DOES MATERNAL NICOTINE EXPOSURE DURING GESTATION AND LACTATION CHANGE THE ANTIOXIDANT-OXIDANT STATUS OF THE LUNGS OF THE OFFSPRINGS AND IS THE TOMATO JUICE SUPPLEMENTATION PROTECTING THE LUNGS OF THE OFFSPRINGS?

By

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ABSTRACT

Nicotine exposure to the fetus through tobacco smoking or nicotine replacement therapy during the whole period of gestation and lactation causes diverse effects on fetal and neonatal lung development, integrity and maturation which compromise the gas exchange function of the lungs and renders this vital organ susceptible to gradual damage and different diseases in latter life. Maternal nicotine exposure during gestation and lactation results in gradual destruction of the lung parenchyma, and this leads to the combination of many small air sacs in one bigger alveoli which is a sign of emphysema. Many researchers speculated that the way in which, nicotine causes emphysema and other damage, is by inducing the formation of many reactive oxygen species (ROS), and creating an imbalance between the oxidants and the antioxidants of the body, which is termed oxidative stress.

The aim of this study was to assess the effects of nicotine exposure on the lung of the fetal and neonate rat during gestation and lactation as gas exchanger, and also to see whether the supplementation of tomato juice containing lycopene, a powerful carotenoid antioxidant could protect the lungs against these effects of maternal nicotine exposure.

In this study pregnant rats have been divided into 4 groups: a group which received nicotine (1mg/kg body weight/day) subcutaneously, a group that received the tomato juice only (6mg/kg body weight/day per os), a third group that received the combination of tomato juice (6mg /kg body weight/ day per os) and nicotine (1mg/kg body weight /day subcutaneously) . The control group that received saline (1mg/kg body weight /day)

subcutaneously and water. The injections were done during pregnancy and lactation until weaning at postnatal day 21.

The results showed that maternal nicotine exposure during gestation and lactation leads to a gradual damage of the lung parenchyma and slower formation of the alveoli during the equilibrated phase of the lung growth leading to a decrease in the internal surface area required for gas exchange. Supplementation with tomato juice during gestation and lactation prevents all the adverse effects of maternal nicotine exposure on the lungs of the offspring. Since nicotine induce an increase in the oxidant levels of the mother and the fetus, my results imply that lycopene protected the lungs of the offsprings against the oxidants and thus against changes in the program that controls lung development as the animals age. This is supported by the observation that at postnatal day 84 the antioxidant capacity of the lungs of the nicotine exposed rats was normal.

KEY WORDS: tobacco smoking, maternal nicotine, tomato juice, lycopene, lung development, emphysema, foetal programming.

DECLARATION

I declare that **"Does maternal nicotine exposure during gestation and lactation change the oxidant-antioxidant status of the lungs of the offsprings and is tomato juice protecting the lungs of the offsprings?"** is my own work and has not previously in its entirety, or in part, been submitted at any university. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

KAYIGIRE XAVIER ABDULKARIM

DEDICATION

For my both parents died in 1997

Acknwoledgement

I would like to thank:

First Almighty God to keep me alive so that I can accomplish this work.

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LIST OF ABREVIATIONS

DNA: 2’-deoxy-5’ribonucleic acid

Cu: Copper

COPD: Chronic obstructive pulmonary disease

ANOVA: Analysis of variance

Gr: gram

kDa: kilodalton

ml: milliliter

mm: millimeter

IL-1: interleukin- 1
mRNA :messenger ribose nucleic acid

Fas: apoptosis stimulating factor

Cyp 450: cytochrome p 450

RDS: respiratory distress syndrome

CLD: chronic lung disease

TNF-α: tumor necrosis factor alpha

FGF: fibroblast growth factor

Shh: sonic hedgehog

TGF-b: transforming growth factor beta

VEGF: vascular endothelial growth factor

AAT: alpha-1 antitrypsin

A-1AD: alpha-1 antitrypsin deficience

P1, P4, P5, P14: postnatal day 1, day 4, day 5, day14

BC: before Jesus Christ

CDC: the center for disease control

$C_{10}H_{14}N_2$: 1-methyl-2-(3-pyridyl-pyrrolidine)

