritz, 1986). It has been suggested that maternal nicotine, through irreversible inhibition of

glycolysis and a persistent high level of AMP in the lungs of the offspring, induce premature aging in the lungs of the offspring (Maritz, 1986; Maritz & Burger, 1992; Maritz & Harding, 2011) which may also result in the gradual change in the lung parenchymal structure.

Fatty acids are also important energy substrates for both adults and the developing lung. During fasting the blood fatty acid levels are elevated, replacing glucose as a primary energy source. Glucose is then preserved by the lung for α-glycerophosphate synthesis and ultimately surfactant formation by type II alveolar epithelial cells (Rhoades, 1974). This demonstrates the importance of glucose in maintaining lung structural integrity and function.

Past studies have shown that maternal nicotine exposure irreversibly suppresses glycolysis and glycogenolysis in lung tissue of the rat fetus and neonate (Maritz, 1986; 1987). This can conceivably affect lung growth and development because glucose uptake and metabolism is essential for the proliferation and survival of cells. Glucose is therefore essential for the functional development of the lung (Gilden et al. 1977).

3.3.4 Effects of maternal nicotine exposure on lung structure: A summary

Several studies have shown that maternal nicotine exposure during pregnancy/or lactation results in:

1. Compromised lung connective tissue framework as reflected in:
    a) Decreased elastin staining of lung parenchyma (Maritz & Woolward, 1992; Maritz & Dennis, 1998; Maritz & Thomas, 1994; Maritz et al. 1993).

b) Altered fibrillar collagen expression in developing primate lungs serving as reference of how maternal nicotine exposure can lead to impaired pulmonary function in neonates (Sekhon et al. 1999).

c) Increased the staining of collagen surrounding the larger airways and vessels (Sekhon et al. 1999).

d) Increased type I and III collagen mRNA s and protein expression in airway and alveolar walls and increased airway wall area (Sekhon et al. 2002).

e) Decreased lung copper content, resulting in a lower activity of the copper–requiring enzyme lysyl oxidase in the lungs of the offspring, which is required for crosslinking elastin and collagen in the extracellular matrix (Maritz & Woolward, 1992; Maritz & Dennis, 1998; Maritz et al. 2000) with a consequent compromised connective tissue framework and impaired support of the lung parenchyma.

f) Inhibiting lysyl oxidase activity by copper starvation (Kida & Thurlbeck, 1980) or use of β-aminoproprionitrile, an inhibitor of lysyl oxidase (Das, 1980), irreversibly suppresses alveolar development in the neonatal rat.

2. Compromised lung structure as illustrated by the:

    a) Increased mean linear intercept (Maritz & Woolward, 1992; Maritz & Dennis, 1998; Maritz & Thomas, 1994; Maritz et al. 1993).

    b) Decreased radial alveolar counts and increased alveolar volume indicative of emphysema-like changes in the neonatal lung (Maritz & Woolward, 1992; Maritz & Dennis, 1998; Maritz & Thomas, 1994; Maritz et al. 1993).

    c) Bleb formation of type I cells, flattening of the alveoli as they age and disappearance of alveolar walls leading to larger alveoli (Maritz & Windvogel, 2003).

d) An increase in the type II/Type I cell ratio due to Type II cell proliferation in response to type I cell damage as well as mitochondrial swelling in type II alveolar epithelial cells (Lieberman et al. 1992; Maritz & Thomas, 1995).

e) Increase in the intensity of immunoreactivity of α7 receptor in cartilginous airway wall cells, blood vessel walls, and airway epithelial cells (Sekhon et al. 1999).

f) Increase in the number and size of neuroendocrine cells and the number of alveolar type II epithelial cell (Sekhon et al. 1999).

g) Increase of type I and III collagen mRNA s and protein expression in airway and alveolar walls and increased airway wall area (Sekhon et al. 2002).

3.3.5 Effects of maternal nicotine exposure on lung function

Because of the structure-function relationship in the lung, changes in lung structure will be accompanied by changes in lung function. Maternal nicotine exposure during gestation and lactation results in metabolic and structural changes in the lungs of the offspring as they age (Maritz, 1988; Elliot et al. 2001; Gao et al. 2008). This means that functional changes related to the structural changes will also become apparent as the animal ages. This was supported in studies by Sekhon et al. (2001) who showed that prenatal nicotine exposure of rhesus monkeys altered pulmonary function and:

a. Decreased the lung weight and lung volume with a significant decrease in $FEV_{0.2}$, peak tidal expiratory flow.

b. Decreased mid-expiratory flow (FEF).

c. Increased pulmonary resistance leading to decrease in all other forced expiratory flow rates and dynamic lung compliance..

3.4 Motivation for the study

It has been well documented that maternal nicotine exposure increases the incidence of premature delivery, spontaneous abortions, low birth weight, and neonatal morbidity and mortality (Andres & Day, 2000; Haustein, 1999). Yet, 15 to 20% of women smoke during pregnancy (Coleman et al. 2004; Nelson & Taylor, 2001; Owen & Penn, 1992 - 1999). NRT is widely promoted by health professionals as a safe smoking cessation aid for pregnant women (Ontario Medical Association, 1999), despite the fact that many studies using various animal models showed that maternal nicotine exposure during pregnancy and lactation compromise lung structure and function in the offspring later in life (Maritz et al. 1993; Maritz, 1996; Elliot et al. 2001).

It has been suggested that the "program" that directs normal growth are vulnerable to external factors during critical periods of growth and development, rendering the offspring more susceptible to respiratory disease. This can be attributed to the genotoxic effects of nicotine as well as the production of oxidants during a phase in which the protection mechanisms of the fetal lung is inadequate. Thus, the overall objective of this study is to re-establish the balance between oxidant/antioxidant of the developing lungs by using lycopene in tomato juice as the antioxidant that restores oxidant damage caused by maternal nicotine exposure.

3.5 Aims and objectives

The main objectives of this study are therefore:

- Firstly, to investigate the effects of maternal nicotine exposure during pregnancy on a developing lung of an offspring.

- Secondly, to explore whether tomato juice will have a protective effect against the adverse effects of nicotine, and whether it can restore the function of the developing lung.

CHAPTER 4

Materials and Methods

4.1 Animal preparation

The effect of maternal nicotine and tomato juice exposure during gestation on lung development was performed using female White Wistar rats. The breeding program was maintained in the animal house of the Department of Medical Biosciences at the University of the Western Cape, South Africa. The rats received water and animal food as required. The rats were checked regularly to maintain their health, wellbeing and survival at room temperature of 22 ± 1˚C with a day-night cycle of 12 hours. The rats were mated for a period of seven days. The animals were divided into four different groups, namely:

Group 1: Controls – received water in water bottles

Group 2: Pregnant females received tomato juice only in water bottles. The tomato juice was freely available. The brand of tomato juice that was used was the *All Gold Tomato juice* (containing 8mg of lycopene per 100ml).

Group 3: Pregnant animals received nicotine only subcutaneously. Water was freely available in water bottles.

Group 4: Received both tomato juice + nicotine. Nicotine was administered subcutaneously and the tomato juice was freely available in water bottles.

Seven (7) female rats were assigned to each of the 4 groups. Nicotine and tomato juice was administered as illustrated in (Fig. 4.1). Nicotine and tomato juice was given to the mothers only. The offspring received nicotine and tomato juice supplements via the placenta only. The nicotine solution was prepared weekly by diluting 1mg of nicotine with 100 ml distilled H_2O.

The (1 mg/kg body weight/day) nicotine was administered to the pregnant rats subcutaneously using sterile 1ml tuberculin syringes. Nicotine was administered between 17:00 and 18:00 during gestation only. This dose was kept constant throughout the experiment and not adjusted for increase in body weight (BW) during gestation. The other group was introduced to tomato juice only during the gestational period and this was achieved by replacing their daily water intake with tomato juice. The tomato juice was diluted 50:50 with water. The total volume of the tomato juice and water intake was measured daily for each rat.

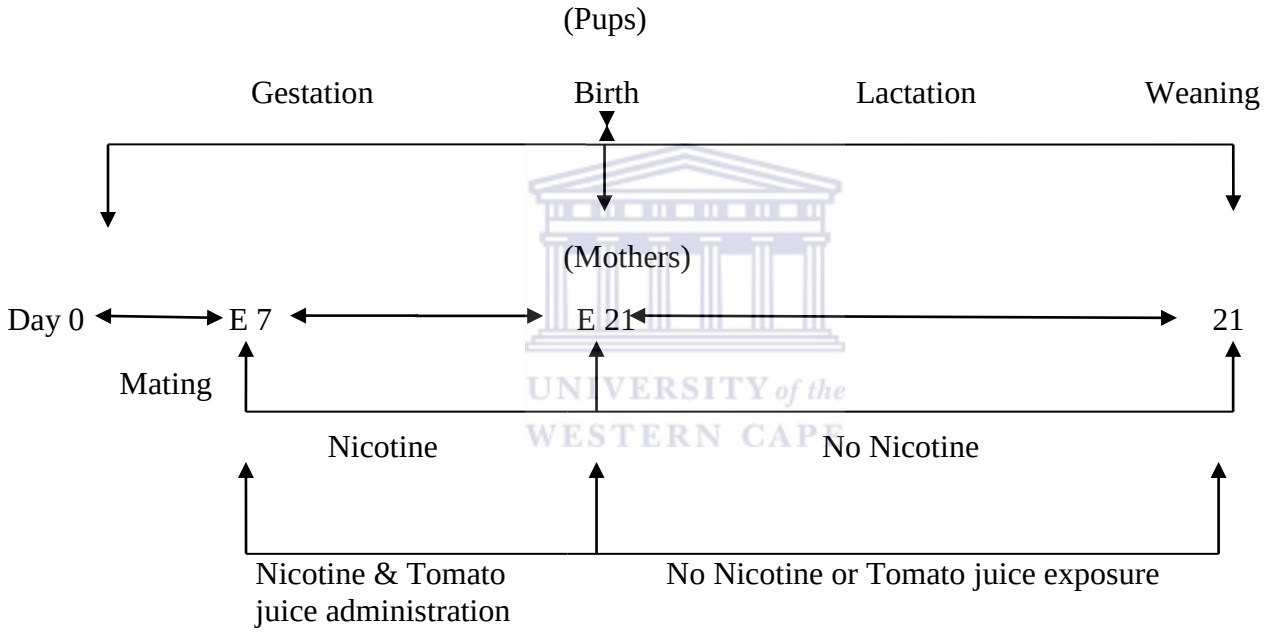


Figure 4.1. Treatment schedule followed -Day 1-7: Mating; Days 7-21: Nicotine or Tomato juice or both N+T, Postnatal day 1-21: No Nicotine or Tomato juice.

4.2 Ethical clearance

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

4.3 Excision of lung tissue

Lung tissue for each of the experimental groups was obtained at postnatal days 14, 21, 42, and 84. In each of the age groups a total of 3 pups where used from 7 different litters in each experimental group. Therefore, a total number of 21 pups were used for each experimental group within each age group.

Body weight was determined by weighing the pups on a top loader laboratory balance (Adam Scale precision balance, PGL 303). Animals were then sacrificed by intraperitoneal injection of an overdose of 6% sodium pentobarbitone solution (100mg/kg of body weight). After the weighing of the pups, the chest circumference and crown-rump length (Fig. 4.2) were measured and recorded. Hereafter the trachea was ligated, and the diaphragm was punctured and lungs were infused with 10% formaldehyde (pH 7.2).

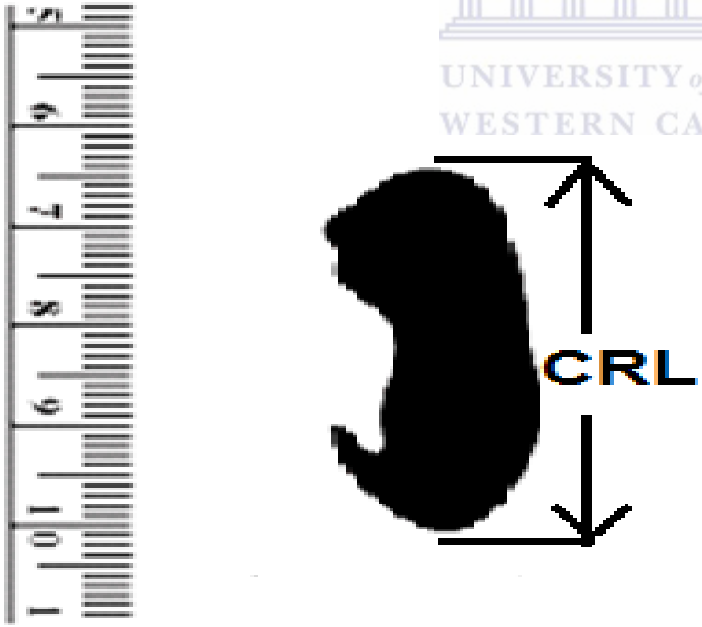


Figure 4.2. Illustration of Crown-rump length.

4.3.1 Intratracheal instillation

Intratracheal installation of a fixative is a reliable, simple 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 (Fig. 4.3) punctured in order to allow the fixative to run into the lungs whilst a transpulmonary pressure gradient of 25 cm fixative was maintained for approximately 30 minutes. The lungs were 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 (Fig. 4.4). Lung tissue was then placed in buffered formalin (pH 7.2) for 24 hours before the histological processing.

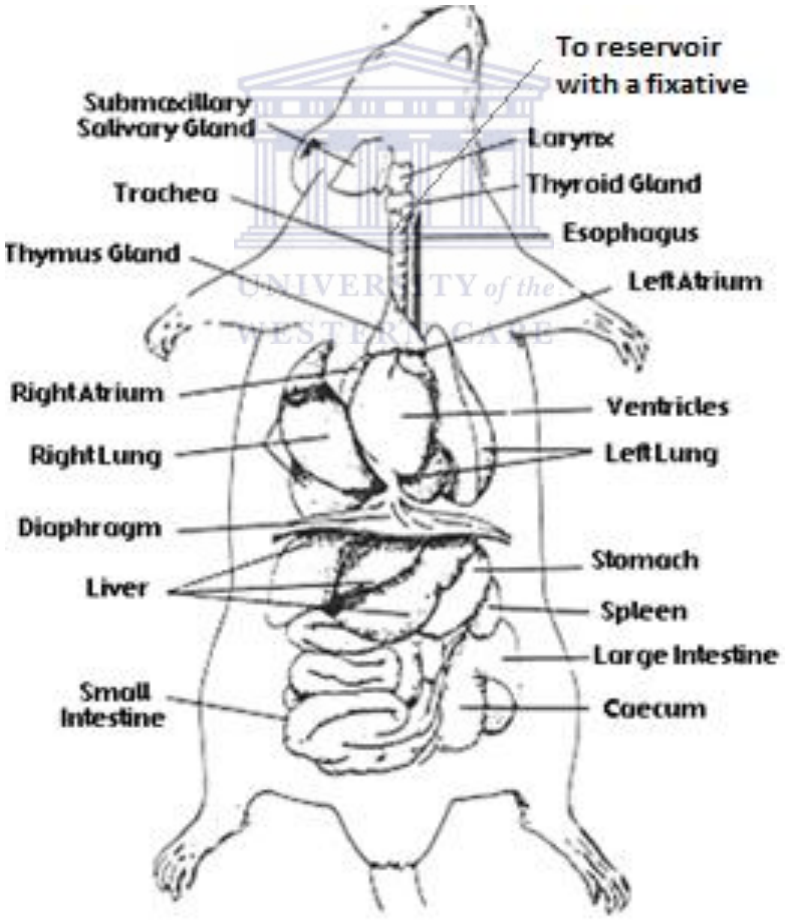


Figure 4.3. Cross-section of a rat (Hickman, 1992).

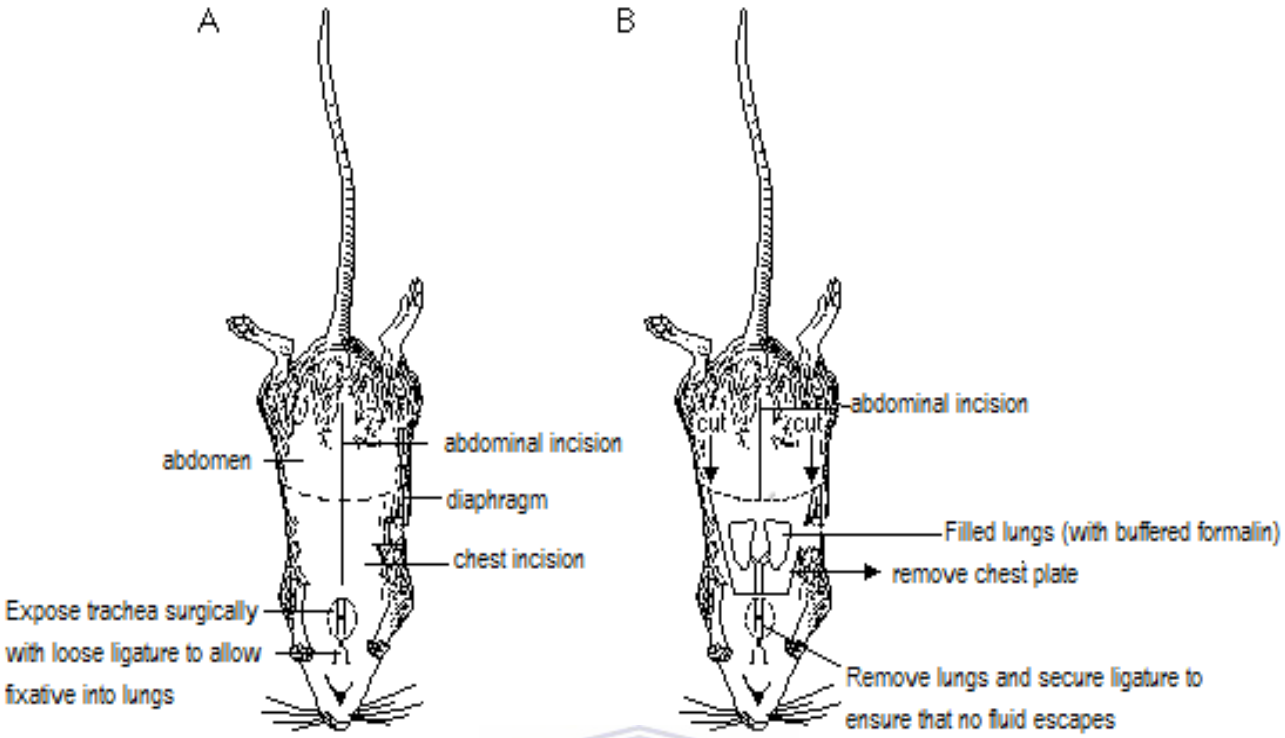


Figure 4.4. Preparation of mouse for collection of lung tissue samples. (A) A midline incision is made to expose the diaphragm and tracheostomize the animal. (B) The diaphragm is then punctured, and lungs filled with a fixative through the trachea. The chest plate is then removed by cutting along the deflection point of the ribs on both sides of the sternum. The lungs are then removed by careful dissection and cutting of the trachea dorsal to the secure ligature.

4.4 Measurement of lung volume

The lung volume was measured by the fluid displacement method of Scherle (1970). A beaker containing physiological saline was placed on a scale (Adam Scale precision balance, PGL 303) which was zeroed. The lung was immersed into the beaker with the aid of a forceps at a level of buoyancy and the initial weight (beaker and saline) was subtracted from the final weight (beaker and saline and 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 10% buffered formalin at the pH of 7.2. The below reagents shown below (table 4.1) were used to make up 10% buffered formaldehyde solution.

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

Table 4.1 Formula for 10% buffered formaldehyde solution.

4.5 Processing and embedding of the lung tissue samples

The lung was carefully removed from the 10 % buffered formaldehyde and placed on a clean glass tile. A sterilized surgical blade was used to cut off the left lobe of the lung. The lobe was then placed in a plastic tissue processing cassette that was properly labeled and placed into a tissue processing rack of the automatic tissue processor (Leica TP 1020). When all the cassettes containing the tissue were ready in the tissue processing rack they were then processed in a programmed tissue processor (Leica TP 1020) using newly prepared reagents (table 4.2) as supplied by manufacturer (Kimix chemicals and laboratory suppliers CC) following an 18 hour cycle.