nAchr: nicotinic acetylcholine receptor

PKC: protein kinase C

Raf-1: Raf protein kinase -1

ROS: reactive oxygen species

NRT: nicotine replacement therapy

NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

MAP: mitogen activating protein

CNS: centre nervous system

SIDS: sudden infant death syndrome

5-HT: 5-hydroxytryptamine

CuZnSOD: copper zinc superoxide dismutase

MnSOD: manganese superoxide dismutase

EcSOD: extracellular superoxide dismutase

Lv: lung volume

Lm: mean linear intercept

Vt: volume density of parenchyma tissue

Va: volume density of airspaces

Sa: internal surface area

Tsept: interalveolar septal thickness

BW: body weight

CC: chest circumference

CR: distance crown-rump

TW: alveolar wall thickness

NEB: neurepithelial bodies

NF-Kb: nuclear factor kappa beta

β: beta

α: alpha

Π: pi=22/7

mm^2:millimetre square

ERK: extracellular signal-regulated kinase

JNK: c- jun-NH_2-terminal kinase

^0C: degree Celsius

$C_{40}H_{56}$: lycopene

O^-_2: oxygen free radical

NO_2: nitrogen dioxide

CO_2: carbon dioxide

SO_2: sulfur dioxide

GJIC: gap-junction intercellular communication

BAX: member of the Bcl-2 family

BCl_2: B- cell Leukemia/lymphoma 2

Akt: serine- threonine-kinase

THmRNA: Tyrosine hydroxylase messenger ribose nucleic acid

MWt: molecular weight

Fe^{2+}: iron ion

OH^{-}: hydroxyl free radical

Zn: zinc

IP: intraperitoneally

WT: wild type, stands for control

Lynx 1: a nAChR modulator protein found in brain of rhesus monkey

CHAPTER 1

INTRODUCTION

1. Introduction

The lungs are situated in the chest cavity on either side of the heart enclosed by pleura with serous fluid, the rib cage and diaphragm. Respiration is the main function of lungs which exchange oxygen and carbon dioxide between the atmosphere and the circulation and cells of the body. This exchange takes place in the respiratory units of the lungs or alveoli. Other role of the lungs is water evaporation: an important factor in the fluid balance and heat of the body.

Different researchers have shown that exposure to tobacco smoke has diverse effects on lungs which impact negatively on gaseous exchange and these effects can become fatal in case of prenatal exposure to tobacco smoke, which leads to many respiratory abnormalities in later life, such as emphysema, chronic bronchitis and impaired lung function (Yarnell and St Leger, 1979; Maritz, 2008; Brčić Karačonji, 2005).

1.1 Lung development

Normal human pregnancy lasts 37-42 weeks, and in rats and rabbits 21 and 28 days respectively, during which time organ development must reach a level of maturation that will allow it to function effectively at birth. Lung development is divided into 5 different phases, namely; the embryonic phase (3-7 weeks), pseudoglandullar phase (7-16 weeks), cannalicullar phase(16-24 weeks), saccullar phase (24-36weeks), and alveolar phase (36 weeks to term) in human.

The *embryonic phase* begins with the first appearance of tracheal bud in the developing embryo, at 26 days of gestation (Fig. 1.1).

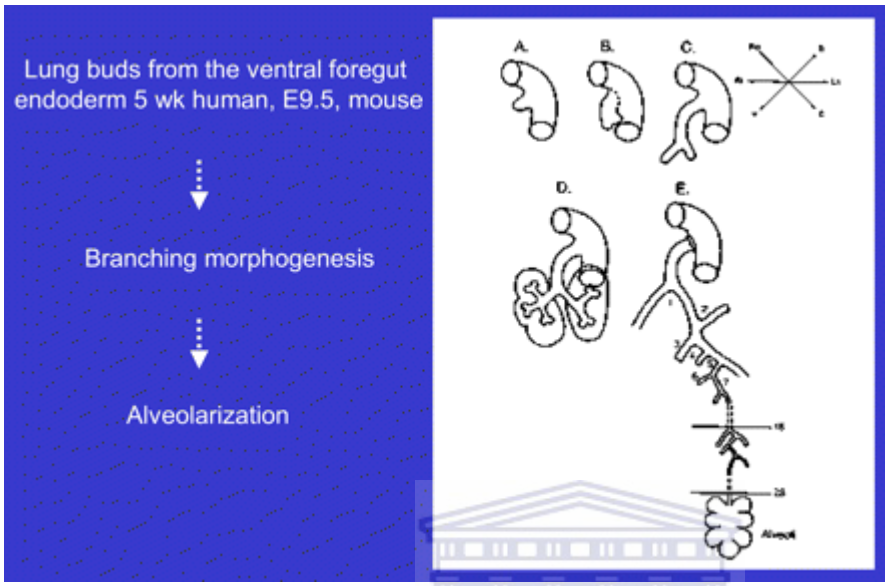


Fig. 1.1**.** Diagram showing key events during lung development. The laryngotracheal groove arises from the primitive foregut to form the primary bronchi, lobar, and segmental bronchi From Warburton et al, 2006).

The endodermal cells from embryonic foregut outpouch ventrally into surrounding mesenchyme. The lung bud elongates and divides dichotomously. At the age of 4.5 weeks there are 5 tiny saccules, 2 on the left hand side and 3 on the right hand side, representing the future lobar bronchi and the corresponding lung lobes (Jeffrey 1998).

The *pseudoglandular phase* of lung development occurs between the 7^{th}-16^{th} weeks of gestation and is marked by further branching of the airways, the vascular network and progressive differentiation of epithelial cells to adult structures of cartilage, sub-mucosal glands, bronchial smooth muscle and epithelial cell types.

By 14 weeks of gestation 70 % of the total airway are formed and by 17 weeks, the formation of conducting airways and terminal bronchioles are complete (Kotecha and Suchita, 2007).

During the *canalicular phase* (16^{th}-24^{th}weeks of gestation) differentiation of the epithelial cells lining the alveolar ducts, respiratory bronchioles and primitive alveoli are formed. There is also differentiation of type I and type II pneumocytes that form the alveolar capillary barriers (Hislop 2002). From weeks 24 to term the terminal phase of lung development occurs: During this phase the capillary networks develop, cells differentiate and the mesenchyme-derived stroma thins down, and the presumptive alveoli expand. Surfactant synthesis and secretion are augmented after the 30^{th} week (Mercus et al., 1996).

The *saccular phase* occurs between 28-36 weeks. During this stage of lung development the division of the airways is almost complete. The growth and the development of lung structures, including enlargement of the peripheral airways, dilatation of acinar tubules to form saccules and thinning of the airways walls occur. There is also differentiation of type II cells into type I cells and increment in surfactant containing lamellar bodies in type II pneumocytes (Kotecha and Suchita, 2007).

The *alveolar phase* occurs from 36 weeks *in utero* to 2 years after birth in humans. During this stage, there is formation of secondary septa and alveoli are shaped into cup-like structures. Gradually low ridge like projections, with double capillary network

appear in the airspaces, which divide the airspaces into alveoli. The multiplication of alveoli and formation of double capillary walled secondary septa continue up to 3 years of age in humans (Burri, 2006).

Postnatal lung growth: At birth the number of alveoli is 20-50 million and increases up to 3 years after birth. The internal surface area (Sa) also increases until after adolescence (Kotecha, 2000). The alveolar number in fully developed adult lung is 300-800 million (Ochs et al., 2004). The alveolar number in boys is higher than in girls of the same age and stature. This means that alveolar surface area is greater in boys than in girls (Thurlbeck, 1982).

The stage of microvascular maturation lasts up to 3 years postnatally. During this phase of maturation of the mirovasculature the initial double capillary networks fuses into a single layer. When that is completed alveolar septation stops (Burri, 2006)

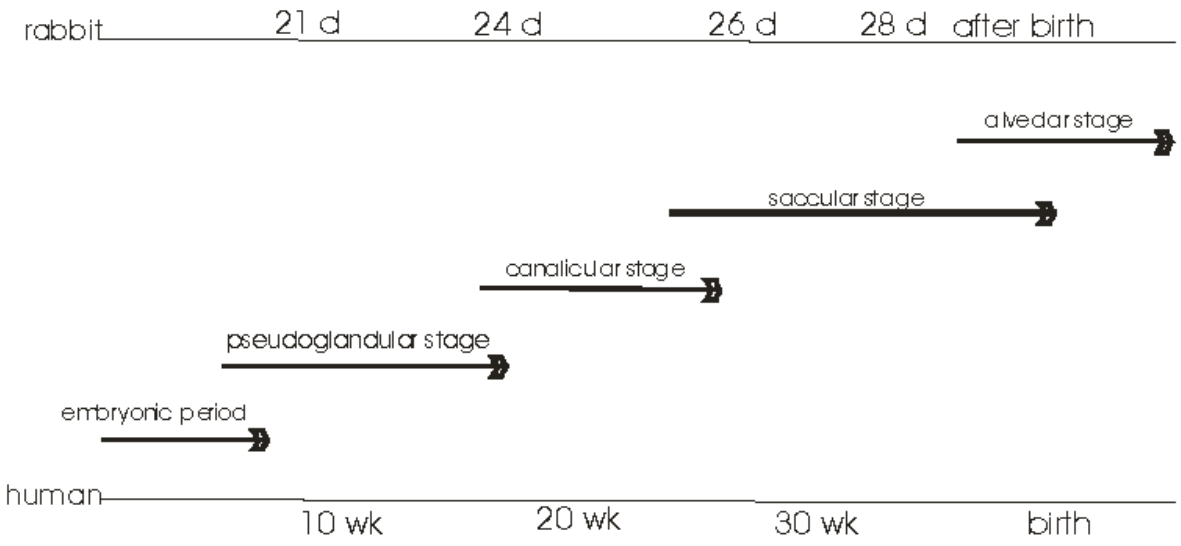


Fig. 1.2: Comparison of human and rabbit lung development (Burri, 1997)

From figure 1.2 it is clear that alveolar formation start before birth in humans and proceeds for up to 6 to 8 years after birth (Warbuton et al.,2006) On the other hand, in rats, mice, and rabbits the phase of alveolarisation starts after birth Fig. 1.2 and 1.3).

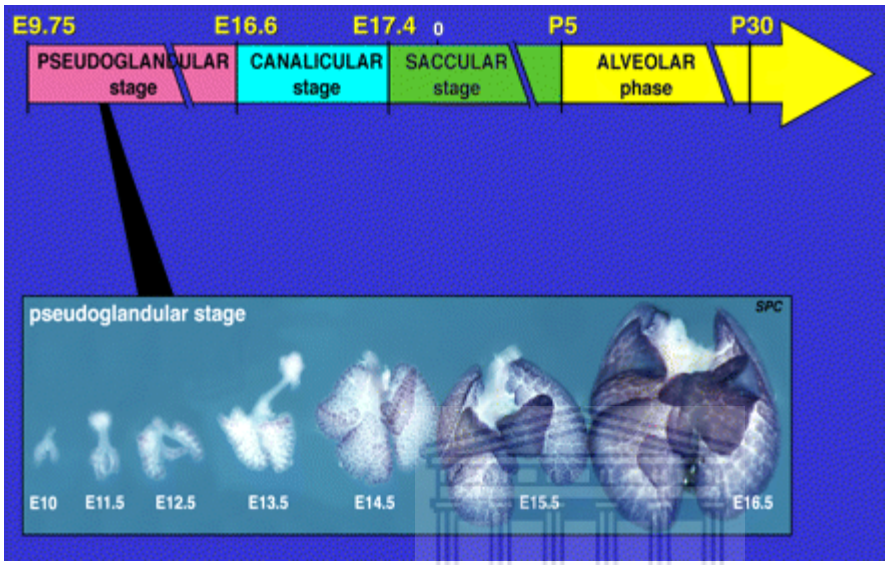


Fig. 1.3**.** Mouse lung development: chronology. The timing of stages of lung development is illustrated in the *upper panel*. In the lower panel, developmental series whole-mount studies are shown (From Warburton et al, 2006.).