1. 70% ethanol	2 hours
2. 80% ethanol	2 hours
3. 90% ethanol	2 hours
4. Absolute ethanol	2 hours
5. Absolute ethanol	2 hours
6. Xylene I	2 hours
7. Xylene II	2 hours
8. Wax bath I	2 hours
9. Wax bath II	2 hours

Table 4.2 Histological tissue processing of rat lung tissue.

An embedding system (Tissue embedding center, Tissue – TEK II) was used for this process, prior to completing the embedding process; care was taken to standardize 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 (±1ml) of wax was then run slowly into the mould, ensuring that the tissue sample was fully covered with wax. The mould was then 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. The mould plus cassette was then filled with sufficient wax to permit proper adhesion to the cassette after the waxed solidified.

4.6 Microtomy

When the wax had solidified, the cassettes were removed from the mould. The tissue blocks were trimmed and sections of 5µm were cut using a Leica RM 2125RT microtome for haematoxylin and eosin (H & E) staining and proliferating cell nuclear antigen (PCNA) staining.

4.7 Microscope slide preparation

Sections were made in such a way 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 sections. The 5 µm sections were obtained from the embedded tissue. The tissue was transferred onto labelled microscope slides. This was done by allowing the cut sections of wax ribbon to float in a warm (60 - 70˚C) water bath (Electothermal paraffin section mounting bath) allowing the wax ribbon with the tissue section to flatten for easy picking with the microscope slide. These slides were left overnight to allow the tissue to be fixed onto the slide. 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. The labelled microscope slide was placed on a slide rack and placed in an incubator (Heraeus) at 80˚C for ± 5 min, to further fix the tissue and melt the wax, after which they were then stored in microscope slide boxes until staining could be executed.

4.8 Staining techniques

4.8.1 Mayer's haematoxylin and eosin staining (H&E) preparation.

4.8.1.1 Reagents that were used

Ethanol: Absolute (as supplied by Kimix chemicals and laboratory suppliers, CC); The following concentrations: 90%; 80% and 70% were used. The dilutions were done using distilled water.

All other chemicals were of analytical grade and obtain from Merk & company, Inc.

1. Eosin: Dissolve 3, 0 grams of Eosin and 2, 0 grams of Phloxine in 1 liter distilled water.
2. Mayer's alum haematoxylin: Combine 1, 0 gram haematoxylin and 50, 0 grams of potassium Alum and dissolve in 20 ml 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, and remove from heat and allow cooling.
3. Scott's Tap water: Dissolve 2, 0 grams Sodium Bicarbonate and 20, 0 grams Magnesium Sulphate in 1 litre distilled water.
4. 1 % Acid Alcohol: combine 1, 5 liters of 96 % Ethanol with 0, 48 liters of distilled water and 0, 02 liters of concentrated Hydrochloric acid. Mix well to form a homogeneous solution.
5. D.P.X.: Use as supplied by the manufacture.
6. Xylene

4.8.1.2. Haematoxylin and Eosin stain protocol.

1	Xylene (X 2)	5 minutes each
2	100% Ethanol (X 2)	5 minutes
3	Ethanol 90 and 80 %	5 minutes
4	Haematoxylin	15 minutes
5	Rinse in tap water	1 minute
6	Scott's tap water	2 minutes
7	Rinse in tap water	50 seconds
8	1% acid alcohol	2 minutes
9	Rinse in tap water	50 seconds
10	Eosin	3 minutes
11	Rinse in tap water	1 minute
12	80, 90, 100 % Ethanol	2minutes each
13	Xylene(X 2)	2 minutes each
14	Mount for Observation	

Table 4.3 Procedure of Haematoxylin and Eosin stain.

1. A slide rack containing labeled microscope slides with tissue section was immersed in fresh xylene for 5 minutes. This process was repeated in a second microscope slide staining dish containing fresh xylene for another 5 minutes to deparaffinize of to de-wax.

2. The sections were then transferred to absolute alcohol to initiate the rehydration process. This process was continued by repeating the procedure described above

3. The rehydration process was continued by placing the slide rack in a series graded percentage alcohol (90%, 80%) as described in table 4.3.

4. The slides were rinsed in running tap water for 5 minutes.
5. Staining was performed in Haematoxylin for 15 minutes and rinsed under running tap water to remove excess stain.
6. Sections were blued in Scott's tap water for 2 minutes, and then rinsed under tap water.
7. They were then differentiated in 1% acid alcohol and rinsed under running tap water.
8. These slides where then stained in Eosin for 3 minutes, rinsed in running tap water to remove the excess stain.
9. Sections were dehydrated for 2 minutes each in 80%, 90% and absolute alcohol.
10. The slides were cleared in two successive xylene baths for 2 minutes each.
11. The slides were then mounted in DPX and used for morphometric techniques using the light microscope

4.8.2 Procedure: Proliferating cell nuclear antigen (PCNA) stain used to measure cell proliferation.

4.8.2.1 Background

PCNA is a 36 kDa nonhistone protein found in the nucleus, and plays a role in the initiation of cell proliferation by mediating DNA polymerase. PCNA levels are elevated in the S, G2, and M phases of cell mitosis in normal and malignant tissues. PCNA expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation. PCNA has

proven useful for proliferative studies of normal and neoplastic tissues both *in vivo* and *in vitro* (Waseem & Lane, 1990; Robbins et al. 1987; Linden et al. 1992).

Invitrogen's PCNA staining kit (Histostain-Plus kit) was obtained from Invitrogen/Life technologies uses a biotinylated PCNA monoclonal antibody (clone PC10), thus eliminating the need for a species-specific secondary antibody. As a result, PCNA staining is exceptionally clean and can be performed in tissue and cell samples from most species, including: human, primate, mouse, rabbit, rat, yeast, and insect without background caused by cross-reactivity with endogeneous immunoglobulins. Streptavidin-peroxidase is used as a signal generator, and DAB as the chromogen, to stain PCNA-containing nuclei a dark brown. This kit also contains 5 PCNA positive control slides for the convenience of the investigator; 1 stained reference slide and 4 unstained slides for use in the procedure.

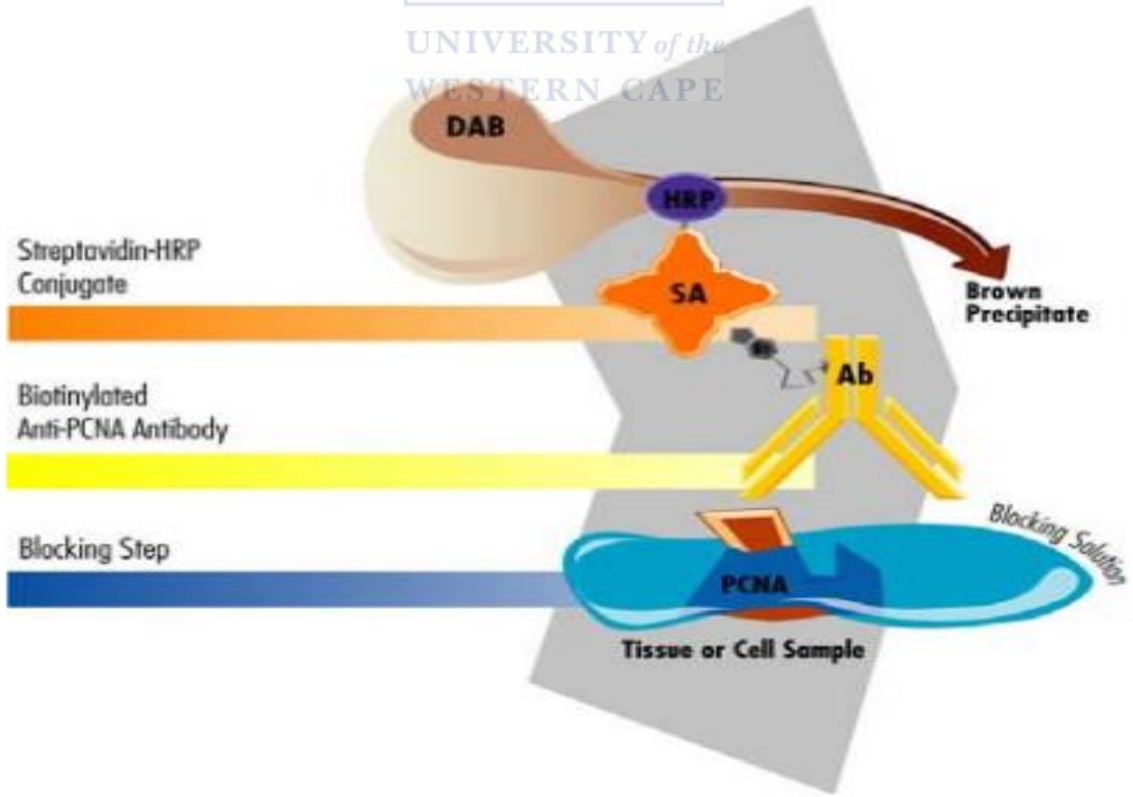


Figure 4.5. Process of staining for PCNA (Invitrogen's PCNA staining kit).

4.8.2.2 PCNA slide preparation

1. Labeled microscope slides, as described in microscope slide preparation on p.44 were deparaffinized in 2 changes of xylene for 5 minutes each. Then the slides were rehydrated in a series of graded alcohol as described in table 4.3.

2. PCNA staining was then performed following the steps as set out in table 4.4.

4.8.2.3 Reagents

- Phosphate Buffered Saline (PBS)
- Alcohol and xylene
- Distilled water
- Quenching solution for endogenous peroxidase
- Coverslips

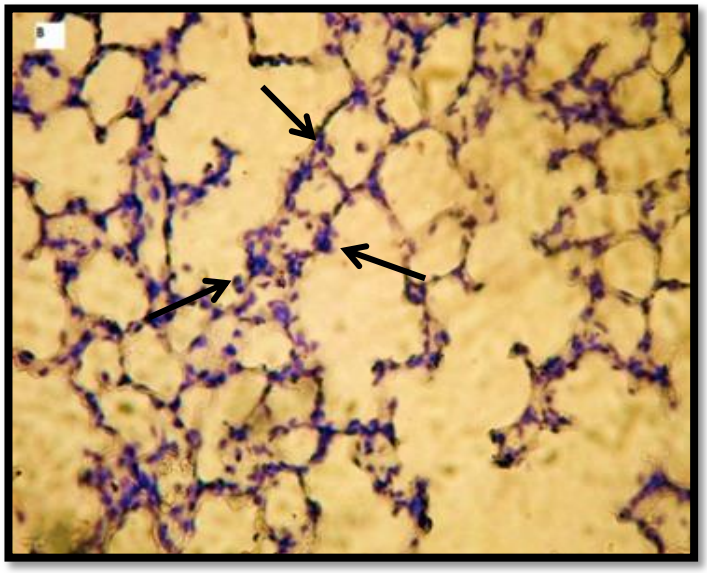


Figure 4.6. PCNA stain illustration of the number of proliferating cells per100µm length of alveolar wall.Arrows show proliferating cells stained dark brown (See arrows).

4.8.2.4 PCNA stain

Reagent	Specimen Preparation	Incubation Time (Min.)
1. BLOCKING SOLUTION	a. Add 2 drops (100 µL) or enough to completely cover tissue, of BLOCKING SOLUTION to each specimen. b. Incubate at room temperature. c. Drain or blot off the solution. Do not rinse	10 minutes
2. BIOTINYLATED MOUSE ANTI-PCNA PRIMARY ANTIBODY	a. Add 2 drops (100 µL) or enough to completely cover tissue, of PRIMARY ANTIBODY to each specimen. b. Incubate in moist chamber c. Rinse with PBS for 2 min., 3 times.	30-60
3. STREPTAVIDIN-PEROXIDSE	a. Add 2 drops (100 µL) or enough to completely cover tissue, of STREPTAVIDIN-PEROXIDASE to each specimen. b. Incubate at room temperature for 10 minutes. c. Rinse with PBS for 2 min., 3 times.	10
4. DAB CHROMOGEN	a. Add 1 drop of Reagent 4A, 1 drop of Reagent 4B and 1 drop of Reagent 4C to 1 mL distilled or deionized water. Mix well. Protect from light and use within one hour. b. Apply 2 drops (100 µL) or enough to completely cover tissue, of DAB CHROMOGEN to each section. Incubate for 2-5 min.	2-5
5. HEMATOXYLIN	a. Apply 2 drops (100 µL) or enough to completely cover tissue, of HEMATOXYLIN to each specimen. Incubate for 1-2 min. b. Wash slides in tap water. c. Put slides into PBS until sections turn blue (approx. 30 seconds) d. Rinse in distilled water	1-2
6. HISTOMOUNT	a. Dehydrate slides in a graded series of alcohol, and clear with xylene b. Add 2 drops (100µL)of histomount and a coverslip	

Table 4.4. Procedure for PCNA stain as indicated by the kit.

4.9 Morphology and Morphometric methods

The following parameters were used to determine the effects of maternal nicotine exposure, exposure of the pregnant mothers to nicotine and tomato juice, or to tomato juice only, on the developing lung of the offspring. Lung tissue from the left lobe at postnatal days 14, 21, 42 and 84 was collected for morphometric tests. Tissue samples were collected for light microscopy according to unbiased techniques described by Weibel (1963).

The morphometric data of males and females growth parameters at postnatal days 14 and 21 was pooled since no differences were observed between the males and females.

4.9.1 Volume density of the airspaces (Va) and the volume density of the tissue of the lung parenchyma (Vt).

4.9.1.1 Introduction.

A gradual breakdown of the alveolar walls is associated with a decrease in the volume density (Vt) of the lung parenchyma, with a concomitant increase in the volume density of the airspaces (Va). A consequence of the destruction of the alveolar walls and the resultant decrease in Vt, will be a merging of adjacent alveolar walls which will result in larger alveoli and a reduction in the surface area available for gas exchange.

4.9.1.2 Method

Standard H&E staining was used to prepare tissues for morphometric techniques. The alveolar air volume density (Va) and the alveolar tissue volume density (Vt) was determined as demonstrated by (Bolender et al. 1993). Vt was achieved by using a point counting technique at 100x magnification. A 122-point eyepiece graticule was used. At least 5 non-overlapping fields were counted for each lung tissue slide. The 5 fields were from 4 female and 4 male animal lung tissue slides within each experimental group. Non-parenchyma tissue included bronchus and blood vessels which had a diameter of >1.1mm.

The alveoli that contributed to the count included:

- Those that were found within the graticule, and

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

The alveoli that did not contribute in the count included:

- Those found outside the square on the upper and left side of the graticule.
- Areas that contained non-parenchymatous tissue were excluded from the counts.

A) Determination of Vt

Example of Vt calculations:

Field	Number of points counted
1	30
2	25
3	29
4	28
5	34
Total	**146**

Where:

Vt = total no. of points counted/total no. of fields

= 146/5

= 29.2/122 points (on the grid)

= 23.93%

B) Determination of Va

The alveolar air volume density (Va) was achieved by subtracting Vt from 100 to determine the percentage of the alveolar air space and the volume available in the lungs of the offspring after maternal nicotine exposure, both nicotine and tomato juice and tomato juice only.

Example of Va calculation: Control day 84 animals had a Vt of 28.79%

Therefore:

Va = 100 –Vt

= 100-28.79%

= 71.21%

4.9.2 Determination of mean linear intercept (Lm)

4.9.2.1 Introduction

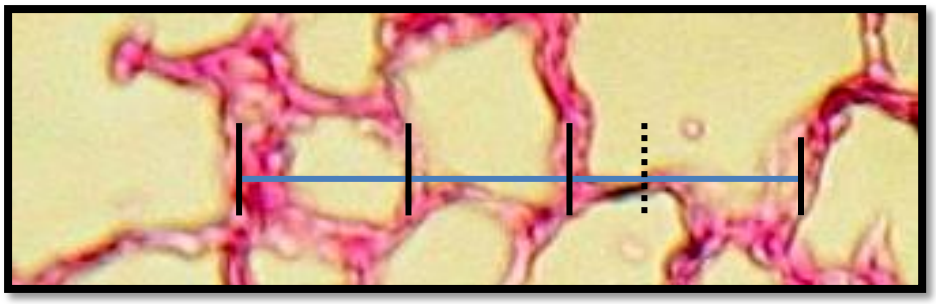
The mean linear intercept (Lm) is the average distance between the walls of an alveolus (Dunnill, 1962). During normal lung maturation, the Lm decreases with decreasing air-tissue interface. In emphysematous lungs the Lm is increased due to loss of alveolar walls (Thurlbeck, 1967). Standard H & E staining was used for morphometric techniques using the light microscope. A public domain software, ImageJ 1.45k Scriptable Java application software for scientific image processing was used to quantify the data.

4.9.2.2 Method

- An intercept where the cross line passes through the alveolar wall = 2 intercepts.

  The line just touches an alveolar wall = 1 intercept.

  Example showing intercepts:



9 intercepts (solid black =2 intercepts, dotted line =1 intercept)

- The number of alveolar intercepts (m) was determined using ImageJ a Java-based image processing program on H&E slides images. For each slide 5 non-overlapping fields were used to determine the mean linear intercept of that sample.

The linear intercept was calculated as follows:

Lm = L x N/m

Where,

N = number of fields counted

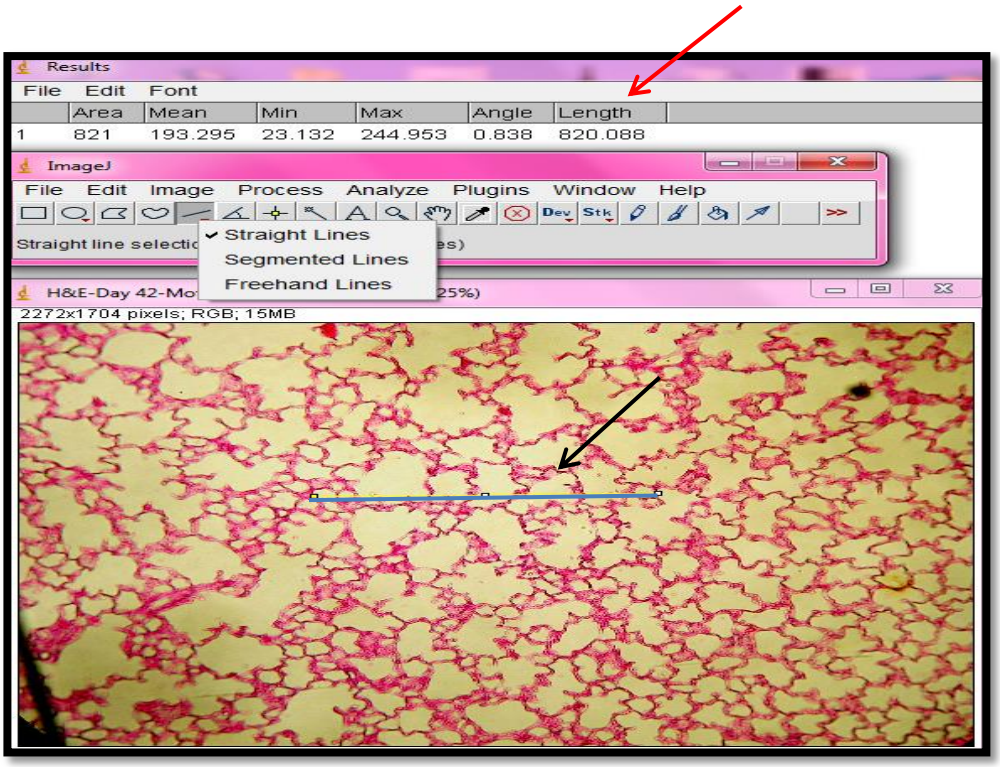
L = length of cross line

m = sum of all intercepts

The alveolar wall that contributed to the intercept count included:

- Those that touched the cross line
- Those that crossed the line (see blue line in figure below).

Example: showing field 1 analysis out of 5:



- Arrow black: Straight line (blue) represent length of cross line.
- Arrow red: Length of area containing the sum of all intercept.

Field	number of intercepts	length of cross line
1	20	820.088
2	26	622.048
3	35	868.021
4	29	760.011
5	32	994.002
Total	142	**Average** 4064.17/5=812.83

Where:
812.84 µm = average length of the cross line
142 = total # of intercepts

Lm =812.83 x 5/142

= 28.62µm

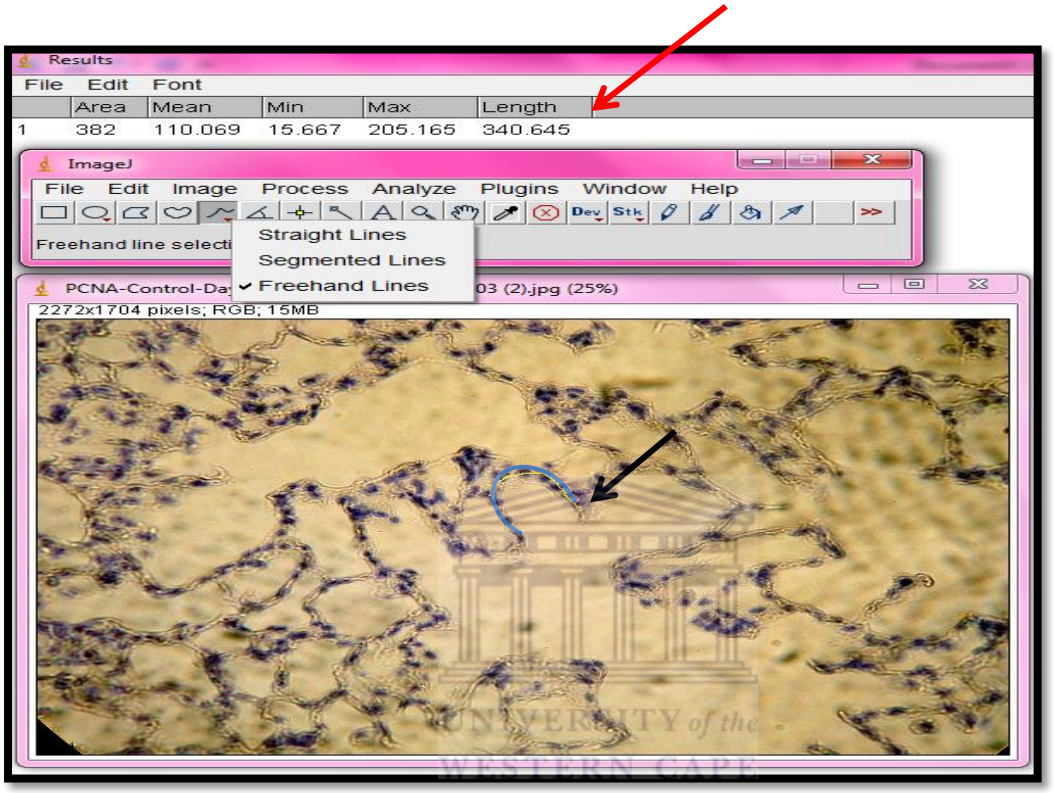
4.9.3 Cell proliferation

4.9.3.1 Method

Standard PCNA staining was used to prepare tissues for morphometric techniques using the light microscope.. The PCNA slides where analyzed using ImageJ a Java-based image

processing program. For each slide 5 fields where used. Freehand lines were drawn along the proliferating cells, and the number of proliferating cells that lied on the line were counted and analyzed.

Example:



- Arrow black: freehand line (blue) drawn along the proliferating cells, and the number of proliferating cells that lay on the line are counted.
- Arrow red: Length of alveolar wall containing proliferating cells
- Proliferating cells were calculated as follows;
- Proliferating cell/100µm=N*100/L

Where,

N= number of proliferating cells counted

L=length of area containing proliferating cells. Lines were drawn starting with a proliferating cell and ending with a proliferating cell. These 2 cells as well as those between it were counted).

Proliferating cells = 6*100/340.645=1.76 cells/100µm.

4.10 Lung compliance

Lung compliance (ml/cmH_2O/kg), as a measure of the ease of expansion of the lungs, was calculated using: lung volume, a constant transpulmonary pressure gradient of 25 cm and the body weight in kg.

4.10.1 Lung compliance was calculated as follows:

Lung compliance = Lv/25/body weight

Where;

- Lv =Maximal lung volume determined as described before; see p 42.

  Distending pressure = transpulmonary pressure gradient of 25 cm H_2O.
- And corrected for body weight

4.11 Average amount of fluids consumed a week.

The following data was collected daily with each pregnant female rat:

- Body weight (g)
- Fluid consumed (ml)

Average amount of liquids consumed = ml/kg body weight/week

Example: Tomato juice/week

Fluid drank per week=97.33ml

Body weight=372.67g

Body weight/1000=372.67/1000=0.37kg

Fluid consumed/week=97.33ml/0.37kg/7days=37.59ml/kg/week.

4.11.1 Lycopene intake is calculated as follows:

Amount of lycopene in 100ml =8mg/100ml tomato juice (lycopene was diluted 50:50 of water; therefore 4mg/100ml).

Example:

How many mg lycopene /kg/week are in 36.19 ml/kg/week where: 4mg lycopene per100ml?

Calculation:

4mg lycopene/100ml (diluted tomato juice)

X / 36.19ml/kg/week

X = 36.19 (ml/kg/week) x 4/100ml

=1.45mg lycopene/kg/week

Where X=the unknown factor (mg lycopene/kg/week)

4.11.2 Energy intake was calculated as follows:

Energy intake=70kJ/100ml tomato juice

Energy intake= 70kJ x amount of liquids consumed x (tomato juice was diluted 50:50 with hence the 35kJ/100ml.

Example:

35kJ/100ml

X /36.19 ml/kg/week

X=35kJ*36.19(ml/kg/week)/100ml

=12.67kJ/kg/week

Where X=the unknown factor (kJ/kg/week)

4.12 Statistical analysis

Results were analyzed using standard error bars and a one-way analysis of variance test (ANOVA) for unpaired data used by Student-Newman-Kuels 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 5

Results

5. A weekly average amount of liquids consumed by pregnant female rats during gestation only.

The volume of liquid consumed per week was recorded for the controls, nicotine rats, those receiving both nicotine and tomato juice, and those receiving tomato juice only. The volume of liquids consumed was recorded to determine whether the fluid consumption by the pregnant rats that received lycopene via tomato juice was any different from those that received water only.

The data show that the average volume of liquids consumed per week (table 5 (i) and Fig. 5 (i)) by pregnant control female rats, those exposed to nicotine only, and those exposed to both tomato juice and nicotine remained constant ($P>0.05$) from week 1 to week 3. On the other hand, the volume of tomato juice consumed by the group that received tomato juice only tend to be higher at week 1 of gestation ($P>0.05$) but only reach significance at week 2 (41.18 ± 3.51ml/kg/week) ($P<0.01$) than the average volume of water consumed by the control group (23.63 ± 1.34 ml/kg/week). The amount of lycopene (1.65 mg lycopene/kg/week) and energy intake (14.41kJ/kg/week) consumed was highest at week 2 to week 3 (1.45 mg lycopene/kg/week) (12.67kJ/kg/week) of tomato exposed rats, which was consistent with the volume of tomato juice consumed. The energy intake of the rats was higher ($P<0.05$) than that of the animals that received both nicotine and tomato juice.

5(i) Amount of liquids consumed a week (ml/kg/week)

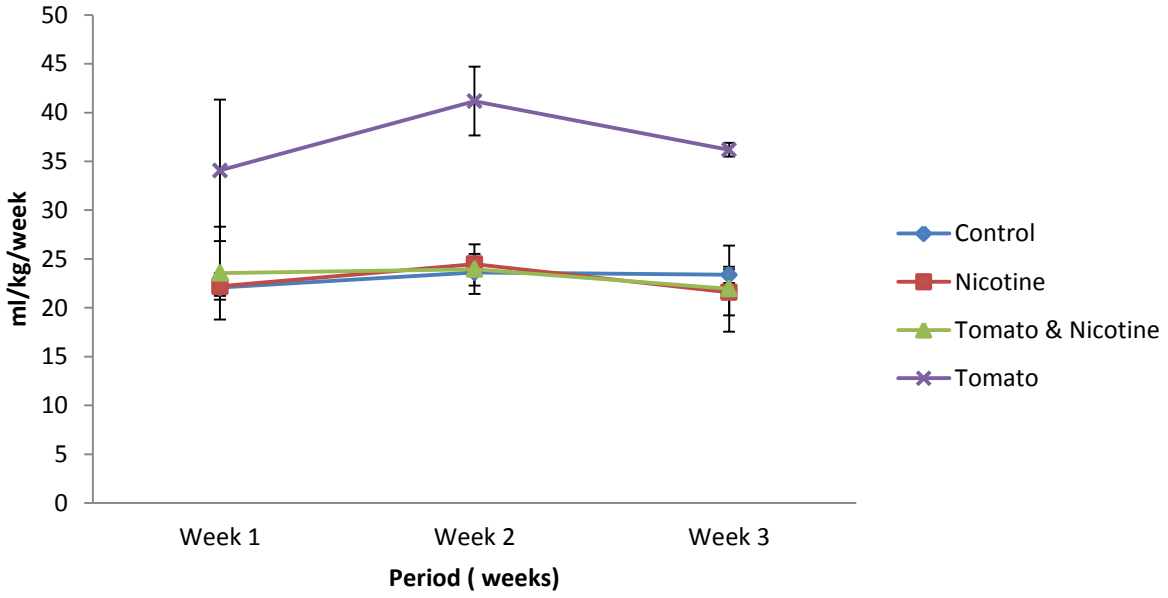


Figure 5 (i). The average volume of fluid by pregnant female rats from week 1 to week 3 of pregnancy (ml/kg/ week).

Amount of liquids consumed a week (ml/kg/week)							
Week	**Control**	**Nicotine**	**T+N**	**Tomato juice**	**Control vs. Nicotine**	**Control vs. T+N**	**Control vs. Tomato juice**
Week 1	22.07±0.83	22.21±1.36	23.56±4.76	34.08±7.26	*P>0.05*	*P>0.05*	*P>0.05*
Week 2	23.63±1.34	24.49±1.05	23.96±2.55	41.18±3.51	*P>0.05*	*P>0.05*	*P<0.01*
Week 1vs. week 2	*P>0.05*	*P>0.05*	*P>0.05*	P>0.05			
Week 3	23.38±0.84	21.60±2.36	21.96±4.41	36.19±0.71	P>0.05	P>0.05	P<0.001
Week 3vs. week 2	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			
Week 3 vs. week 1	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			

Table 5(i). The volume of liquids consumed by pregnant female rats during gestational phase, where the control and nicotine groups consumed water only.

Lycopene intake (mg lycopene/kg/week)		
	N+T	**Tomato juice**
Week 1	0.94	1.36
Week 2	0.96	1.65
Week 3	0.88	1.45

Table 5(ii). A weekly average intake of lycopene in a form of tomato juice by pregnant female rats between week 1 and week 3.

Energy intake (kJ/kg/week)		
	N+T	**Tomato juice**
Week 1	8.25	11.93
Week 2	8.39	14.41
Week 3	7.69	12.67

Table 5(iii). A weekly average energy intake in a form of tomato juice by pregnant female rats between week 1 and week 3.

5.1. The influence of maternal nicotine exposure during gestation, or receiving tomato juice supplementation only, or nicotine + tomato juice supplementation on growth parameters of the offspring.

It is possible that maternal nicotine exposure during gestation may interfere with the placental-fetal blood supply and thus nutrient supply. If this is so, it will affect growth and development of the fetus. This will be reflected in the body weight and other growth parameters at birth and even later in the life of the offspring. Supplementing the diet of the mother with tomato juice may also have an impact on the growth of the fetus and neonate because of the additional energy (35kJ/100 ml tomato juice) that the pregnant animals have access to in addition to the normal dietary intake. Therefore, the growth parameters were determined as the animals aged to determine whether those animals that received tomato juice and thus additional energy, grow faster than the control group as well as the nicotine group.

The body weight (BW), lung volume (Lv), chest circumference (CC), crown rump length (CRL) and other growth parameters of the 14- and 21-day-old male and female rats were not recorded separately since no differences were observed between males and females of the control and experimental groups. No differences in the growth parameters between the control and experimental groups were evident either. Differences in BW only became apparent at postnatal day 42. Therefore, only the changes in BW at postnatal days 42 and 84 are described.

5.1.1 Body weight (BW)

Postnatal day 42

The data show that the BW (table 5.1and Fig. 5.1(a)) of the 42 day-old control rats was the same ($P>0.05$) as the rats receiving nicotine only, rats receiving tomato juice only, and those

receiving both nicotine and tomato juice during gestation (P>0.05) for both male and female rats. The BW of male rats (138.54±6.01g) exposed to nicotine was higher (P<0.05) than that of the female rats (112.25±5.02g) of the same experimental group. This means that nicotine had no effect on the BW of the males but resulted in a lower BW at postnatal day 42 in the females.

Postnatal day 84

At postnatal day 84 the BW (Fig. 5.1(b)) of the male and female control rats was lower (P<0.05) (table 5.1) than the rats that received nicotine only, or tomato juice only, or those receiving both nicotine and tomato juice. The BW of control **female** rats (171.49±11.62g) was 15.46% (P<0.05) lower than the BW of female rats receiving nicotine only (202.85±3.92g) and 19.31% (P<0.005) lower than the BW of the female rats receiving both nicotine and tomato juice (212.54 ±3.42g). The BW of control **male** rats (241.23±12.75g) was 20.79% (P<0.005) lower than the BW of male rats receiving nicotine only via the placenta (304.56±10.22g) and 17.70% (P<0.05) lower than the BW of male rats receiving both nicotine and tomato juice (293.13±17.96g).

Up to postnatal day 42 (table 5.1) the BW of the male and female rats was the same. From postnatal day 42 the BW (table 5.1.1) of the male rats increased faster (2.55 g/day) than that of the females (1.14 g/day) so that at postnatal day 84 the BW of the control males was 40.7% higher (P<0.001) (Fig. 5.1(b)) than that of the control female rats. This trend was the same for all the other experimental groups. It is however, important to note that the daily increase in the BW of the male and female experimental groups was higher than that of the control male and female animals.

5.1.1(a) Body weight (BW)

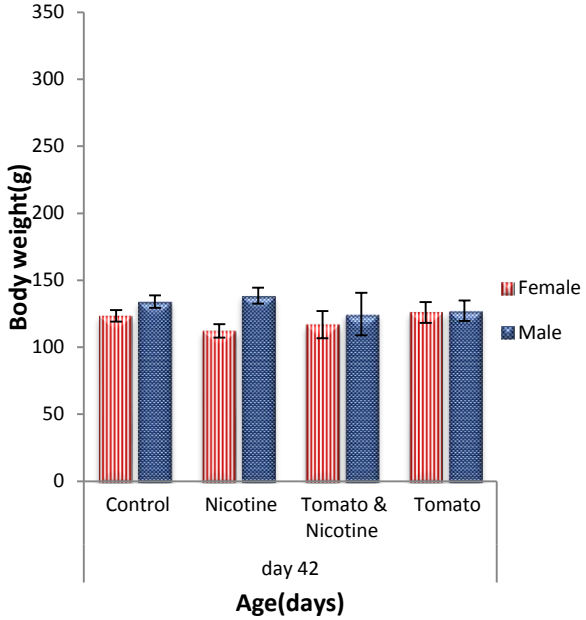


Figure 5.1(a).The effect of nicotine, or nicotine + tomato juice, or tomato juice only on body weight of 42-day old female and male rats

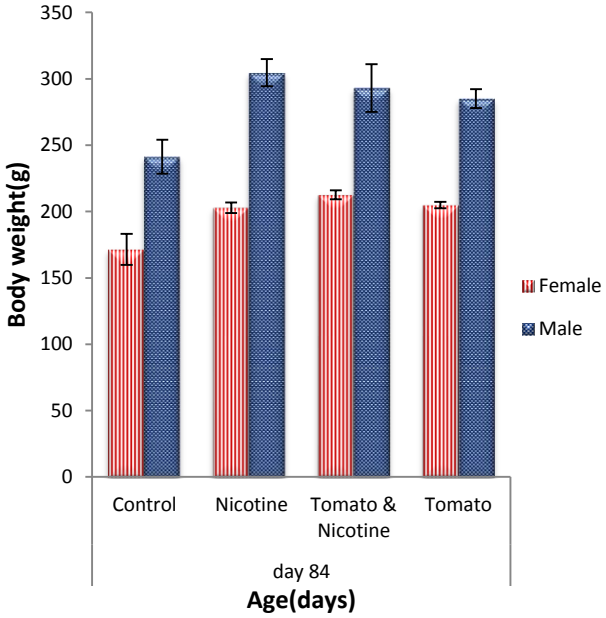


Figure 5.1(b). The effect of nicotine, or nicotine +tomato juice or tomato juice only on body weight of 84 day-old female and male rats.

Body weight (g)								
Age in days	**Body weight**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Tomato +Nicotine**	**Control vs. Tomato**
42	Female	123.49±4.24	112.25±5.02	116.89±10.11	126.03±7.77	*P>0.05*	*P>0.05*	*P>0.05*
	Male	134.13±4.73	138.54±6.01	124.68±15.86	127.28±7.55	*p>0.05*	*P>0.05*	*P>0.05*
F42vs. M42		*P>0.05*	*P<0.05*	*P>0.05*	p>0.05			
84	Female	171.49±11.62	202.85±3.92	212.54±3.42	204.89±2.41	*P<0.05*	*P<0.005*	*P<0.05*
	Male	241.23±12.75	304.56±10.22	292.13±17.96	285.12±7.12	*P<0.005*	*P<0.05*	*P<0.05*
F 84vs. M84		*P <0.001*	*P<0.001*	*P<0.001*	*P<0.001*			

Table 5.1. The effects of maternal nicotine exposure, or tomato juice only, or both nicotine and tomato juice on the body weight (BW) of the male and female offspring.

Daily increase in body weight (g)				
	Control	**Nicotine**	**N+T**	**Tomato**
Female	1.14	2.16	2.28	2.14
Male	2.55	3.24	3.06	3.03

Table 5.1.1. The effects of nicotine, tomato juice, nicotine + tomato juice exposure on daily increase in body weight between days 42 and 84.

5.1.2 Lung volume (Lv)

Postnatal day 42

Although the Lv of the rats that were exposed to nicotine via the placenta, as well as that of the other experimental groups, tends to be higher than that of the control rats, the Lv (table 5.2 and Fig. 5.2 (a)) of the control **female** rats (5.28±0.31ml) at postnatal day 42 was the same as that of the experimental groups ($P>0.05$). The Lv of the nicotine exposed **male** rats (7.13±0.57ml) and those male rats exposed to tomato juice only (6.46±0.42ml) was higher ($P<0.05$) than that of the control male rats (5.34±0.30ml). Contrary to the previous results, the Lv of the control males was the same as that of the rats that were exposed to both tomato juice and nicotine during gestation.

Postnatal day 84

Between postnatal days 42 and 84 (table 5.2 and Fig. 5.2 (b)) the Lv of the **female** control rats increased from 5.28±0.31 ml to 8.08±0.71ml ($P<0.005$), and that of female rats exposed to nicotine from 6.39±0.43 ml to 9.58±0.47ml ($P<0.001$). The Lv of the female rats that were exposed to tomato juice was at 9.73±0.25 ml also higher ($P< 0.05$) than that of the control females. Likewise, at postnatal day 84 the Lv of the offspring exposed to both nicotine and tomato juice during gestation was at 11.41±0.61ml also higher ($P<0.005$) than that of the control females of the same age.

As for the female rats, the Lv of the 84-day old **male** rats that were exposed to nicotine (13.72±1.05ml), or to tomato juice supplement only, or to nicotine and tomato juice was at 12.06±0.47ml and 14.16±0.87ml respectively, significantly higher ($P<0.05$ and $P<0.001$

respectively) than that of the control male rats (10.17±0.58ml). The difference between the control males and females and the respective experimental groups at postnatal day 42 was in general the same at postnatal day 84 (table 5.2.1).

As expected the Lv at postnatal day 84, (Fig. 5.2(b)) of the **female** rats of the control and experimental groups was lower (P<0.05) than that of the **male** rats.