In rats, the animal model used in this study, the phase of rapid alveolarisation starts around postnatal day 4 and last up to postnatal day 13 (Burri , 1974). After postnatal day 13 alveolar formation still proceeds but at a slower rate (Maritz, and Dolley, 1996). The increase in alveolar number results in an increase in the internal surface area available for gas exchange. The size of the surface area is determined by the oxygen demand of the body of the animal (Massaro et al., 1975, Massaro and Massaro, 2004).

1.2 Interference with lung development.

1.2.1 Living near the highway affects the lung development:

Studies showed that children living 500 meters or approximatively a third of a mile from a highway since age 10, had substantial deficits in lung function by the age 18 compared to children living 1500 meters or a mile away (Bayer-Oglesby et al.,2006). According to Gauderman et al., (2007), at the University of Southern California, someone suffering pollution–related deficits in lung function as a child probably will have less than healthy lungs all of his or her life, and poor lung function is to be in later life a major risk factor for respiratory and cardiovascular diseases. It implies that healthy children who are not asthmatic and do not smoke, have a significant decrease in lung function from traffic pollution. According to Schwartz (2004), the effects of childhood exposure to exhaust fumes can last a lifetime. Studies done by Romieu et al., (2007), support the suggestion by Schwartz (2004) namely that long–term exposure to air pollutants in early age increase the risk of developing chronic obstructive lung disease, cardiovascular morbidity and mortality.

Previous studies have found that short-term exposure to pollutants such as; carbon monoxide, lead, nitrogen dioxide, ozone, particulate matter, and sulfur dioxide is associated with acute but reversible deficits in lung function. On the other hand, the effects of long-term exposure to pollutants, like that experienced by residents of heavily polluted urban environments, had not been conclusively characterized (Jedrychowski et al., 1999; Frischer et al., 1999).

1.2.2 Diseases and lung development:

Respiratory Distress Syndrome (RDS) in premature newborn infants is a dyspnea with cynosis heralded by the following signs: diltation of the alae nasi, expiratory grunt and retraction of the suprasternal notch or costal margins. Deficiency of pulmonary surfactant is the main cause of respiratory distress syndrome (RDS) in premature newborn infants, which is often complicated by chronic lung disease (CLD). Preterm infants are born with the lungs that are not fully developed and often with surfactant deficiency. This often requires mechanical ventilation and/or oxygen therapy to assist the respiratory function of the lungs. Hyperoxia and barotraumas cause lung injury, disrupt septation and decrease gas exchanger/surface area (Thebaud and Abman, 2007).

Intrauterine infection result in disordered lung growth in the newborn and is often associated with intra-amniotic infection (IUI), which is characterized by increased pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), in the amniotic fluid. In very preterm birth due to IUI, the incidence of RDS is decreased, while the incidence of CLD is increased. Tumor necrosis factor (TNF) is a cytokines involved in systemic inflammation and can also stimulate an acute phase reaction. The primary role of TNF is the regulation of immune cells, to induce apoptotic cells death, to induce inflammation and inhibit tumorigenesis and viral replication. Dysregulation of this cytokine results in different human diseases as well as cancer (Locksley et al., 2001).

Glucocorticoids are used in imminent preterm birth and as rescue therapy for chronically ventilated preterm infants, but these medicines, even at low dose, cause impairment of lung alveolarization and precocious microvascular maturation (Tschanz et al., 2003).

Some growth factors, such as fibroblast growth factors (FGF) and Sonic hedgehog (Shh) seem to have influential roles in determining the pattern of airway branching. FGFs are also important in type II cell maturation and differentiation as well as secretion of surfactant protein C later in lung development (Maeda et al., 2007; Warburton et al., 2003). On the other hand, transforming growth factor (TGF β) has an opposite but important role of inhibiting cell proliferation and branching morphology, promoting formation of lung matrix and aiding pulmonary repair after injury. Its deficiency can lead to undue lung inflammation and it's over expression may lead to lung fibrosis (Maeda et al., 2007; Warburton et al., 2003). Vascular endothelial growth factor (VEGF) plays a central role in vasculogenesis and angiogenesis and is therefore critical for normal lung development. A part from its primary role on vascular development, VEGF also has positive effects on differentiation of type II cells and stimulation of surfactant production. Furthermore, VEGF also has an important role on endothelial function and inhibition of VEGF in premature lung has been known to reduce NO bioavailability that may lead to the development of chronic lung disease of prematurity (CLD) (Voelkel et al., 2006).