5.1.2(a) Lung volume (Lv)

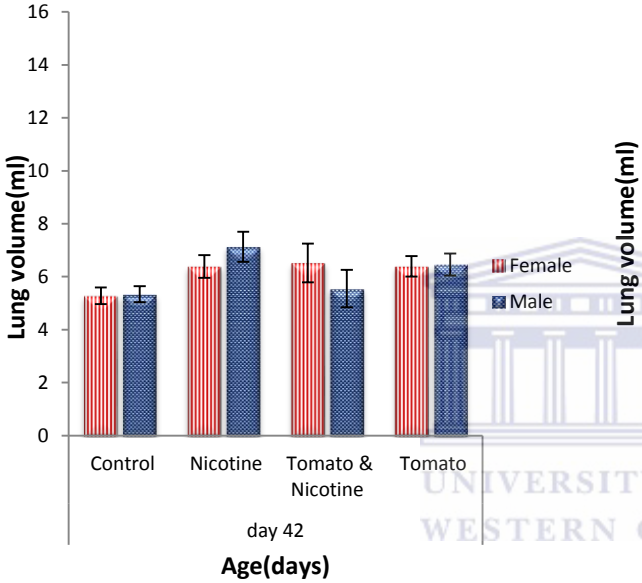


Figure 5.2(a). The effect of nicotine, or nicotine + tomato juice, or tomato juice only on lung volume of 42-day old female and male rats.

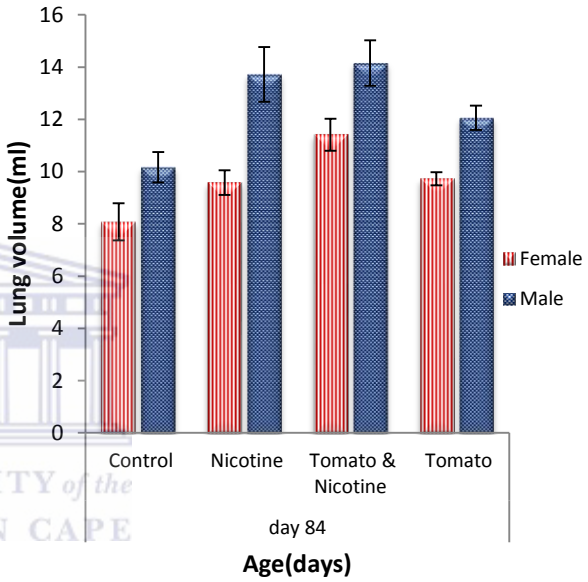


Figure 5.2(b). The effect of nicotine, or nicotine +tomato juice or tomato juice only on lung volume of 84 day-old female and male rats.

		Lung volume (ml)						
Age in days	**Lung volume**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Tomato +Nicotine**	**Control vs. Tomato**
42	Female	5.28±0.31	6.39±0.43	6.52±0.73	6.39±0.39	*P>0.05*	*P>0.05*	*P>0.05*
	Male	5.34±0.30	7.13±0.57	5.55±0.71	6.46±0.42	*P<0.05*	*P>0.05*	*P<0.05*
F42vs. M42		*P >0.05*	*P >0.05*	*P >0.05*	p>0.05			
84	Female	8.08±0.71	9.58±0.47	11.41±0.61	9.73±0.25	*P<0.05*	*P<0.005*	*P<0.05*
	Male	10.17±0.58	13.72±1.05	14.16±0.87	12.06±0.47	*P<0.05*	*P<0.001*	*P<0.05*
F 84vs. M84		*P <0.05*	*P<0.001*	*P<0.05*	P<0.001			

Table 5.2. The effects of maternal nicotine exposure or tomato juice supplementation, or both nicotine exposure and tomato juice supplementation on the lung volume (Lv) of the male and female offspring.

	Daily increase in Lung volume (ml)			
	Control	**Nicotine**	**N+T**	**Tomato**
Female	0.07	0.08	0.12	0.08
Male	0.12	0.16	0.21	0.13

Table 5.2.1. The effects of nicotine, tomato juice, nicotine + tomato juice exposure on daily increase in lung volume between days 42 and 84.

5.1.3 Lung volume to body weight ratio (Lv/BW).

Both the BW and Lv of the rats increased over time as the animals mature. After postnatal day 42 the BW and Lv of the female rats increased at a slower rate. There were also differences between the control and experimental groups. The increase in Lv is determined by the increase in BW. Therefore, correcting the Lv for BW should give an Lv/BW ratio that is the same for all groups unless the increase in BW or Lv is not proportional.

Postnatal day 42

At postnatal day 42 the Lv/BW ratio (table 5.3 and Fig. 5.3(a)) of the control **female** rats (4.35±0.37ml/g) was lower ($P<0.05$) than that of the nicotine exposed female rats (5.67±0.27 ml/g. This can be ascribed to the higher Lv of the nicotine exposed female rats than that of the control rats of the same age. The Lv/BW ratio of the control female rats was the same ($P>0.05$) as the Lv/BW ratio of the animals exposed to both nicotine and tomato juice, or tomato juice only.

At postnatal day 42 the Lv/BW ratio of the control male rats (4.02±0.24 ml/g) was lower ($P<0.05$) than that of the nicotine exposed male offspring (5.13±0.34 ml/g). It was also lower than those exposed to the tomato juice supplement only (5.16±031 ml/g). However, the Lv/BW ratio of the rats exposed to both nicotine and tomato juice was the same as for the control male rats.

Postnatal day 84

Small but significant differences were seen in the Lv/Bw ratios at postnatal day 42, the Lv/BW ratio of the control **male** and **female** rats and the experimental groups (table 5.3) at

postnatal day 84 was the same (P>0.05). There was no difference (P>0.05) seen between the control male and female rats, rats exposed to nicotine only, or to both a combination of tomato juice and nicotine at postnatal day 84.

5.1.3(a) Lv/BW

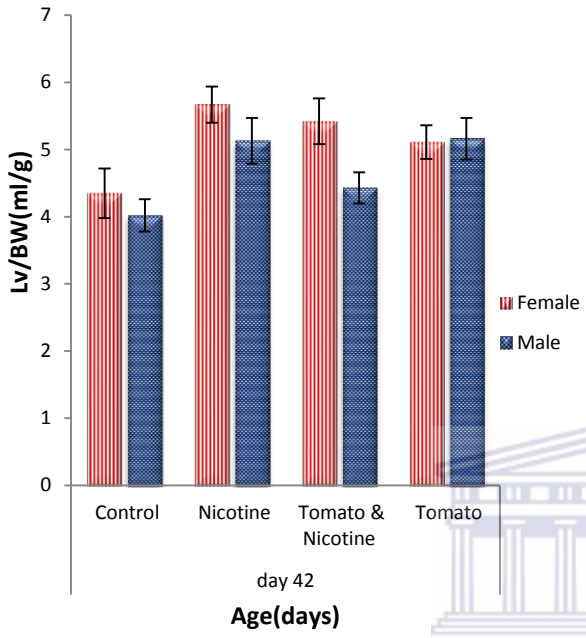


Figure 5.3(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the Lv/BW of 42-day old female and male rats.

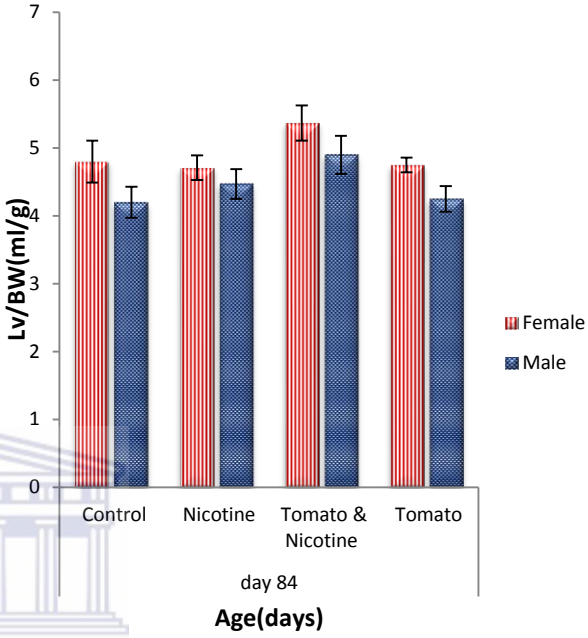


Figure 5.3(b). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the LV/BW of 84-day old female and male rats.

Lv/BW (ml/g)								
Age in days	**Lv/BW**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine + Tomato**	**Control vs. Tomato**
42	Female	4.35±0.37	5.67±0.27	5.42±0.34	5.11±0.25	*P<0.05*	*P>0.05*	*P>0.05*
	Male	4.02±0.24	5.13±0.34	4.43±0.0.23	5.16±0.31	*P<0.05*	*P>0.05*	*P<0.05*
F42vs. M42		*P >0.05*	*P >0.05*	*P <0.05*	p>0.05			
84	Female	4.8±0.31	4.71±0.18	5.36±0.26	4.75±0.13	*P>0.05*	*P>0.05*	*P>0.05*
	Male	4.26±0.23	4.47±0.22	4.90±0.28	4.25±0.19	*P>0.05*	*P>0.05*	*P>0.05*
F 84vs. M84		*P >0.05*	*P>0.05*	*P>0.05*	P>0.05			

Table 5.3. The effects of maternal nicotine exposure, or tomato juice only, or both nicotine and tomato juice on the on lung volume to body weight ratio (Lv/BW) of the male and female offspring.

5.2 The effect of maternal exposure to nicotine during gestation and supplementing the diet with tomato juice on chest circumference (CC) and crown-rump length (CRL) of the offspring.

Various growth parameters are used to determine growth of humans and animals over time. These include biparietal diameter (BPD), head circumference (HC), abdominal circumference (AC), mid-thigh circumference (MTC), and femoral diaphysis length (FDL) (Lee et al. 2009).

In this study the chest circumference (CC) and crown-rump-length (CRL) are the indicators of growth that were used. The CC/CRL and CC/Lv ratios were used in this study to establish whether growth and development were proportional. For example, an increase in CC is followed with an equal growth in lung volume as the animal age. If growth of any one of these ratios was affected it will affect the ratio.

Postnatal day 42

The CC (table 5.4) and CRL (table 5.5) of the **female** and **male** rats was the same at postnatal day 42, and was also not affected by maternal nicotine exposure during gestation, or by supplementing the diet of the rats with tomato juice (Fig. 5.4 (a)) and (Fig. 5.5 (a)).

Postnatal day 84

As at postnatal day 42, maternal nicotine exposure during gestation had no effect on the CC of the 84-day-old female and male offspring. On the other hand, at postnatal day 84 the CC (table 5.4) of the control **female** rats was at 117.73±3.27 mm, lower ($P<0.001$) than the CC of the female rats that were exposed to both nicotine and tomato juice (138.08±2.71 mm). The CC of 84-day-old male rats followed the same trend where the CC of the control males were

123.00±4.26 mm and that of the 84-day-old males exposed to both nicotine and tomato juice was 146.75±3.61 mm (P<0.001). The data showed no male to female differences between the control group, those exposed to nicotine only and those exposed to both nicotine and tomato juice.

At postnatal day 84 the control **female** CRL (table 5.5) was lower than the CRL of those rats exposed to nicotine only (P<0.005), or those receiving tomato juice only (P<0.05), or those rats receiving both nicotine and tomato juice (P<0.001).

Similarly, the CRL of the control **male** rats was lower (P<0.05) (Fig. 5.5 (b)) than those of the nicotine exposed rats, as well as those male rats that received tomato juice as dietary supplement and those exposed to a combination of nicotine and tomato juice (P<0.005)

The higher CC and CRL of the nicotine exposed offspring is to be expected since the BW of these animals was higher than that of the control animals.

5.2.1(a) Chest circumference (CC)

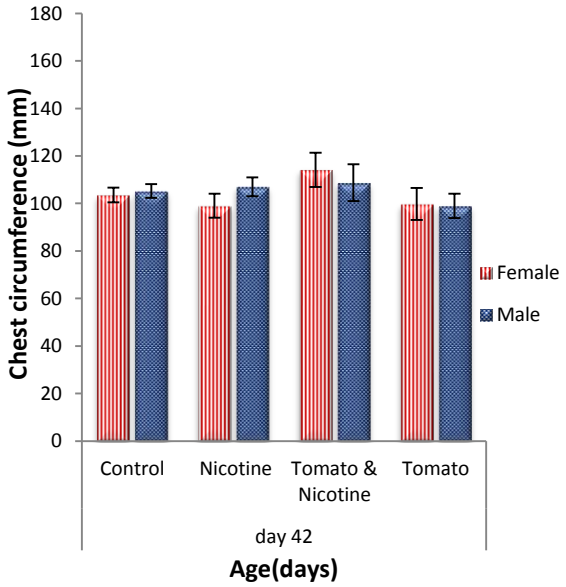


Figure 5.4(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CC of 42-day old female and male rats.

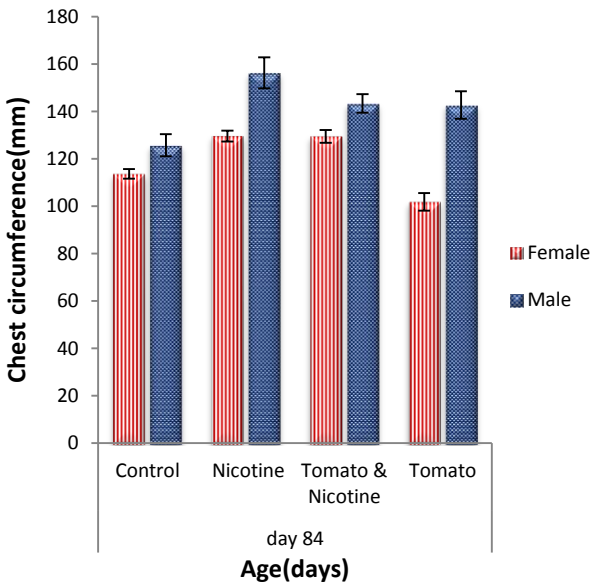


Figure 5.4(b). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CC of 84-day old female and male rats

		CC(mm)						
Age in days	**Chest Circumference**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine +Tomato**	**Control vs. Tomato**
42	Female	103.5±3.11	99±5.03	114.2±7.21	99.75±6.77	*P>0.05*	*P>0.05*	*P>0.05*
	Male	105.21±2.88	107±4.03	108.7±7.75	99±5.15	*P>0.05*	*P>0.05*	*P>0.05*
F42vs. M42		*P >0.05*	*P >0.05*	*P>0.05*	P>0.05			
84	Female	117.73±3.27	124.85±5.83	138.08±2.71	110±5.38	*P>0.05*	*P<0.001*	*P>0.05*
	Male	123.00±4.26	125.00±5.33	146.75±3.61	139.64±7.20	*P>0.05*	*P<0.001*	*P<0.05*
F 84vs. M84		*P>0.05*	*P>0.05*	*P>0.05*	*P<0.005*			

Table 5.4. The effect of maternal nicotine exposure during pregnancy, or receiving tomato juice supplementation only, or nicotine + tomato juice on the chest circumference (CC) of the offspring.

5.2.2(a) Crown-rump length (CRL)

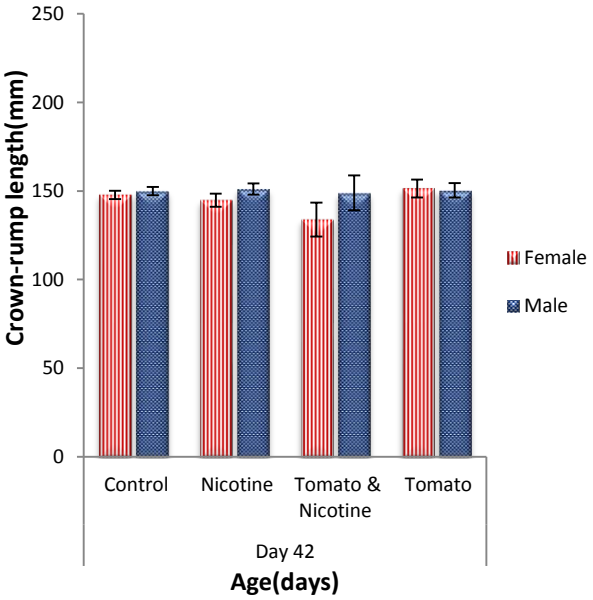


Figure 5.5(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CRL of 42-day old female and male rats.

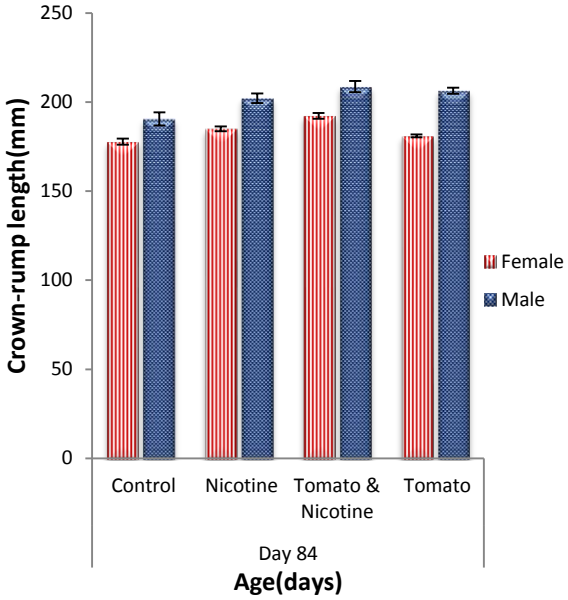


Figure 5.5(b). The effect of nicotine, or nicotine + tomato juice, or tomato juice only on the CRL of 84-day old female and male rats

		CRL(mm)						
Age in days	**Crown -Rump length**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine + Tomato**	**Control vs. Tomato**
42	Female	147.8±2.39	144.75±3.74	133.9±9.58	151.38±4.99	*P>0.05*	*P>0.05*	*P>0.05*
	Male	149.93±2.35	151.13±3.14	148.9±9.89	150.36±4.04	*P>0.05*	*P>0.05*	*P>0.05*
F42vs. M42		*P >0.05*	*P >0.05*	*P>0.05*	P>0.05			
84	Female	177.82±1.73	185.08±1.38	192.31±1.65	181.06±0.81	*P<0.005*	*P<0.001*	*P<0.05*
	Male	190.64±3.73	202.25±2.76	208.75±3.17	206.5±1.7	*P<0.05*	*P<0.005*	*P<0.005*
F 84vs. M84		*P<0.05*	*P<0.001*	*P<0.001*	*P<0.001*			

Table 5.5. The effect of maternal nicotine exposure during pregnancy, or receiving tomato juice supplementation only, or nicotine + tomato juice on the crown-rump distance (CRL) of the offspring.

5.3 The effect of maternal exposure to nicotine during gestation and supplementing the diet with tomato juice on the chest circumference to lung volume (CC/Lv), crown-rump length to chest circumference (CRL/CC) and chest circumference to body weight (CC/BW) ratios of the offspring.

5.3.1 Chest circumference to lung volume (CC/Lv)

Postnatal day 42

At postnatal day 42 the CC/Lv ratio (table 5.6) of the **female** control rats was higher ($P<0.05$) than that of the nicotine exposed female rats as well as the rats whose diets were supplemented with tomato juice. This difference ($P<0.05$) was also seen between the control **male** rats and the nicotine exposed male rats and those male rats exposed to tomato juice only. The ratio was not affected in the rats whose mothers received both tomato juice and nicotine during gestation. Supplementing the diet of the mother with tomato juice thus prevents the effect of maternal nicotine exposure on the CC/Lv of the offspring ($P>0.05$). A comparison of the male and female data shows that there were also no male to female difference seen ($P>0.05$) Fig. 5.6(a) between the CC/Lv of the control groups and the other experimental groups at postnatal day 42.

Postnatal day 84

From the data in table 5.6 it appears that the CC/Lv ratio of the 84-day-old control **female** and **male** rats was the same ($P>0.05$) as the CC/Lv ratio of nicotine exposed rats. This means that between postnatal day 42 and 84 the effect of nicotine on one of the component of this ratio returned to normal. At postnatal day 84 the CC/Lv ratio of control **female** rats was higher ($P<0.001$) than the CC/Lv ratio of animals exposed to tomato juice only and those

exposed to both nicotine and tomato juice (P<0.05). There were no difference (P>0.05) seen between the CC/Lv of control **male** rats and other experimental groups.

There were no **male** to **female** differences (P>0.05) (Fig. 5.6(b)) seen between those rats exposed to both nicotine and tomato juice and those exposed to tomato juice only. It is interesting to note that the CC/Lv ratio of males and females decreased (P<0.05) between postnatal days 42 and 84 due to a faster increase in Lv than CC.

5.3.2 Crown rump length to chest circumference ratio (CRL/CC)

Postnatal days 42 and 84

The CRL/CC ratio (mm/mm) (table 5.7) of 42 and 84-day-old offspring was not affected by maternal nicotine exposure during gestation. The CRL/CC ratio of the female and male offspring whose mothers consumed tomato juice during gestation was also not different from that of the control animals (P>0.05).

5.3.3 Chest circumference to body weight ratio (CC/BW)

Postnatal day 42

The CC/BW ratio (table 5.8, Fig. 5.8 (a)) of 42-day old **female** and **male** offspring was not affected (P>0.05) by nicotine exposure via the placenta. The CC/BW ratio of those rats whose mothers consumed tomato juice as a supplement to their diets, or those whose mothers consumed tomato juice and was exposed to nicotine via the placenta, was the same as that of the controls of the same age (P>0.05). On the other hand, male offspring exposed to nicotine

via the placenta only (0.88±0.03mm/g) had a higher (P<0.05) CC/BW ratio than the female offspring (0.77±0.02 mm/g) of the same experimental group.

Postnatal day 84

From the data in table 5.8 the CC/BW ratio of the **female** offspring was significantly affected (P<0.05) by maternal nicotine exposure, and not affected (P>0.05) by a combination of nicotine and tomato juice. The CC/BW ratio of female offspring whose mothers received a tomato juice as a supplement (0.54±0.02mm/g) was lower (P<0.001) than of the control female rats (0.71±0.04mm/g). Similarly, the CC/BW ratio of **male** offspring expose to nicotine via the placenta (0.41±0.02mm/g) was lower (P<0.05) than the CC/BW ratio of the control male offspring (0.52±0.03mm/g). There were no differences (P>0.05) seen between the CC/BW ratio of the male rats and those expose to both nicotine and tomato juice and tomato juice only.

The CC/BW ratio (table 5.8, Fig. 5.8 (b)) of the control **female** offspring was significantly higher (P<0.05) than the CC/BW of the **male** control group. A more pronounced difference (P<0.001) was seen between the **female** and **male** offspring that were exposed to nicotine only, and those exposed to both nicotine and tomato juice. There were no female to male differences (P>0.05) seen in the CC/BW of rats exposed to tomato juice only.

5.3.1(a) CC/Lv

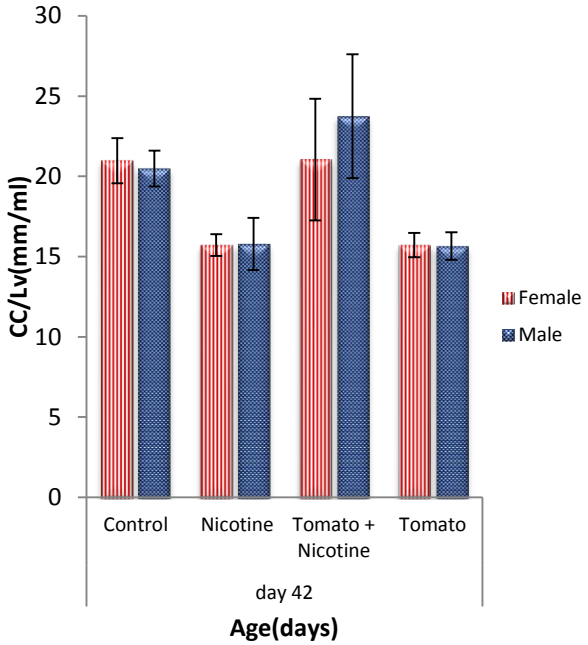


Figure 5.6(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CC/Lv of 42-day old female and male rats.

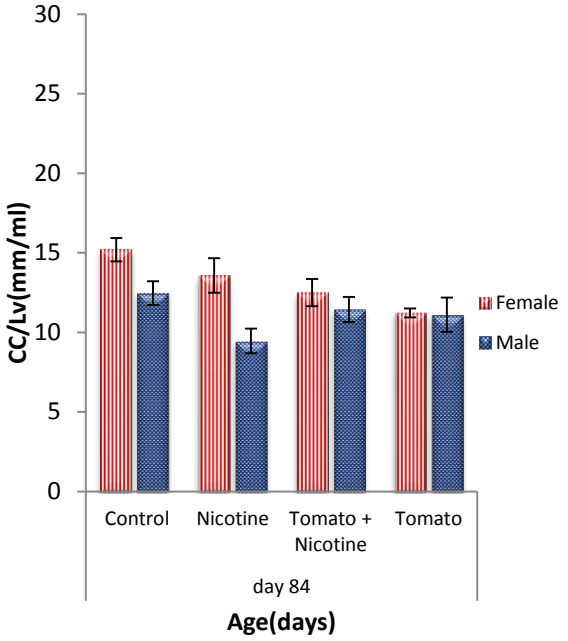


Figure 5.6(b). The effect of nicotine, or nicotine + tomato juice, or tomato juice only on the CC/Lv of 84-day old female and male rats.

		CC/Lv(mm/ml)						
Age in days	**CC/Lv**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine +Tomato**	**Control vs. Tomato**
42	Female	20.97±1.40	15.72±0.67	21.04±3.79	15.72±0.76	*P<0.05*	*P>0.05*	*P<0.05*
	Male	20.49±1.12	15.79±1.63	23.74±3.86	15.66±0.85	*P<0.05*	*P>0.05*	*P<0.05*
F42vs. M42		*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			
84	Female	15.20±0.74	13.57±1.09	12.44±0.57	11.22±0.28	*P>0.05*	*P<0.05*	*P<0.001*
	Male	12.47±0.74	9.46±0.77	10.58±0.54	11.82±0.89	*P>0.05*	*P>0.05*	*P>0.05*
F 84vs. M84		*P<0.05*	*P<0.05*	*P>0.05*	*P>0.05*			

Table 5.6. The effect of nicotine, tomato juice, nicotine + tomato juice exposure during different lung development phases on neonatal ratio of CC/Lv (mm/ml).

5.3.2(a) CRL/CC (mm/mm)

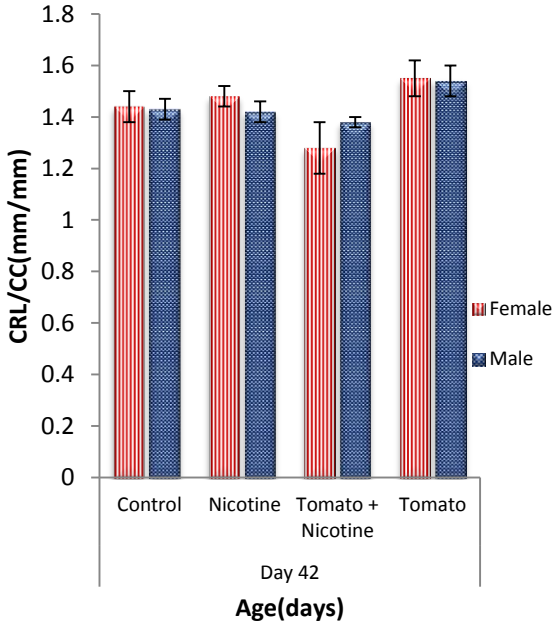


Figure 5.7(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CRL/CC of 42-day old female and male rats.

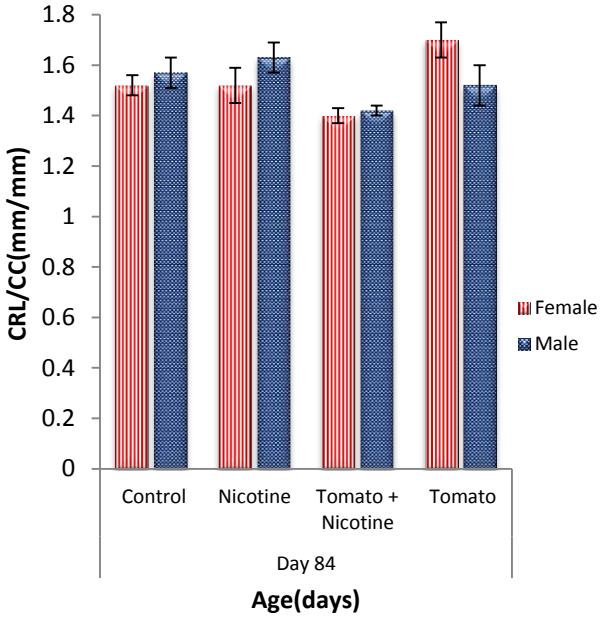


Figure 5.7(b). The effect of nicotine, or nicotine + tomato juice, or tomato juice only on the CRL/CC of 84-day old female and male rats.

CRL/CC(mm/mm)								
Age in days	**CRL/CC**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine +Tomato**	**Control vs. Tomato**
42	Female	1.44±0.06	1.48±0.04	1.28±0.10	1.55±0.07	*P>0.05*	*P>0.05*	*P>0.05*
	Male	1.43±0.04	1.42±0.04	1.38±0.02	1.54±0.06	*P>0.05*	*P>0.05*	*P>0.05*
F42vs. M42		*P>0.05*	*P>0.05*	*P>0.05*	P>0.05			
84	Female	1.52±0.04	1.52±0.07	1.40±0.03	1.70±0.07	*P>0.05*	*P<0.05*	*P>0.05*
	Male	1.57±0.06	1.63±0.06	1.43±0.02	1.51±0.08	*P>0.05*	*P>0.05*	*P>0.05*
F 84vs. M84		*P>0.05*	*P>0.05*	*P>0.05*	P>0.05			

Table 5.7. The effect of nicotine, tomato juice, nicotine + tomato juice exposure during different lung development phases on neonatal ratio of CRL/CC (mm/mm).

5.3.3(a) CC/BW

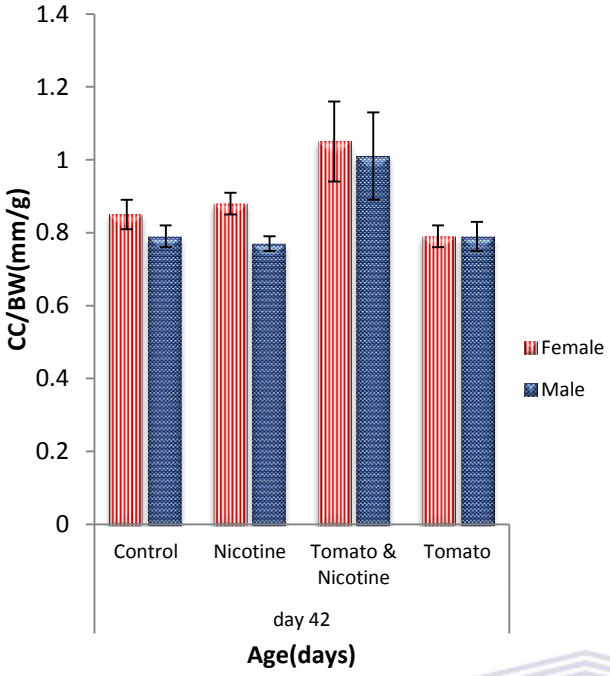


Figure 5.8(a). The effect of nicotine, or nicotine + tomato juice or tomato juice only on the CC/BW of 42-day old female and male rats.

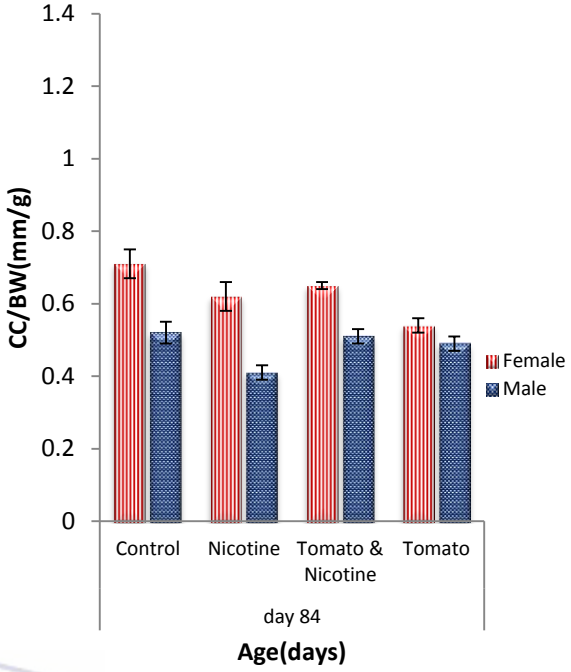


Figure 5.8(b). The effect of nicotine, or nicotine + tomato juice, or tomato juice only on the CC/BW of 84-day old female and male rats.

CC/BW(mm/g)								
Age in days	**CC/BW**	**Control**	**Nicotine**	**Tomato +Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine + Tomato**	**Control vs. Tomato**
42	Female	0.85±0.04	0.88±0.03	1.05±0.11	0.79±0.03	*P>0.05*	*P>0.05*	*P>0.05*
	Male	0.79±0.03	0.77±0.02	1.01±0.13	0.79±0.04	*P>0.05*	*P>0.05*	*P>0.05*
F42vs. M42		*P >0.05*	*P <0.05*	*P>0.05*	P>0.05			
84	Female	0.71±0.04	0.62±0.04	0.65±0.01	0.54±0.02	*P<0.05*	*P>0.05*	*P<0.001*
	Male	0.52±0.03	0.41±0.02	0.51±0.02	0.49±0.02	*P<0.05*	*P>0.05*	*P>0.05*
F 84vs. M84		*P<0.05*	*P<0.001*	*P<0.001*	*P>0.05*			

Table 5.8. The effect of nicotine, tomato juice, nicotine + tomato juice exposure during different lung development phases on neonatal ratio of CC/BW (mm/g).

5.4 The effect of maternal exposure to nicotine during gestation on the volume densities of the airspaces (Va) and parenchymal tissue (Vt) of the offspring.

The Va and Vt data of male and female animals of the various age groups were pooled since no gender differences were evident. Tables 5.9 and 5.10 as well as figures 5.9 and 5.10 show the volume densities of the air spaces (Va) and parenchymal tissue (Vt) from postnatal day 14 up until postnatal day 84.

Postnatal days 14 and 21

Comparison between the control group and experimental groups:

The Va and Vt of the control offspring at postnatal days 14 and 21 was the same (P>0.05) as that of the other experimental groups. Between postnatal days 14 and 21 the Va and Vt of control group and those born to mothers that received tomato juice during gestation remained constant. However, the **Va** of nicotine only exposed offspring increased between postnatal days 14 and 21 from 68.29±0.76% to 77.47±2.24% (P<0.005). Consequently the **Vt** of nicotine only exposed offspring decreased between postnatal days 14 and 21. The same trend was also followed by animals exposed to both nicotine and tomato juice within the Va and Vt

Postnatal days 42 and 84

Comparison between the control group and experimental groups:

At postnatal days 42 and 84 the **Va** of the control rats was lower (P<0.05) (table 5.9) than that of the offspring that received nicotine via the placenta only, but was the same (P>0.05) as that of the of the rats whose mothers received a tomato juice as a dietary supplement during gestation only. It also resembled those born to mothers who received both nicotine and

tomato juice between postnatal days 42 and 84. A consequence of this is that the Vt of the control offspring of the same age was higher ($P<0.05$) than that of the offspring that were exposed to nicotine during gestation.

The **Va (**table 5.9 and Fig. 5.9) of rats that were exposed to nicotine during gestation increased from 68.29±0.76 % at postnatal day 14 to 82.44±0.81% at postnatal day 84 ($P<0.001$). At the same time the Vt of the control offspring decreased with age from 33.19±3.06% at postnatal day 14 to 28.79±2.22% at postnatal day 84 ($P>0.05$). During the same period of time the Vt of the nicotine exposed rats decreased from 31.71±0.76% to 17.56±0.81% ($P<0.001$).

From the data it is evident that the Vt **(**table 5.10 and Fig. 5.10) of the control as well as that of the experimental animals exposed to tomato juice and to both nicotine and tomato juice remained constant ($P>0.05$) from postnatal day 21 to postnatal day 84. It is important to note that the maternal intake of tomato juice during gestation prevented the lungs of offspring of from the harmful effects of nicotine on the lung parenchyma of the offspring.

5.4.1(a) Volume density Va (%)

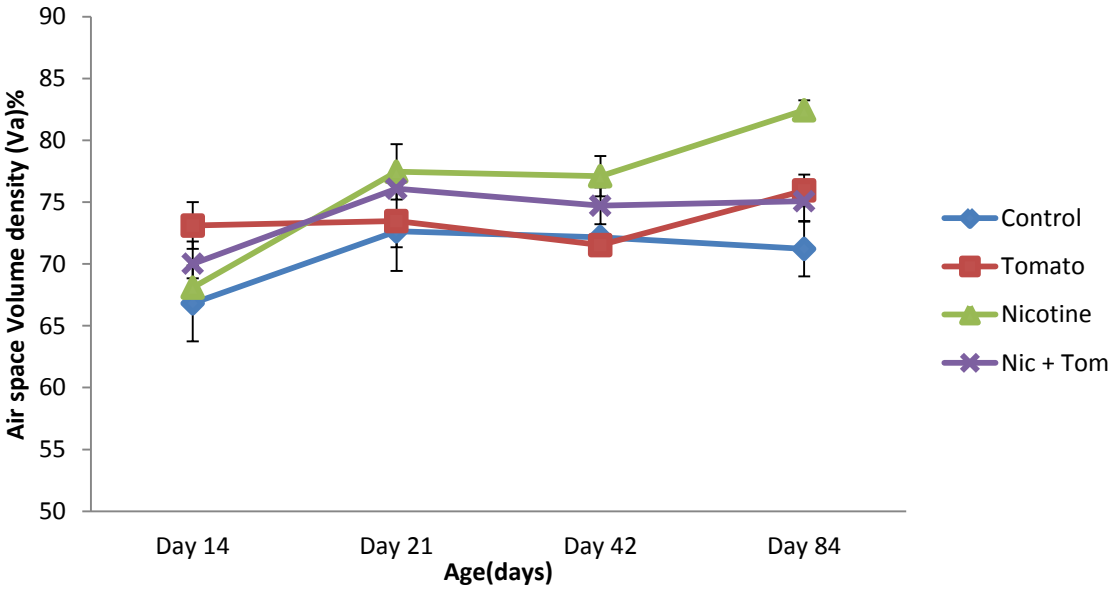


Figure 5.9. The effects of maternal exposure to nicotine during gestation, and supplementing the diet with tomato juice on air space volume density (Va) (%) of the lungs of the offspring.

Va (%)							
Age in days	**Control**	**Nicotine**	**T+N**	**Tomato juice**	**Control vs. Nicotine**	**Control vs. T+N**	**Control vs. Tomato juice**
14	66.81±3.063	68.29±0.76	70.03±1.81	73.11±1.89	*P>0.05*	*P>0.05*	*P>0.05*
21	72.65±1.29	77.47±2.24	76.08±0.21	73.47±4.04	*P>0.05*	*P>0.05*	*P>0.05*
14 vs.21	*P>0.05*	*P<0.005*	*P<0.05*	P>0.05			
42	72.17±0.49	77.10±1.63	74.71±1.49	71.53±0.77	*P<0.05*	*P>0.05*	*P>0.05*
42vs.14	P>0.05	P<0.005	P>0.05	P>0.05			
42vs.21	P>0.05	P>0.05	P>0.05	P>0.05			
42vs.84	P>0.05	P>0.05	P>0.05	P>0.05			
84	71.21±2.22	82.44±0.81	75.08±1.59	75.95±1.28	*P<0.05*	*P>0.05*	*P>0.05*
84 vs.14	*P>0.05*	*P<0.001*	*P>0.05*	*P>0.05*			
84 vs.21	*P>0.05*	*P<0.05*	*P>0.05*	P>0.05			
84 vs.42	*P>0.05*	*P>0.05*	*P>0.05*	P>0.05			

Table 5.9. The effect of maternal nicotine exposure during pregnancy, or receiving tomato juice supplementation only, or nicotine + tomato juice on the volume density (Va) % of the offspring.