1.2.3 Effect of tobacco smoke on lung development

The studies done by Maritz (1986, 1987, 1988, 1992, 1997a&b, 2000, 2001) showed that maternal nicotine exposure has a number of effects on lung development in the offspring.

Nicotine provokes accumulation of AMP and ROS. Nicotine itself has an oxidave function. This results in DNA damage that in turn may change the program that controls glycolysis and as a consequence result in a decrease of glycolysis which is the main

pathway by which lung procures energy (Maritz, 1986). All of these consequences of nicotine on normal metabolism will provoke insufficient energy for normal lung development and maintenance of lung integrity in the long-term and cause diverse effects in later life such as premature aging, characterized by microscopic emphysema and obstructive chronic diseases later in life (Maritz, 1997a).

1.3. Emphysema

Emphysema is characterized by damage to the small air sacs and small airways in the lungs in absence of fibrosis (Shapiro et al, 2005). This damage obstructs airways resulting in reduced FEV_1. When emphysema is advanced, the patient must work so hard to expel air from the lungs, that just the simple act of breathing can consume a great amount of energy. Unfortunately, because the emphysema develops gradually over many years, the patient may not experience symptoms such as a shortness of breath until irreversible damage has already occurred (Shapiro et al., 2005).

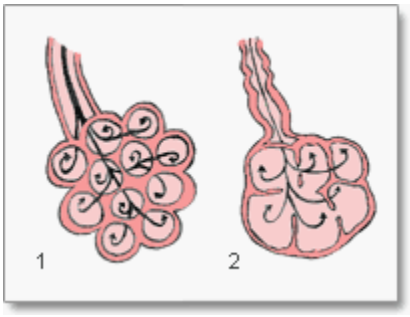
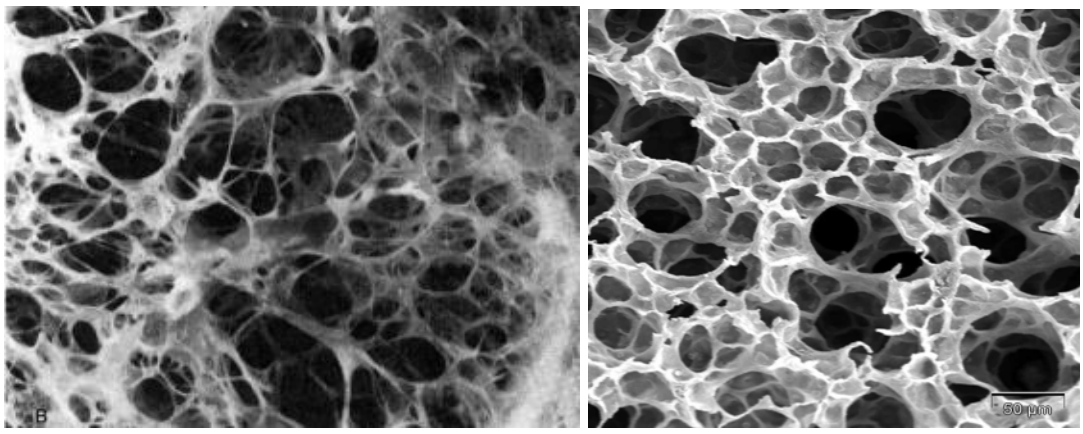


Fig. 1.4. Illustration of changes in alveolar size as a result of emphysema where 1) shows normal healthy alveoli and 2) illustrates enlarged alveoli as seen in emphysematous lung.

1.3.1 Mechanisms whereby emphysema develops.

In emphysema inflammation destroys the fragile walls of air sacs causing them to lose their elasticity. As a result the bronchioles collapse and air becomes trapped in the air sacs which overstretches them and interferes with the ability to exhale. The overstretching may cause more air sacs to rupture, forming larger air sacs instead of many small ones. This result in less elasticity, an increase in compliance and a reduced area available for gas exchange (Stephen et al., 2006).



Emphysema A control B

Fig. 1.5: damage caused by emphysema (A) compared to control (B) (Stephen et al., 2006)

1.3.2 Causes of emphysema

Cigarette smoking is the major cause of emphysema, accounting for more than 80 percent of all cases. Emphysema occurs most often in people older than 40 who have smoked for many years. Long-term exposure to second hand smoke may also play a role. Smoking stresses the natural antioxidant defence system of the lung, allowing free radicals to damage tissue down to the cellular level. When cigarette smoke is inhaled, 80 to 90 per cent remains in the lungs and causes irritation and increased mucous production and damage to the deep parts of the lungs. Eventually mucous clog up the smaller airways and causes chronic bronchitis and emphysema (Seagrave, 2000).