5.4.2(a) Parenchymal tissue Vt (%)

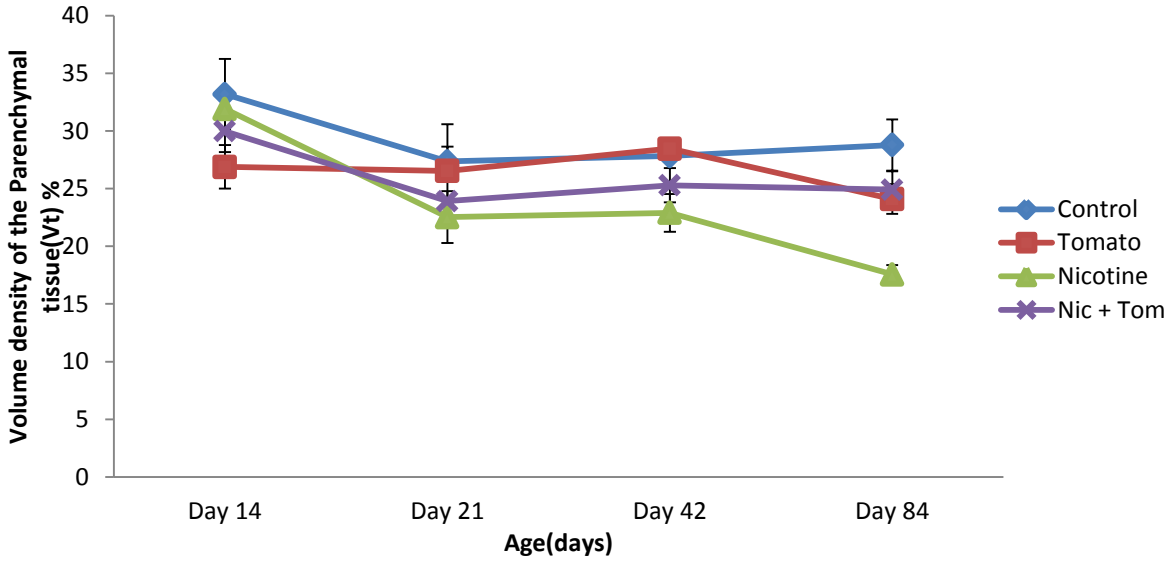


Figure 5.10. The effects of maternal exposure to nicotine during gestation, and supplementing the diet with tomato juice on parenchymal tissue volume density (Vt) (%) of the lungs of the offspring.

	Vt (%)						
Age in days	**Control**	**Nicotine**	**T+N**	**Tomato juice**	**Control vs. Nicotine**	**Control vs. T+N**	**Control vs. Tomato juice**
14	33.19±3.063	31.71±0.76	29.97±1.81	26.89±1.89	*P>0.05*	*P>0.05*	*P>0.05*
21	27.35±1.29	22.53±2.24	23.91±0.21	26.53±4.04	*P>0.05*	*P>0.05*	*P>0.05*
14 vs.21	*P>0.05*	*P<0.005*	*P<0.05*	P>0.05			
42	27.83±0.49	22.90±1.63	25.29±1.49	28.47±0.77	*P<0.05*	*P>0.05*	*P>0.05*
42vs.14	*P>0.05*	P<0.005	P>0.05	P>0.05			
42vs.21	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			
84	28.79±2.22	17.56±0.81	24.92±1.59	24.05±1.28	*P<0.05*	*P>0.05*	*P>0.05*
84 vs.14	*P>0.05*	*P<0.001*	*P>0.05*	*P>0.05*			
84 vs.21	*P>0.05*	*P<0.05*	*P>0.05*	*P>0.05*			
84 vs.42	*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			

Table 5.10. The effect of maternal nicotine exposure during pregnancy, or receiving tomato juice supplementation only, or nicotine + tomato juice on the volume density (Vt) % of the offspring.

5.5 The effect of maternal nicotine exposure during gestation, and supplementation of the diet with tomato juice on lung morphology and the alveolar linear intercepts (Lm) of the lungs of the offspring.

5.5.1 Lung morphology.

From figure 5.11 A, and B it is evident that supplementing the mother's diet with tomato juice had no effect on the structure of the lungs of the offspring. However, figure 5.11 C clearly show enlarged airspaces of the lungs of the nicotine exposed offspring compared to that of the controls (A) and the tomato juice (B) groups. The lungs of the offspring of the mothers who received nicotine subcutaneously during pregnancy and received a tomato juice supplement during gestation (D), resembles that of the control and tomato juice groups. This means that the supplementing the diet of the mother with tomato juice prevented the adverse effects of nicotine on the lungs of the offspring and thus ensures that the alveolar volume of the lungs of the offspring was maintained even when the mother was exposed to nicotine during gestation.

5.5.2 Mean linear intercept (Lm).

The Lm is a measure of the distance between alveolar walls of an alveolus and thus an indication of the volume of an alveolus. The Lm of the male and female animals of the various age groups were pooled since no gender differences were evident.

Except for a transient increase in the Lm between postnatal days 14 and 21 (table 5.11 and Fig. 5.12). The Lm of those mothers that were receiving tomato juice supplementation during gestation as well as those that were receiving both nicotine and tomato juice decreased with aging from postnatal day 21. No significant differences were evident ($P>0.05$) between the control, tomato juice and both nicotine + tomato juice groups from postnatal day 42. On the

other hand, while the Lm (table 5.11) of the control, tomato juice and both nicotine and tomato juice groups decrease to lower levels at postnatal day 84 than at postnatal days 14, 21 and 42, the Lm of the rats that were exposed to nicotine via the placenta only was at postnatal day 21, 35.2 % higher than the control ($P<0.001$). This difference increased to 91% at postnatal day 84 ($P<0.001$) when compared to the control of the same age group. This change in the Lm induced in the lungs of the offspring by maternal nicotine exposure during gestation was prevented by supplementing the nicotine exposed mother's diet with tomato juice.

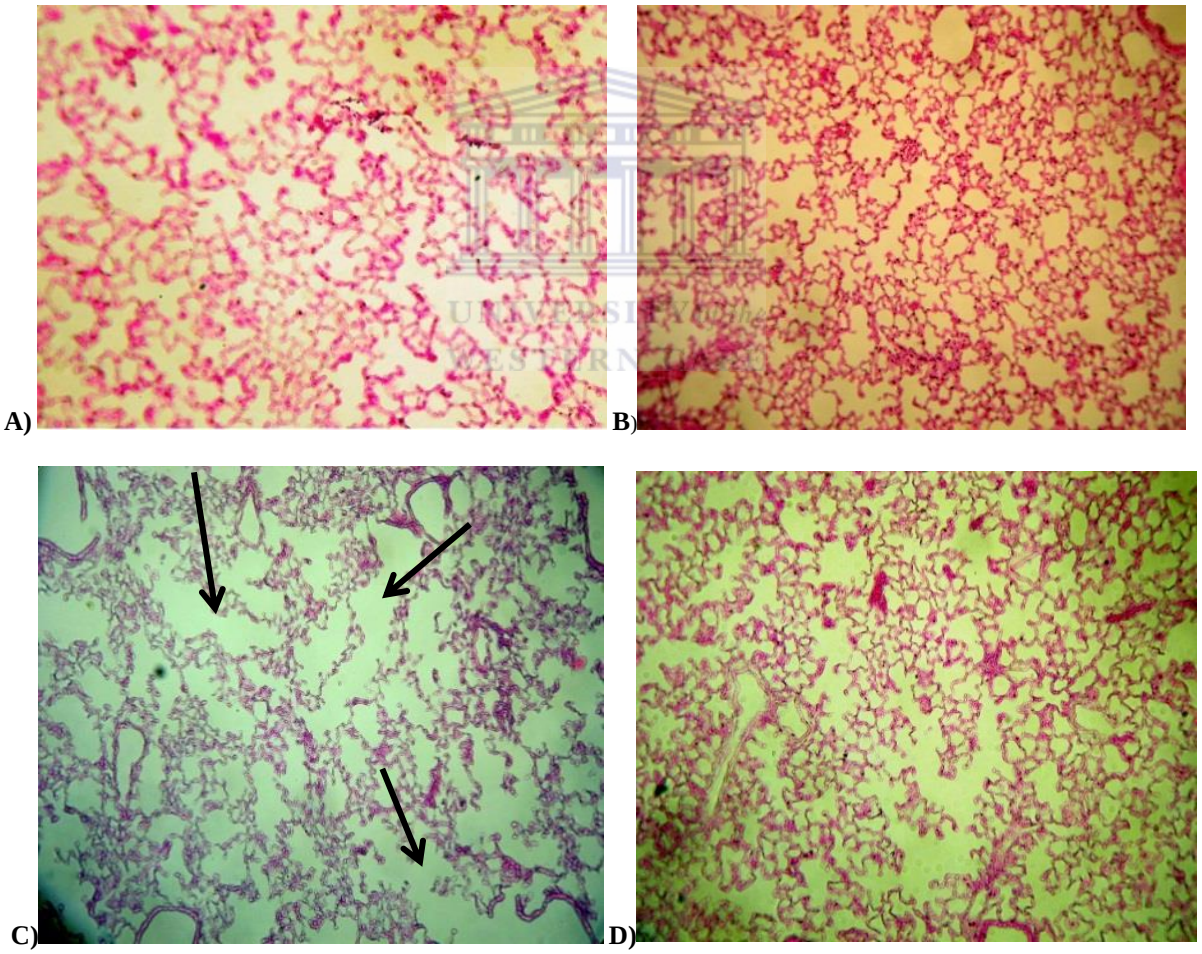


Figure 5.11. The effect of maternal nicotine exposure during gestation as well as the effect of supplementing the mother's diet with tomato juice on the structure of the lungs of the 84-day-old offspring. A) Control, B) Tomato juice only, C) Nicotine only. D) Tomato juice + Nicotine. Arrows indicate enlarged alveoli.(H&E, 10x)

5.5.1(a)Alveolar linear intercept (µm)

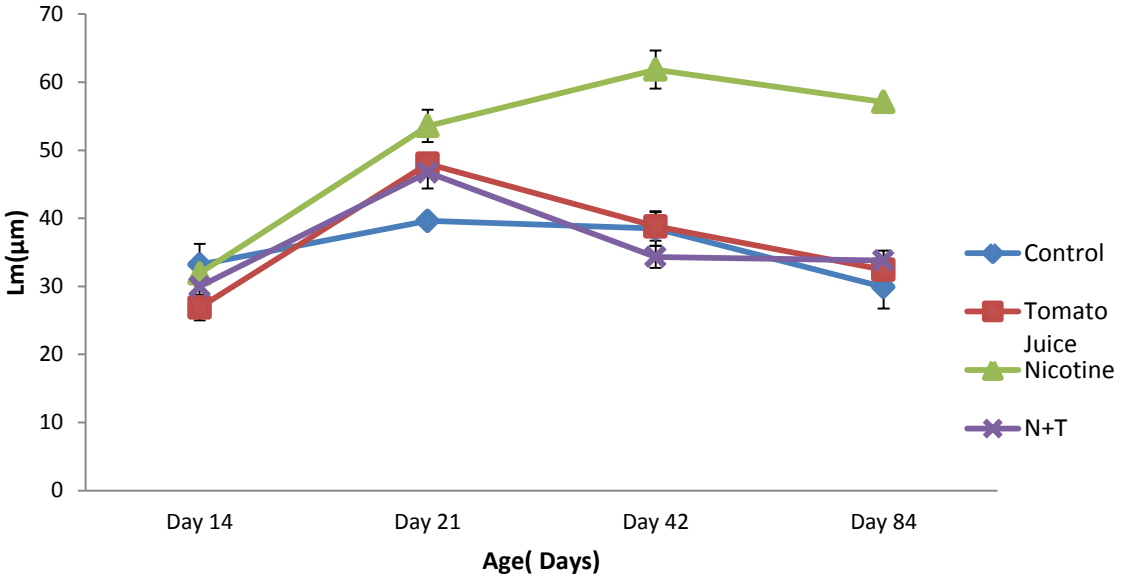


Figure 5.12. The effects of maternal exposure to nicotine during gestation, and supplementing the diet with tomato juice on alveolar linear intercept (Lm) of the lungs of the offspring.

	Alveolar linear intercept (µm)						
Age in days	**Control**	**Nicotine**	**T+N**	**Tomato juice**	**Control vs. Nicotine**	**Control vs. T+N**	**Control vs. Tomato juice**
14	33.19±3.06	31.91±0.76	29.98±1.81	26.90±1.89	*P>0.05*	*P>0.05*	*P>0.05*
21	39.63±0.84	53.58±2.38	46.71±2.31	48.01±0.99	*P<0.001*	*P<0.05*	*P<0.05*
14 vs.21	*P>0.05*	*P<0.001*	*P<0.01*	P<0.001			
42	38.50±2.55	61.86±2.78	34.33±1.63	38.81±2.08	P<0.001	P>0.05	P>0.05
42 vs.14	*P>0.05*	*P<0.001*	*P>0.05*	P<0.005			
42 vs.21	*P>0.05*	*P>0.05*	*P<0.05*	P<0.05			
84	29.9±3.15	57.09±0.29	33.81±1.45	32.45±1.30	P<0.001	P>0.05	P>0.05
84 vs.14	*P>0.05*	*P<0.001*	*P>0.05*	P>0.05			
84 vs. 21	*P<0.05*	*P>0.05*	*P<0.05*	P<0.001			
84 vs. 42	*P>0.05*	*P>0.05*	*P>0.05*	P<0.05			

Table 5.11. The effects of maternal nicotine exposure during gestation and tomato juice supplementation during pregnancy on alveolar linear intercept (Lm) of the offspring.

5.6 The effect of maternal nicotine exposure during gestation, and supplementation of the diet with tomato juice on the number of proliferating cells in the alveolar walls of the lungs of the offspring.

5.6.1 Effects on aging.

Apoptosis, cell proliferation and differentiation is important for the maintenance of the alveolar walls and thus of the lung parenchyma. Cell proliferation is important to replace damaged and senescent cells in the alveolar walls. Interference with this process, for example due to pollutants in the inhaled air, may result in compromised alveolar wall integrity and lung damage. Aging might also impact on cell proliferation. In this study there was a 42.27% decrease ($P<0.01$) in the number of proliferating cells (table 5.12 and Fig. 5.13) in the alveolar walls of control animals between postnatal days 14 and 84. The number of proliferating cells remained constant ($P>0.05$) from postnatal day 21 to 84. This shows that the number of proliferating cells gradually decrease with aging up to postnatal day 21 when the animals are weaned. Thereafter the rate of cell proliferation in the alveolar walls remained constant.

5.6.1(a) Effects of aging on the number of proliferating cells/100µm

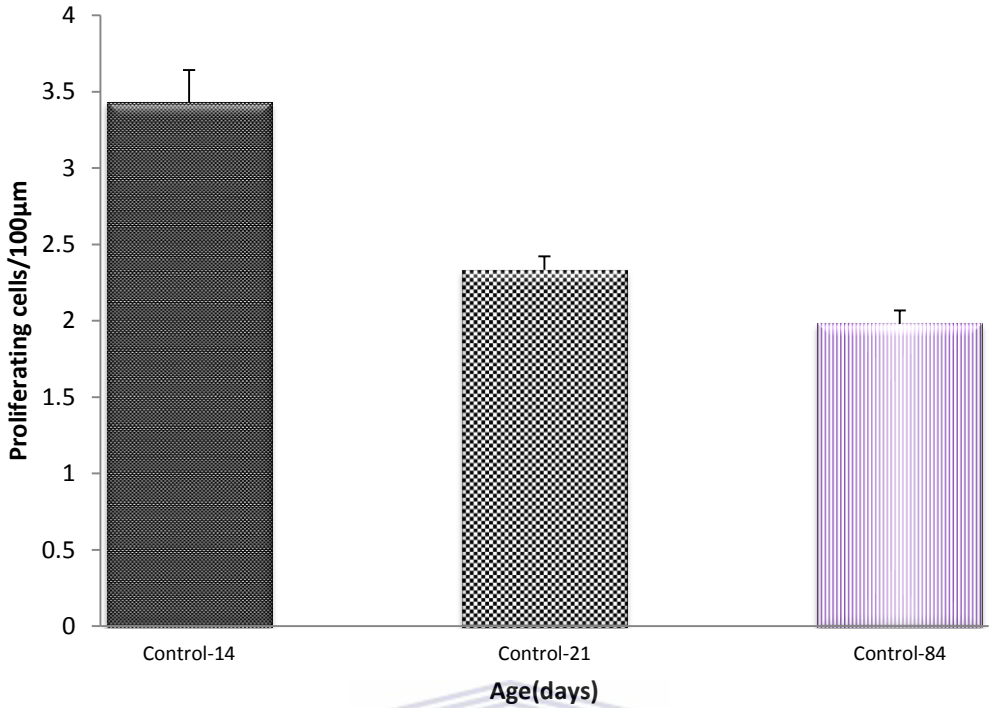


Figure 5.13. The effects of aging on the number of proliferating cells/100µm alveolar wall.

Proliferating cells/100µm	
Age in days	Control (µm)
14	3.43±0.21
21	2.3±0.09
14 vs. 21	*P<0.001*
84	1.98±0.09
84 vs. 14	*P<0.01*
84 vs.21	*P>0.05*

Table 5.12. The effects of aging on different phases of maternal lung development on the number of proliferating cells/100µm.

5.6.2 The effects of maternal exposure to nicotine during gestation, and supplementing the diet with tomato juice on cell proliferation in the alveolar walls.

The number of proliferating cells (Fig. 5.14) in the alveolar walls of the 14-day-old control rat pups was at (3.43±0.21 proliferating cells/100µm), 41.73% higher ($P<0.01$) than that of the rat pups that received nicotine via the placenta (2.42±0.10 proliferating cells/100µm). The number of proliferating cells in the alveolar walls of 14 day-old control animals and those born to mothers who received only tomato juice supplementation during gestation, and those who received both nicotine and tomato juice during gestation, was the same ($P>0.05$). At postnatal day 21 (Fig. 5.14) the number of proliferating cells of control rat-pups was 37.07 % ($P<0.001$) higher than rat-pups exposed to nicotine during gestation, and 22.63% higher ($P<0.05$) than rat-pups exposed to both tomato juice and nicotine during gestation.

It is interesting to note that although the number of proliferating cells in the alveolar walls of the 14-day-old nicotine exposed rat pups was 29.4% lower ($P<0.01$) than that of the control animals. This difference is less pronounced at postnatal day 84 since the number of proliferating cells in the alveolar walls of the 84-day old nicotine exposed rats was only 10.6% lower ($P<0.05$) than in the alveolar walls of the control animals.

5.6.2(a) Number of proliferating cell/100µm

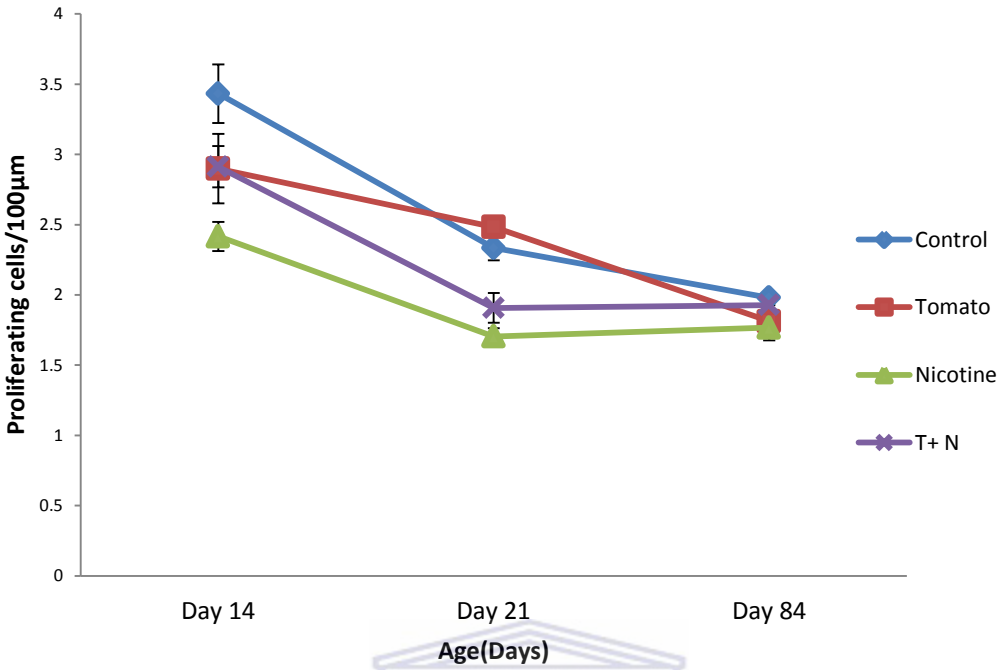


Figure 5.14. The effects of Maternal Nicotine and Tomato Juice exposure on Proliferating cells/100µm alveolar wall.