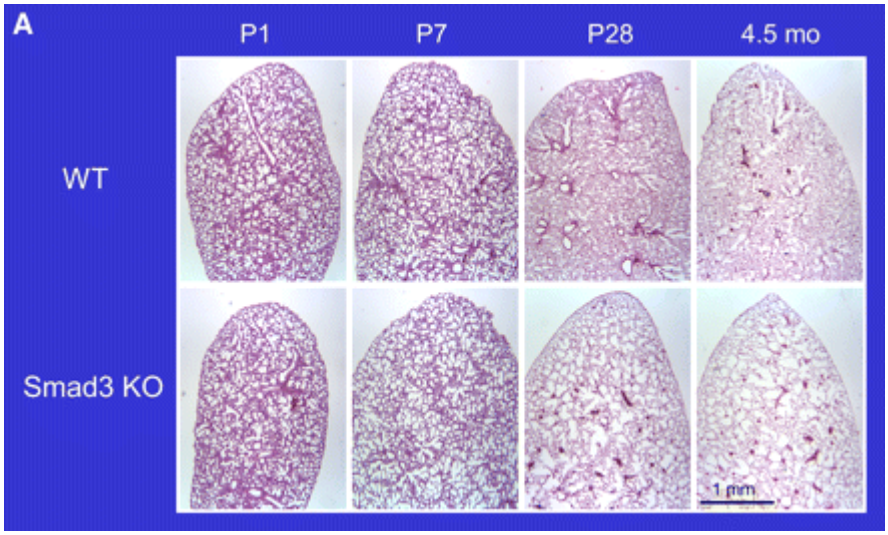


Fig. 1.6. Morphologic changes in *Smad3* knockout (KO) mouse lungs. Alveolarization fails to complete properly in null mutants, followed by progressive centrilobular emphysema. The onset of progressive emphysema is associated with an increase in matrix metalloproteinase-9 activity (From Warburton et al, 2006).

People who have a deficient of alpha-1 antitrypsin (AAT) activity are at a higher risk of developing severe emphysema. Alpha-1 antitrypsin deficiency (AAT deficiency) is an inherited condition and occurs in varying degrees. The deficiency leads to A1AD-related emphysema when the liver produces insufficient AAT to control a natural enzyme known as neutrophil elastase. Though neutrophil elastase plays an important role in fighting bacteria and cleaning up dead lung tissue, it eventually causes irreversible damage to the alveoli by damaging or destroying their elastic fibres if there is not enough AAT to neutralize it. For AAT-deficient individuals who smoke, the risk of developing emphysema is much greater than for the general population. A1AD-related emphysema usually strikes people in their thirties or forties and is very rarely seen in children (Shapiro, 1995).

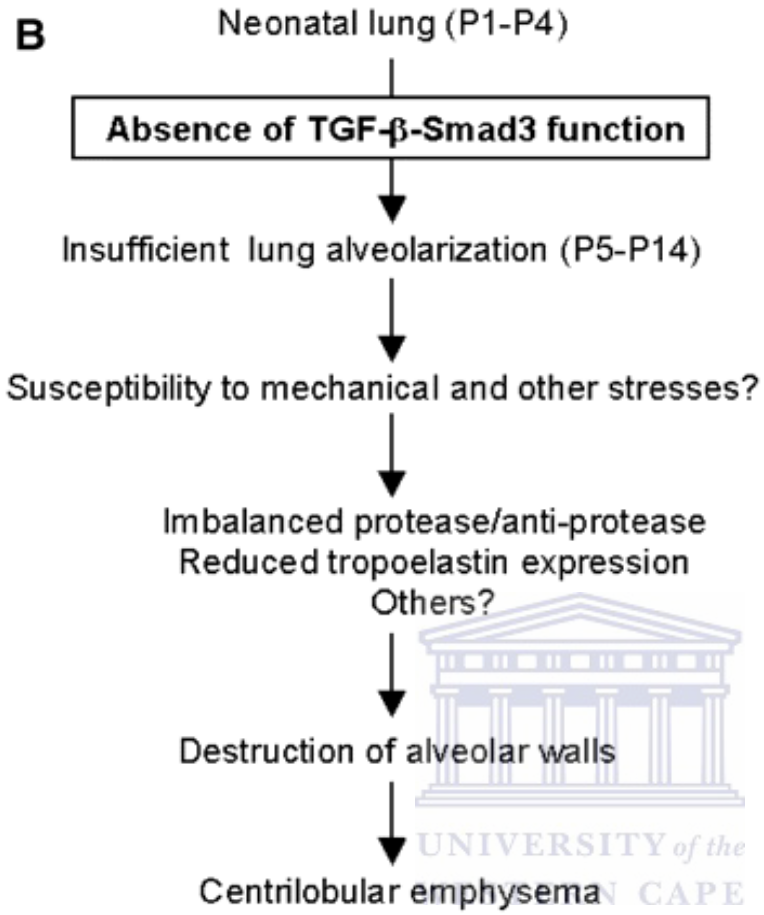


Fig.1.7. Concept diagram explaining the proposed sequence of events in *Smad3*-null mutants, leading to early-onset centrilobular emphysema (From Warburton et al, 2006).

Among other causes of emphysema are industrial pollutants, aerosol sprays, non-tobacco smoke, internal-combustion engine exhaust, and physiological atrophy associated with old age (senile emphysema). Physical damage caused by an accident and followed by scarring can give rise to scar emphysema; severe respiratory efforts can rupture alveoli in cases of near suffocation, whooping cough, labour (child-bearing), and acute bronchopneumonia. Tuberculosis and asthma can also give rise to lung overstretching, severely damaging the elastic fibres of the alveoli walls and bringing on emphysema.

Areas with high rate of poverty also experience higher mortality rates among those suffering from chronic obstructive airway disease, possibly a reflection of inadequate medical care (Montgomery et al., 1996).

The mechanisms leading to tobacco smoke induced emphysema are not so simple, and recent advances revealed that not only various cells and mediators but also different biological processes are involved in pathogenesis of emphysema, including the imbalance between oxidant stress and antioxidant defences in the lungs (Christopher and Robert, 2006).

1.4. Smoking

1.4.1 History of smoking

For thousands of years people have smoked or chewed the leaves of the tobacco plant Nicotiana tabacum. Tobacco was first found and cultivated in the America perhaps as early as 6000 B.C. Following the discovery and colonisation of North and South America, tobacco was exported widely to Europe and the rest of the civilized world. Even in its early days tobacco use was controversial. Some hailed its medicinal properties. For example, tobacco was supposed to be protective against the ravages of plague. As early as in the 1600s people speculated that there might be a link to the diseases like cancer and tobacco use (Doll and Bradford, 1954).

Since then modern research methods have provided evidence of this link and public service announcements that warn of tobacco' health risks and addictive nature are regularly seen in the media.

1.4.2 Effects of cigarettes smoking in general

Tar in cigarettes smoke can cause lung and throat cancer in smokers. It is responsible of yellow-brown staining on smoker's fingers and teeth. In addition to tar, cigarette smoke contains more than 4000 different chemicals (Stedman, 1968, Dube et al., 1982). Among those chemicals are known to be carcinogens like naphthalene which participates in cancer development by damaging the genes which control cell growth (Cooper, 2007).

According to world health organization (2007) tobacco related deaths in India may exceed 1.5 million annually by 2020 (Rani et al., 2003). The leading causes from smoking are cardiovascular diseases, chronic obstructive pulmonary diseases and the lung cancer (Ezzati and Lopez, 2003). The tar phase and the gas phase of tobacco smoke both contains more than 10^{14} free radicals which cause oxidative damage in the form of lipid peroxidation (Pryor et al., 1983). Tobacco smoke is a major cause of atherosclerosis and is considered to be one of the major risk factors for coronary heart diseases (McBride, 1992). Tobacco smoke also may cause type 2-diabetes because it reduces insulin sensitivity due to lower peripheral glucose uptake (Attival et al.; 1993).Also, it destroys the peribronchiolar alveoli and this contributes to a loss of elastic recoil and emphysema (Skurnik et al., 1998). It may also cause gastric and duodenal ulcer and cirrhosis (Johnsen et al., 1994).

1.4.3 Effects of cigarettes smoke on each part of the body

Hair: smell and staining

Brain and mental effects: stroke (cerebrovascular accident), addiction/withdrawal, altered brain chemistry, anxiety about harm caused by smoking, and stimulation of sympathetic nervous system.

Eyes: eyes sting, water blink and more, blindness (macular degeneration), cataracts.

Nose: less sense of smell

Thyroid: graves disease, thyroid disease.

Lung: lung cancer, emphysema, chronic bronchitis.

Heart: rapid heart beat, blood pressure increased, stroke.

Vessels: atherosclerosis and risk of developing clots in big vessel like aorta.

Mouth, Pharynx, Larynx and oesophagus (for cigars, pipes smokers and those who chew tobacco and snuff).

Cigarette smoke causes cancer of kidney, bladder, stomach and pancreas

Teeth: periodontis and brown stains on teeth

The skin: tend to be pale and deeper wrinkles as they age

Bones: risk of osteoporosis, spinal injuries, hip fractures, and low bone density.

White blood cells: high incidence of acute amyeloid leukemia and immune system compromised in general.

Apart from the above it is important to note that smoking during pregnancy holds additional risks for the mother, fetus and newborns. These include spontaneous

abortion/miscarriage, ectopic pregnancy, abruption placenta, placenta previa, and premature rupture of the membranes, premature birth. In addition to that infants are smaller for gestational age. Stillbirths, birth defects can also occur. There are also more nicotine receptors in the foetal brains and an increased likelihood that they will start smoking as teenagers. Maternal smoking and nicotine exposure also result in adverse effects on brain development (Slotkin et al., 1987b).

1.4.4 Light or low tar cigarettes

Research has shown that there is a little difference regarding the amount of chemicals inhaled by people who smoke regular cigarettes as oppose to those who smoke light cigarettes. People who smoke light cigarettes have the same risks of developing smoking related disease as those who smoke regular cigarettes (Hoffmann and Hoffmann, 1997; Doull et al., 1994).

1.4.5 Immediate effects of tobacco smoking

After smoking tobacco the following effects may be experienced:

a. Initial stimulation of tobacco smoking, followed by reduction in brain and nervous system activity.
b. Enhanced alertness and concentration
c. Mild euphoria
d. Feeling of relaxation
e. Increased blood pressure and heart rate
f. Decreased blood flow to the body extremities like fingers and toes

g. Dizziness, nausea, watery eyes, and acid in the stomach
h. Decreased appetite, taste and smell (US Department of Health and Human Services, 1988).