	Proliferating cells/100µm alveolar wall						
Age in days	**Control**	**Nicotine**	**T+N**	**Tomato juice**	**Control vs. Nicotine**	**Control vs. T+N**	**Control vs. Tomato juice**
14	3.43±0.21	2.42±0.10	2.91±0.15	2.90±0.25	*P<0.01*	*P>0.05*	*P>0.05*
21	2.33±0.09	1.70±0.06	1.90±0.11	2.48±0.07	*P<0.001*	*P<0.05*	*P>0.05*
14 vs.21	*P<0.005*	*P<0.001*	*P<0.001*	P>0.05			
84	1.98±0.03	1.77±0.03	1.93±0	1.81±0.14	*P<0.05*	*P>0.05*	*P>0.05*
84 vs.14	*P<0.05*	*P<0.05*	*P>0.05*	*P<0.05*			
84 vs.21	*P>0.05*	*P>0.05*	*P>0.05*	P<0.005			

Table 5.13. The effects of maternal nicotine exposure during gestation and tomato juice supplementation during pregnancy on proliferating cells/100µm of the alveolar wall of the offspring.

5.7 The effect of maternal nicotine exposure during gestation, and supplementation of the diet of the mother during gestation with tomato juice on static lung compliance (Cst) of the offspring.

Lung compliance is a measure of the ease of expansion of the lungs and the thorax. It is defined as the change in volume over the change in the intrapleural pressure. There is a decrease in compliance with increasing age and in a diseased lung due to its inability to recoil. In this study the Cst was measured at weaning on postnatal day 21 and at postnatal day 42 (3 weeks after nicotine withdrawal) and at postnatal day 84 (9 weeks after nicotine withdrawal).

Postnatal day 21

The data (table 5.14, Fig. 5.15 (a) and (b)) show that there was no difference ($P>0.05$) in the Cst seen between the female and male control groups and other experimental groups ($P>0.05$). There were no gender differences seen between the experimental groups.

Postnatal day 42

Between postnatal days 21 and 42 the lung compliance of all the animals increased with age. At postnatal day 42 the Cst (table 5.14; Fig. 5.15 (a) and (b)) of animals exposed to nicotine only and those exposed to tomato juice only was significantly higher ($P<0.05$) than the control group. There were no significant differences ($P>0.05$) seen between rats exposed to both nicotine and tomato juice when compared to the control group. There were no male to female differences ($P>0.05$) seen within this age group.

Postnatal day 84

At postnatal day 84 (table 5.14, Fig. 5.15 (a) and (b)) the Cst of the control female and male rats was lower ($P<0.001$) than the Cst rats of nicotine exposed rats. Male and female rats exposed to nicotine only showed a much higher Cst. This change in the Cst induced in the lungs of the offspring by maternal nicotine exposure during gestation was prevented by supplementing the diet of nicotine exposed mother's with tomato juice.

5.7.1(a) Female static lung compliance (ml/cmH_2O/kg)

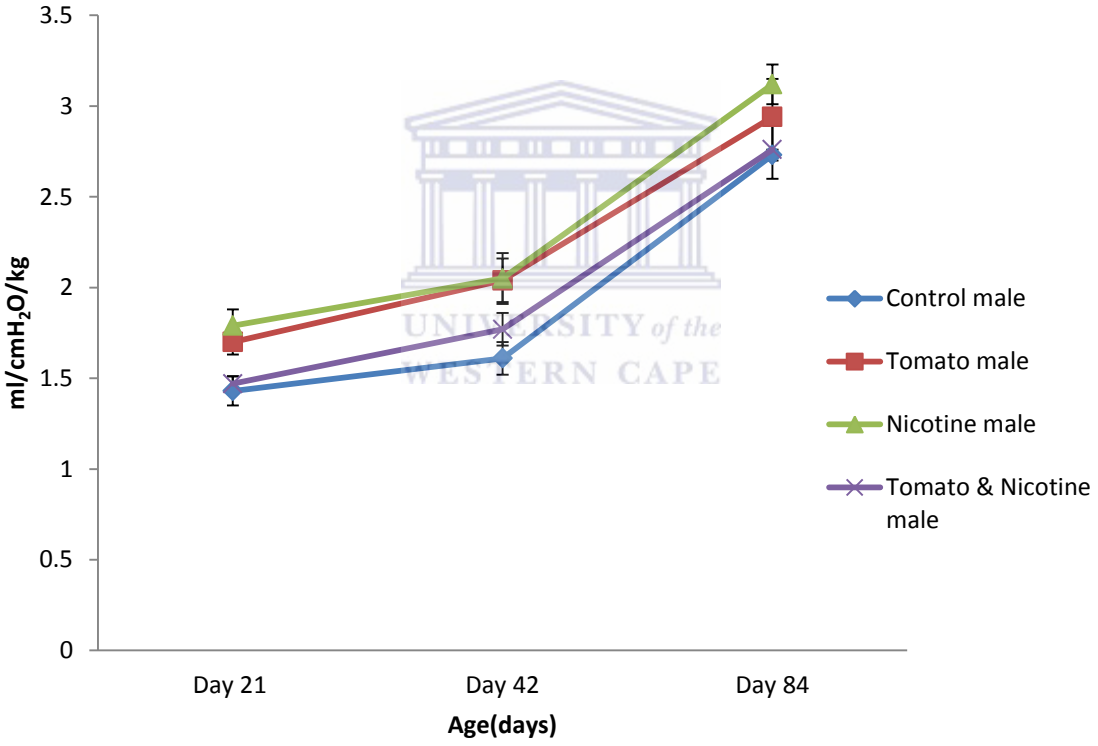


Figure 5.15(a). The effects of Maternal Nicotine and Tomato Juice exposure on female lung compliance.

5.7.2(a) Male static lung compliance (ml/cmH_2O/kg)

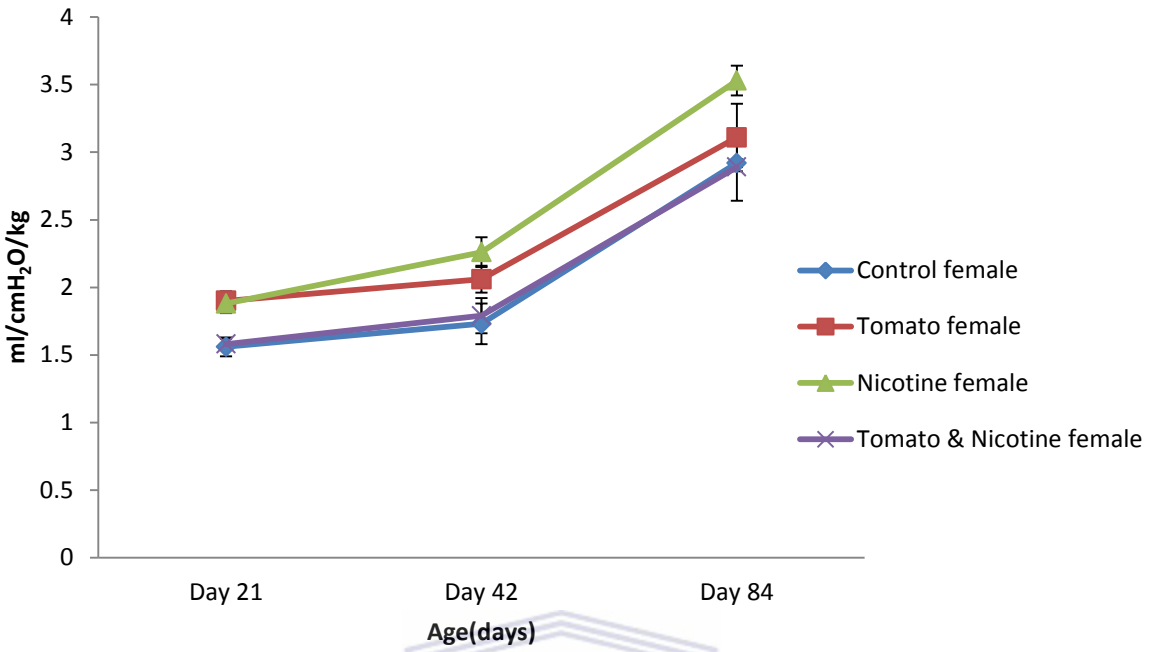


Figure 5.15(b). The effects of Maternal Nicotine and Tomato Juice exposure on male lung compliance.

		ml/cmH_2O/kg						
Age in days	**ml/cmH_2O/kg**	**Control**	**Nicotine**	**Tomato + Nicotine**	**Tomato**	**Control vs. Nicotine**	**Control vs. Nicotine +Tomato**	**Control vs. Tomato**
21	Female	1.56±0.07	1.88±0.07	1.58±0.04	1.71±0.07	*P>0.05*	*P>0.05*	*P>0.05*
	Male	1.43±0.08	1.79±0.09	1.47±0.04	1.70±0.07	*P>0.05*	*P>0.05*	*P>0.05*
F21vs. M21		*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			
42	Female	1.73±0.15	2.26±0.11	1.79±0.13	2.04±0.10	*P<0.05*	*P>0.05*	*P<0.05*
	Male	1.61±0.09	2.05±0.14	1.77±0.09	2.06±0.12	*P<0.05*	*P>0.05*	*P<0.05*
F42vs. M42		*P>0.05*	*P>0.05*	*P>0.05*	*P>0.05*			
84	Female	2.92±0.03	3.53±0.11	2.89±0.25	3.11±0.25	*P<0.001*	*P'>0.05*	*P>0.05*
	Male	2.73±0.03	3.12±0.11	2.76±0.16	2.94±0.21	*P<0.001*	*P>0.05*	*P>0.05*
F84vs. M84		*P<0.05*	*P<0.05*	*P>0.05*	*P>0.05*			

Table 5.14. The effects of maternal nicotine exposure during gestation and tomato juice supplementation during pregnancy on lung compliance of the offspring.

CHAPTER 6

Discussion

6.1 Introduction

Nicotine has been associated with causing adverse effects such as respiratory disorders and low birth weights in the fetus and in newborns (Rubin et al. 1986; Martin & Bracken,. 1986), and although approximately 20–30% of pregnant woman successfully abstains from smoking during pregnancy, half of these women relapse within 6 months of parturition (Ebert & Fahy, 2007). This implies that the developing neonate of these mothers will be exposed to nicotine early in life which may affect the development of the respiratory system. This is plausible since alveolar formation and thus cell division in humans is rapid at this early stage of development.

Nicotine induces formation of oxygen radicals and also reduces the antioxidant capacity of the lungs of the offspring (Hecht, 1999; Maritz & Wyk, 1997; Windvogel et al. 2008). The oxidant/antioxidant ratio imbalance associated with nicotine exposure cause point mutations in the DNA molecule (Jablonka & Lamb, 2005; Hecht; 1999), thereby changing the program that controls lung growth and maintenance of lung structure in the longer term. It has been further suggested that maternal nicotine exposure induces an irreversible inhibition of glycolysis and a drastically increased AMP levels (Maritz, 1986; Maritz & Burger, 1992). These metabolic changes are thought to contribute to the premature aging of the lungs of the offspring of mothers that are exposed to nicotine via the placenta and mother's milk (Maritz et al. 2011) and consequently an increased risk of respiratory disease.

It is known that changes in the *in utero* environment within which the fetus develops, such as during maternal smoking or NRT, may change the program that controls lung growth and development (Barker, 1995, 1998; Lucas, 1991; Barker, 1998). One of the consequences of smoking during pregnancy or the use of NRT to quit smoking is that it induces changes to the genetic program that controls lung development, maintenance and aging in the offspring. Smoking and nicotine decrease the antioxidant levels of the mother and at the same time increases the oxidant levels of the mother and of the *in utero* environment. It is known that the fetus is unable to protect itself against oxidants (Asikainen & White, 2004; Valachovicova et al. 2003) and is therefore dependent on the mother for protection. Therefore, if the mother's ability to protect the fetus is maintained, for example by maintaining her antioxidant status, the *in utero* environment within which the fetus develops will not be compromised. This can potentially be achieved by supplementing the mother's diet with food that is rich in anti-oxidants.

Tomato juice used as a dietary supplement in this study, contains an antioxidant called lycopene, which is a carotenoid (Di Mascio et al. 1989; Kaplan et al. 1990). It has been documented that carotenoids have an antioxidant effect and thus prevents oxidant-induced cellular damage (Bendich, 1993). Apart from lycopene tomato juice also contains vitamin C and other phytonutrients with antioxidant activity, making it a good supplement to improve or maintain the antioxidant capacity of the mother and to remove oxidants from her body and thereby preventing it to accumulate in the amniotic fluid or developing lung. Since tobacco smoke contains more than 10^{15} oxidant molecules/puff (Church and Pryor, 1985), it is conceivable that smoking will reduce the antioxidant status of the body and thus induce an oxidant-antioxidant imbalance in the body. It is therefore plausible that dietary carotenoid intake may improve the antioxidant capacity of the body and thus counter the tobacco smoke-

associated changes in the body. Maternal smoking and use of nicotine may compromise the antioxidant capacity of the mother and thus her ability to protect the developing fetus against oxidants and consequently its impact on fetal organ development. The hypothesis of this study is therefore that maternal nicotine exposure during pregnancy compromise the antioxidant capacity of the mother and thus her ability to protect the fetus. Consequently, the overall objective of this study is to restore the oxidant/antioxidant balance of the mother and developing lungs by supplementing the diets of the mothers during gestation only with tomato juice. The restoration of the mother's antioxidant capacity may protect the fetus against the harmful effects of oxidants such as that in smoking or induced by nicotine.

6.2 Body Weight (BW)

Adequate nutrition is essential for fetal and neonatal growth and development because it supplies the building blocks and the energy that is required to support tissue growth. Inadequate nutrition results in stunted fetal and neonatal growth; this will then result in a decrease in body size of the young at birth (Jamison et al. 2006; Ramakrishnan, 2004). The fetal-placental blood flow is essential for the sufficient supply of nutrients to the growing fetus as well as the removal of waste produced during metabolism. It has been suggested that maternal smoking during pregnancy leads to a reduction of more than 40% in the utero-placental blood flow due to placental vasoconstriction (Birnbaum et al. 1994). Therefore, the nutrient supply to the fetus of smoking mothers will be compromised (Larsen et al. 2002). A consequence of this is a reduction in the birth weights of the offspring (Bassi et al. 1984; Philipp et al. 1984; Birnbaum et al. 1994), with the associated long term consequences such as high blood pressure later in life (Gao et al. 2008; Brion et al. 2008).

In contrast to the previous findings Bainbridge & Smith (2006) showed that nicotine does not affect the feto-placental blood flow and thus the nutrient supply to the fetus. Since the BW of the 14- and 21-day-old offspring in this study was not affected by maternal nicotine exposure during pregnancy, it implies that the nutrient supply to the fetus was adequate to support the normal growth and development of the offspring. There is no information in the literature showing that the response of the male and female offspring of mothers that smoke is different regarding growth and development. It is, however, possible that male and female offspring of smoking mothers is responding differently to nicotine in the long term. Consequently I compared the responses of male and female offspring of mothers that were exposed to nicotine during pregnancy to establish whether males and females respond differently to maternal nicotine exposure during pregnancy.

6.2.1 Male and Female differences:

In the current study the BW of the control male rats was, as expected, higher than the BW of control females at postnatal day 84. Differences in BW only became apparent after postnatal day 42 (6 weeks after birth) because from postnatal day 42 up to postnatal day 84 the BW of the male rats increased faster than the female rats. It is important to note that male and female rats reach sexual maturity from day postnatal 49 (7 weeks after birth) (Quinn, 2005) and is conceivably the reason for the later onset of difference in the increase in BW between males and females. Kim & Ivy (1952) showed that the difference in body weight between male and female rats was attributed to the fact that male rats consumed a greater amount of food or there may be a difference in the absorption or utilization of the food after consumption between males and females. In this study it is unlikely that the increase in body weight of male animals can only be attributed to the amount of food intake because the BW of both

male and female rats remained constant from birth to up to postnatal day 42. The faster increase in BW in males may be due to hormonal and metabolic changes that come into play at this stage of growth, development and maturation.

Secondly the BW of male and female control rats was lower than all the other experimental groups. The BW of those rats whose mothers were exposed to nicotine and also received tomato juice in addition to the normal diet, during gestation, as well as those whose mothers received only the tomato juice supplement in addition the normal diet was higher than that of the control offspring at postnatal day 84.

6.2.2 Effect of energy intake

It is important to note that supplementing the diet during pregnancy with tomato juice also exposed the rats to higher levels of energy than those rats that were exposed to the normal diet. However, the higher level of energy intake during gestation had no effect on the BW of the 42-day-old rats whose mothers received tomato juice supplementation during gestation. It is also unlikely that the higher energy intake, mostly in the form of carbohydrate, of the mothers during gestation had any impact on the BW of the offspring later in life as recorded in this study.

6.2.3 Role of hypothalamus

It has been shown that the signals for body weight regulation and energy balance are integrated in the hypothalamus, suggesting that it is the most important center in the brain for the regulation of appetite and body weight homeostasis (Hillebrand et al. 2002; Kalra et al. 1999; Wilding, 2002). Jo et al. (2002) showed that nicotinic acetylcholine receptors (nAChR)

are widely distributed in the hypothalamus. Since nAChR are widely distributed in the hypothalamus it is plausible that it will impact in control of growth. Grove et al. (2001) also showed that *in utero* exposure to nicotine in the rhesus macaque can alter hypothalamic regulators responsible for appetite and satiety in the neonate. It is therefore possible that the nicotine-induced alterations in body weight homeostasis, as seen in this study, may be induced by changes in hypothalamic control of metabolism during fetal life and possible compromise later in life.

6.2.4 Effects of nicotine

A single exposure per day of the pregnant female rat to nicotine during gestation had no effect on the BW of the offspring up to postnatal day 42. This meant that the nutrient intake of rats exposed to nicotine was sufficient during gestation and after weaning to sustain normal growth of the offspring up to postnatal day 42. Since nicotine was given once per day to the pregnant mother, and since it is rapidly removed from the circulation of the mother (Dempsey et al. 2002), it is highly likely that the length in which placental vasoconstriction occurred was too short to suppress nutrient supply for long enough to have a significant difference on the growth of the offspring during pregnancy and thereafter. However, at postnatal day 84 the BW of the nicotine exposed male and female offspring was higher than that of the control animals of the same age. Since the difference in BW only becomes apparent around postnatal day 84, it implies that it was due to a change in the control of metabolism and growth of the nicotine exposed offspring. This is in contrast to studies which demonstrated that exposure of adult rats to nicotine results in a decrease in BW probably due to a diversion of fats away from storage in the fat tissue towards utilization in muscle tissue (Sztalryd et al. 1996).

It is important to note that the increase in BW was observed from 6 weeks after nicotine withdrawal at birth. This means that the faster increase in BW after postnatal day 42 took place at a time when nicotine was no longer present in the body since the half-life of nicotine is only 90 minutes (Obach et al. 2006; Kyerematen et al. 1988). It is also interesting to note that this faster increase also started at a time when the differences in male/female body weight becomes apparent. It is plausible that this late onset of the faster increase in BW of the nicotine exposed rats was programmed *in utero*. The health consequence of the nicotine-induced faster increase in BW is not known. Studies conducted by Gao et al. (2005), Newman et al. (1999), and Somm et al. (2008) showed that prenatal nicotine exposure of rats has resulted in an increase in postnatal body weight, which is maintained into adulthood. This is contrary to Philipp et al. (1984) and Birnbaum et al. (1994) who recorded a lower BW with offsprings that were exposed to nicotine via the placenta. It is possible that the doses of nicotine of (6 and 3 mg/kg body weight/day) used by Philipp et al. (1984) on pregnant women and Birnbaum et al. (1994) on pregnant rats were too high. In this study I used 1 mg/kg body weight/day which, resulted in maternal plasma nicotine concentration that is similar to the concentration observed for human smokers (Benowitz et al. 1982) and thus more realistic than that of Birnbaum et al. (1994). Also, studies conducted by Pausova et al. (2003) showed that maternal nicotine exposure during gestation and lactation had no effect on litter size and body weights of the offspring and supports my findings up to postnatal day 42. The conflicting data is difficult to explain but it may, in addition to the different concentrations of nicotine used by researchers, be experimental conditions, or even species differences. In a more recent study Pausova (2010) showed that girls exposed to nicotine in the womb were more likely to put on weight late in adolescence. This finding is supported by my research. It is therefore conceivable that NRT during pregnancy may program the offspring to become more prone to obesity later in life.

6.3 The effects of Maternal Nicotine exposure and Tomato Juice supplementation during gestation on the CC and CRL of the offspring.

In the current study, the chest circumference (CC) and crown-rump length (CRL) of the 42-day-old control male and female rats were the same as that of the rats that were exposed to nicotine during gestation. The CC and CRL of the rats whose mothers received tomato juice supplementation during pregnancy was also not affected. No gender differences were seen in the CC and CRL from birth up to postnatal day 42. This means growth and development of the offspring whose mothers were exposed to nicotine during pregnancy and/or who received tomato juice in addition to the normal diet, was proportional.

Although growth and development of the 84-day-old offspring, like that of the 42-day-old offspring, was not affected by maternal nicotine exposure, the female and male offspring whose mothers were exposed to nicotine **and** received tomato juice in addition to the normal diet during pregnancy, had a higher CC than the control. When analysing the CC/BW ratios it is interesting to note that the CC/BW ratio of the nicotine exposed offspring at postnatal day 84 was lower than the control group of the same age. This can be attributed to a higher BW of the offspring that were exposed to nicotine via the placenta. This was prevented by supplementing the mother's diet with tomato juice during gestation. The increase in the CC could conceivably be attributed to an accumulation of fat around the internal organs and under the skin (Pausova et al. 2013).

On the other hand, the results in this study show that the CRL of 84-day-old females and males was higher for the offspring whose mothers were exposed to nicotine during pregnancy than for the controls of the same age. A comparison of the CRL's of the other experimental groups of the same age also show a higher CRL than for the controls of the same age. This was different to the results reported by Sekhon et al. (1999) that showed that maternal

nicotine exposure showed no difference in the CRL. Furthermore, Lindley et al. (2000) showed a lower crown-heel length (CHL) with maternal nicotine exposure in humans. Our results are also different to studies done by Maritz & Windvogel (2003) who showed that nicotine exposure during gestation and lactation had no effect on the CRL of the animals. The different outcome in CRL based on the different exposure times is difficult to explain because there is no evidence to suggest that exposure during lactation will prevent the effect of maternal nicotine exposure during pregnancy. This is further complicated by the observations of Windvogel (2006) namely that exposure of the offspring to nicotine during pregnancy and lactation, or only after birth, had no effect on BW thereof.

The bigger CRL, taken together with the bigger BW, means that the 84-day-old nicotine exposed rats as well as the offspring of mothers that received tomato juice in addition to their normal diets, was bigger that the control rats of the same age. Maternal nicotine exposure or tomato juice supplementation during gestation had no impact on CRL/CC ratio. This means that although the nicotine exposed rats, and those whose mothers received tomato juice supplements, was bigger that the controls, growth was proportional.

The reason for the faster growth after postnatal day 42 is not clear, but might be due to programming *in utero*. This is likely since up to postnatal day 42 there was no differences between control rats and the experimental animals. Although no data is available to support this, it is possible that more fat is deposited in the fat stores of these rats.

6.4 The effect of Maternal Nicotine Exposure on Lung development in the offspring.

6.4.1 Lung volume (Lv)

The increase in the lung volume of the control rats followed the same pattern as the increase in body weight. This is important because it is expected that the increase in body weight results in an increase in oxygen demand by the body, consequently the Lv and internal

surface area of the lung increases to satisfy the increased demand for O_2 (Massaro & Massaro, 1996). Between postnatal days 42 and 84, the Lv of the male rats increased faster than the female rats. This supports studies done by Becklake & Kauffman (1999) as well as Hyde et al. (2007) who showed that adult males have a larger Lv than females and that the increase in Lv is proportional to the increase in BW (Tenney & Remmers, 1963). The Lv of 84-day-old nicotine exposed male and female rats was bigger than of the control rats of the same age. This could potentially be attributed to the increase in BW because an increase in body size, as reflected by an increased BW and CRL, will result in an increased demand in O_2. Studies supporting these findings were done by Gehr & Ammann, (1981) who showed that larger animals require a larger internal surface area in the lungs to meet the higher demands of the bigger body and to transfer oxygen to the blood. Therefore, as expected, the Lv of the lungs of the male rats increased faster than that of the females because of the faster increase in BW between postnatal days 42 and 84.

The Lv/BW ratio at postnatal day 84 was the same for both male and female rats. There was no difference seen between the control rats and experimental groups at postnatal day 84. This means that the increase in BW and Lv was proportional. The CC/Lv of rats was also not affected by nicotine exposure. This showed that the increase in Lv was proportional to the increase in CC as the animals grow and therefore maternal nicotine exposure during gestation had no effect on proportional growth of the body.

6.4.2 Mean linear intercept (Lm), volume densities of the airspaces (Va) and parenchymal tissue (Vt).

The Lm is a measure of the distance between alveolar walls in the lung and is an indication of the volume of an alveolus. The Lm increases in emphysematous lungs due to loss of alveolar

walls and elastic recoil (Thurlbeck, 1967). During normal lung development it is expected to decrease due to secondary septation and thus the formation of smaller alveoli. The formation of new alveoli and the consequent increase in alveolar number causes an increase in the internal surface area (Thulbeck, 1967; Weibel, 1963; Mascaretti et al. 2009) available for gas exchange. The increase in alveolar number and thus the internal surface area available for gas exchange is important because, as the BW of an animal increases, more energy yielding nutrients will be oxidized to meet the energy demands of the growing animal. This implies that more oxygen will be required for oxidation of energy substrates to meet the increased demand for energy. To meet the increase in the oxygen demand, lung growth and alveolar formation must therefore be proportional to the growth and development of the rest of the body. Alveolar damage, such as in emphysema, result in a smaller internal surface area available for gas-exchange due to destruction of the alveolar walls and the subsequent fusion of adjacent alveoli which result in fewer but bigger alveoli. The latter is accompanied by an increase in the Lm of the lungs.

It is important to note that in this study maternal nicotine exposure, as well as supplementing the diet with tomato juice, occurred during pregnancy only. The phase of rapid alveolar formation only commences at postnatal day 4 in rats and lasts until postnatal day 13 (Burri et al. 1974; Burri, 2006). This implies that it is unlikely that any changes in Lm after birth and after the onset of rapid alveolarisation, was due to the direct effect of nicotine. Changes in the Lm of the offspring that were exposed to nicotine were seen only after weaning at postnatal day 21; that is 3 weeks after nicotine was withdrawn from the lactating female. At this stage the rat lung has fully developed into a functional lung (Bolle et al. 2006).

As expected the Lm of the control offspring, as well as that of the rats whose mothers received tomato juice during pregnancy, decreased gradually as more alveoli are formed to increase the internal surface area available for gas exchange, and thus to meet the gradual

increase in the oxygen demand of the rats as their BW increases. Contrary to the decrease in the Lm of the control and tomato juice groups, the Lm of the nicotine exposed rats increased so that at postnatal day 84 it was significantly bigger than that of the control animals of the same age. This can be attributed to a gradual deterioration of the lung parenchyma of the nicotine group. The late onset of the deterioration of the lung parenchyma and the associated increase in Lm takes place when no nicotine is present in the lungs of the animals. It is therefore plausible that the lungs of these nicotine exposed rats were programmed during pregnancy to start a late onset of parenchymal deterioration. This is supported by Sekhon et al (1999) who suggested that nicotine can alter fetal lung development by crossing the placenta to interact with the nicotinic receptors on the developing lung, therefore altering the programme that control normal lung development. These findings were similar to those done by Collins et al. (1985) that showed that exposing pregnant rats to cigarette smoke resulted in enlarged and fewer saccules in fetal rat lungs. Similarly, Maritz et al. (1993) and Maritz & Thomas (1994) found a decreased radial alveolar count and increased mean linear intercept in neonatal rats that were exposed to nicotine via the placenta and mother's milk. Also, in studies by Foronjoy et al. (2005) it was shown that postnatal cigarette smoke exposure for one year in mice increase the Lm of the rat lung. They reported no change in lung compliance though. Based on findings from the literature and my own findings it is conceivable that the nicotine present in tobacco smoke contributed to the increase in Lm reported by Foronjoy et al. (2005). This is plausible because each puff of smoke that is inhaled contains 10^{15} molecules of oxidants. In addition, nicotine itself also induces oxidant formation in the lungs (Church & Pryor, 1985) thereby contributing to an oxidant overload in the exposed lungs. This enhances cell membrane damage (Halima et al. 2010) and thus a gradual breakdown of alveolar walls.

A consequence of the gradual breakdown of the alveolar walls of those rats that were exposed to nicotine via the placenta is the decrease in the volume density (Vt) of the lung parenchyma followed by an increase in the volume density of the airspaces (Va). An increase in Vt of control rats, the rats exposed to both tomato juice and nicotine and those rats exposed to tomato juice only, is a normal response of the lung to development and aging and is a reflection of the increase in alveolar number as the lung develops.

Supplementing the diets of the nicotine exposed pregnant females with tomato juice prevented the effect of nicotine on alveolar volume and parenchymal tissue volume density. Thus, if nicotine induces an imbalance in the oxidant/antioxidant status of the mother, and thereby compromised the protection of the developing fetus, it is conceivable that this was prevented by tomato juice by restoring the oxidant/antioxidant status of the mother and in this way prevented the programming of the lungs parenchyma to deteriorate prematurely. This is supported by studies of Balakrishnan & Menon (2006) who showed that hesperidin, a low molecular weight polyphenolic antioxidant that occurs in citrus fruit, protect rats against nicotine toxicity.

6.5 The effect of Maternal Nicotine Exposure during gestation on cell proliferation in the lungs of the offspring.

6.5.1 Cell proliferation during normal lung development

Cell proliferation is rapid during pre- and postnatal lung growth and development, and is a tightly controlled process. The coordination and control of cell proliferation and differentiation is important in organogenesis to ensure that the lung develops into an efficient gas exchanger. The various phases of lung development from the initial out-pouching of the embryonic foregut to the appearance of a functional blood–air barrier, and of a big enough

area for gas exchange to maintain tissue oxygenation, is largely controlled by endocrine, paracrine, and epithelial–mesenchymal interactions. This is illustrated by a study which showed that corticotropin-releasing hormone–deficient mice, which, as a consequence are also glucocorticoid-insufficient, result in death of the neonates due to respiratory insufficiency (Muglia et al. 1999).

The histology of the lungs of corticotropin-releasing hormone–deficient mice is normal up to day 17.5 of gestation. Thereafter thinning of the alveolar walls and the development of an adequate internal surface area for gas exchange, as part of lung maturation, fails. These morphologic alterations in the corticotropin-releasing hormone–deficient mouse lung are the result of continued cell division in cellular compartments that, by this time of lung development, have stopped in normal mice. The failure of the alveolar walls to thin in these mice is rather due to excessive cell proliferation over a longer period of time than as a result of a failure of apoptosis. The lungs of the corticotropin-releasing hormone–deficient mice also exhibits delayed induction of type II pneumocyte biochemical parameters, such as messenger RNAs (mRNAs) for surfactant protein-A (SP-A) and surfactant protein B (SP-B), and fatty acid synthase. According to Muglia et al. (1999) Clara cell maturation is also delayed. Their findings indicated an essential role for endogenous glucocorticoids in pulmonary maturation *in utero* and thus control of lung development by requesting alveolar epithelial cell proliferation and differentiation. This then ensure the normal development of the lung into a gas exchanger that will meet the oxygen demands of the offspring after birth. Any interference with this process will, therefore, also compromise normal lung development and function. Interference with epithelial-mesenchyme interaction will also compromise lung development and maintenance of parenchyma structure in the long term.

Formation of alveolar septa is an important process in lung development. In lung development fibroblasts play an important role in the transition from the saccular to the alveolar stage since elastic fibers, produced by the fibroblasts, are also thought to be involved in septation. These fibers provide structural support for the cells of newly emerging secondary septa. This was illustrated by studies of Kida & Thurlbeck (1980) that indeed showed that the inhibition of elastic fiber assembly was linked to impaired septation and alveolarization. Any interference with these cells will, therefore, compromise lung structure and function, especially since the type II pneumocytes and fibroblasts communicate to ensure proper control of alveolar formation (Shannon et al. 2001; Griffin et al. 1993). This means that interference with the integrity of any one or both of these cell types, or with the communication between them, will adversely affect alveolar formation and maintenance.

The phase of lung development that is known as the phase of rapid alveolarisation (Noguchi & Samaha, 1991; Post & Copland, 2002) occurs between postnatal days 4 and 13 in rats, and corresponds with the onset of alveolarization at week 36 of pregnancy in humans (Post & Copland, 2002). As expected, in the present study the numbers of proliferating cells of the control animals were highest at postnatal day 14, which is at the end of the phase of rapid alveolarization and thus rapid cell formation in rat lung. During this phase of lung development interstitial fibroblasts indeed undergo rapid proliferation (Post & Copland, 2002) to support lung growth and maturation. After postnatal day 14, proliferation of the cells in the alveolar walls gradually becomes slower as the lungs mature because alveolar formation slows down.

6.5.2 The effect of maternal exposure to nicotine on cell proliferation in developing lung

The rate of cell proliferation in the alveolar walls of all the experimental animals also decreased from postnatal day 14 and then leveled at postnatal day 21. It also shows that tomato juice supplementation, as can be expected, had no effect on cell proliferation in the alveolar walls. The decrease in the rate of cell proliferation is attributed to the fact that between postnatal days 13 and 21 the number of fibroblasts and type II pneumocytes decreases (Bruce et al. 1999; Noguchi & Samaha, 1991): the decrease in fibroblasts and type II pneumocytes occurs by means of programmed cell death or apoptosis, which peaks between postnatal days 17 and 19 in rats. Apoptosis plays a key role in the thinning of the alveolar septa that occurs after the cessation of alveolarization (Bruce et al. 1999; Wright et al. 1993). On the other hand, cell proliferation in the lungs of the suckling 14 and 21-day-old offspring of the mothers that were exposed to nicotine during gestation was slower than that of the control animals as well as those whose mothers received a tomato juice supplement during gestation. It is interesting to note that cell proliferation in the alveolar walls of the offspring of mothers that were exposed to nicotine during gestation, and also received a tomato juice supplementation, was the same as that for the 14-day-old control offspring. This means that tomato juice protected the lungs of the offspring against the adverse effects of nicotine on cell proliferation in the alveolar walls of the lungs of these offspring. Previous studies showed that type II pneumocyte proliferation increased when rats were exposed to nicotine (Collins et al. 1985; Cunningham et al. 1994; Curet et al. 1983, Hanrahan et al. 1992; Lieberman et al. 1992). It was also shown that nicotine induces type I cell damage which serves as a stimulus for type II pneumocytes proliferation and differentiation of one of the daughter cells into a type I cell (Berthiaume et al. 2006; Maritz & Thomas, 1995). This is a mechanism whereby the lung replace damaged type I cells to maintain alveolar integrity (Rehan et al. 2007). This means that rapid repair of the damaged alveolar surface after injury

is clearly essential for the maintenance of the internal surface area of the lung, which is very large (i.e., 143 m^2), Gehr et al.1978 in adult humans. The size and spatial restrictions of the alveolar surface therefore suggest that at least one progenitor cell per alveolus is required to achieve rapid repair of the alveolar epithelial cells. Thus, a large number of cells must function as a ‘‘ready reserve’’ to repair damaged alveolar surface (Warburton et al. 2008).

In a recent study it was shown that cell proliferation in the alveolar walls was permanently suppressed in the lungs of those animals that were exposed to nicotine via the placenta and mother's milk (Maritz & Mutemwa, 2013). This was supported by this study; maternal nicotine exposure during gestation only, resulted in a slower cell proliferation in the alveolar walls during the phase of rapid alveolarisation and also in adulthood. This means that maternal nicotine exposure during pregnancy only, might interfere with early lung development, and will compromise the maintenance of lung structure and function in the longer term. Therefore, nicotine exposure during pregnancy and lactation or pregnancy only, programs the cells of the alveolar walls of the offspring to proliferate slower permanently and in this compromise maintenance of lung parenchyma in the long term. The mechanism whereby nicotine suppresses cell proliferation during early lung development is not known.

It has been shown that nicotine exposure interferes with glucose metabolism and inhibits apoptosis in fibroblasts (Fischer et al. 1990). It is, therefore, plausible that inhibition of glucose metabolism, such as inhibition of glycolysis in lungs of the offspring by nicotine (Maritz, 1986), will adversely affect cell proliferation in the alveolar walls. Since nicotine-induced inhibition is permanent (Maritz, 1986), it is conceivable that over time it will result in a slow but gradual breakdown of the lung parenchyma with a consequent development of larger but fewer alveoli and a decrease in the surface area available for gas exchange.

In this study the use of tomato juice as an antioxidant was intended to reduce the effects of nicotine by preventing a decrease in the oxidant capacity of the mother and protecting against anti-oxidant damage. Tomato juice counteracts the effects of nicotine on lung development by preventing programming of the lungs of the offspring to be more susceptible to disease later in life. This is achieved through maintaining the oxidant/anti-oxidant balance in the mother and developing fetus. Further studies may have to be conducted to determine the optimum amount of tomato juice intake per day is required to protect the developing lung against the adverse effects of maternal nicotine intake during pregnancy and lactation.

6.6 Lung Function

There is a close association between lung structure and function (Zosky et al. 2001). Any change in the structure will therefore adversely affect lung function. Interference with fetal and neonatal lung development will also impact on lung function. In this study it was showed that maternal nicotine exposure during pregnancy programmed the lungs to become more susceptible to disease as the animal age. This implies that maternal smoking or use of NRT to quit the habit during pregnancy will adversely affect the respiratory health of the offspring later in life. The ability of the lungs to function is also largely dependent upon it being able to reversibly change shape. This can be quantified using two parameters, namely compliance and elastance. Compliance is the ability of a hollow organ such as the lung, to distend and increase volume with increasing transmural pressure, such as during inhalation, or to resist recoil toward its original dimensions on removing the distending force, such as during exhalation. Elastance, is a measure of how readily the lung returns to its original shape during exhalation (elastic recoil). Elastic recoil is inversely related to lung compliance. This is largely dependent on the connective tissue framework of the lung which is important for

stretching of the lungs when air is inhaled and for the lungs to fully recoil when air is exhaled. It also maintains the 3-D structure. Any damage to the connective tissue framework may, therefore, compromise lung compliance and recoil, and in this way lung function. In this study static compliance of the lungs of all animals increased with age. Deterioration of lung function is an important factor in age-related morbidity and mortality and elastic fibre degeneration appears to play a central role in many age-related pulmonary disorders (Sherratt, 2009). Windvogel (2004) showed a gradual but faster deterioration of the elastic tissue in the alveolar walls of rats exposed to nicotine during gestation and lactation compared to the control rats with the concomitant increase in alveolar volume. This breakdown of the elastic tissue component of the connective tissue framework results in an increased compliance and thus impaired lung function. From postnatal day 21 to postnatal day 84, the static compliance of all groups showed an age related increase in the static compliance. This is in agreement with the findings of Ranga et al. (1979) who showed that there was a loss of elastic fibers and increased static compliance with aging in mice. Furthermore the results of this study showed that the static compliance of rats that were exposed to nicotine during pregnancy increased faster from postnatal day 42 to postnatal day 84 than of the control animals. This is contradictory to studies by Milner et al. (1999); Lodrup Carlsen et al. (1997) and Brown et al. (1995) who showed that infants of mothers who smoked during pregnancy demonstrated decreased lung compliance. This can conceivably be attributed to an increase in the connective tissue of the alveolar walls because smoking is one of the risk factors for 'idiopathic' pulmonary fibrosis (Oh et al. 2012).

Apart from the ability of the lungs to expand and retract during inhalation and exhalation, the gas exchange function of the lungs also depends on the internal surface area available for gas exchange. Since maternal nicotine exposure induces a gradual deterioration of the lung

parenchyma, and thus consequent development of emphysema-like lesions, the gas exchange function will be compromised too.

In conclusion maternal nicotine during gestation only resulted in the breakdown of elastic tissue, a loss of the connective tissue framework which resulted in the increase in compliance and therefore an impairment of lung function. Although it is accepted that prevention of maternal smoking during pregnancy is important, evidence of the adverse effects of the nicotine on the lung of the offspring also suggest that it is not suitable to prescribe NRT to pregnant woman. In this study supplementing the mother's diet with tomato juice prevented nicotine induced damage in the lung of the offspring.

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