Although rare, it is possible to overdose on the nicotine in tobacco. Very large doses of nicotine can results in an increase of unpleasant effects, including a feeling of faintness and confusion and a rapid decrease in blood pressure and breathing rate. In some cases it can lead to convulsions and death from respiratory failure, 60 milligrams taken orally can be fatal for an adult (Gosselin, 1988). It is estimated that more than 140,000 hospital episodes and 19,000 deaths in Australia can be attributed to tobacco use every year. The principal diagnoses are cancer, heart disease and chronic obstructive pulmonary disease (Ridolfo and Stevenson, 2001; Doll et al., 1995). In the USA the tobacco smoking is responsible for 433,000 deaths per year or nearly one of every 5 deaths, whereas alcohol result in 75,766 premature deaths and illicit drugs for 20,950 premature deaths (The Centers for Diseases Control and Prevention [CDC], 2008). South Africa has 45 millions of people and a third of adults are smokers. Among smokers 52% are men and 17% are women. In 1998, 8% of the adult deaths were attributed to tobacco smoking in South Africa which is equal to 21500 people (Sitas et al., 2004).

1.4.6. Passive smoking

Passive smoking can cause a number of health problems including heart disease, lung cancer, chronic obstructive diseases and irritation of the eyes and nose. It involves breathing tobacco smoke from other people's cigarettes: smokes that have been exhaled

or smoke from the end of cigarettes (Pinkerton and Joad, 2006). Environmental exposure to tobacco or passive smoking is associated with the development of chronic obstructive diseases in children in United States of America (Eisner et al., 2005). It is also associated with a decreased lung function, an airflow limitation and chronic bronchitis (Gilliland et al., 2000).

Fifty Australians die every day from passive smoking compared to 10 who die from alcohol related conditions and 4 who die as results of road accidents (Ridolfo and Stevenson, 2001; Doll et al., 1995).

1.4.7 Tolerance and dependence

People who use tobacco tend to develop tolerance to the effects of nicotine in the tobacco very quickly. This means they need to smoke more and more in order to get the same effects. With repeated use of tobacco, the risk of dependence on nicotine is high. Dependence on nicotine can be physiological, psychological or both. People who are physically dependent on nicotine find their body has become used to functioning with the nicotine present and may experience withdrawal symptoms when they reduce nicotine intake (Johnston and Glasg 1941; Finnegan et al., 1945).

People who are psychologically dependent on nicotine may find they feel an urge to smoke when they are in specific surroundings such as pubs or in particular situations such as during their lunch time or when socializing with friends (Russell, 1971, 1991). Researches have shown that smoking is often associated with specific roles and meanings for smokers such as:

1. Social roles such as enjoyment of company of friends, the drinking of coffee or alcohol, and promoting social confidence and feelings of independence (particularly for young women).
2. Emotional roles; caring for the self such as helping to deal with stress and anxiety weighty control and providing companionship.
3. Temporal roles such as connecting the flow of events or time in the smoker's day, providing a break from work or activities and relieving boredom.

This is may be why smoking is sometimes referred to as the most difficult drug to give up (US Department of Health and Human Services, 1988).

1.4.8. Withdrawal

If a person who is dependent on nicotine suddenly stops using it, or reduces the amount they use, they will experience withdrawal symptoms because their bodies have to readjust to functioning without the drug. Most of these symptoms will disappear within days or weeks after quitting smoking but cravings can persist for years. Withdrawal symptoms include, craving, irritability, agitation, depression, anxiety, insomnia and disturbed sleeping patterns, increased appetite and weight gain, restlessness and loss of concentration, headaches, coughing and sore throats, body aches and pains, stomach and bowel upsets (Ellenhorn, 1988).

1.4 .9. Benefits of quitting smoking

- Within about 2 hours of stopping, there is no more nicotine in the blood system. However, it may take 2 days for nicotine byproducts to leave the body.
- Within 6 hours your heartbeat slows down and your blood pressure drops slightly. It may however take 3 to 30 days for blood pressure to return to normal.
- Between 12 and 24 hours carbon monoxide is eliminated from the body. Lung efficiency improves, tastes buds and sense of smell improves after 2 days.
- Blood flow to the hands and feet improves after 2 months.
- After a few months the lungs work efficiently and are able to remove the mucus.
- The incidence and progression of lung disease including acute and chronic bronchitis are reduced.
- After 15 years the risks of heart attacks and stroke are almost the same as those of a person who have never smoked.
- The person saves a lot of money (White, 2007).
- The body weight increases once tobacco smoking is stopped, because of increased appetite and increasing activity of lipoprotein lipase (Pasupathi et al., 2009).

1.5. Nicotine

It is an alkaloid found in family of plants called the solanaceae. It occurs predominantly in tobacco, and coca, and lower quantities in tomato, eggplant, potato, and green pepper (Doolittle et al., 1995; Domino et al., 1993; Sigmund et al., 1999). However the main source of nicotine in the body remains the use of tobacco. Nicotine replacement therapy such as transdermal nicotine patches and nicotine containing gum (Heisheman et al 1994)

is another source. Nicotine is named after tobacco plant Nicotiana tabacum which in turn is named after Jean Nicot French Ambassador in Portugal who sent tobacco and seeds from Brazil to Paris and promoted their medicinal use (Tomizawa and Casida, 2003). Nicotine was first isolated from tobacco plant in 1828 by German Posselt & Riemann and its empirical formula was described by Melsens in 1848. Its structure was discovered by Garry Pinner in 1893 and it was synthesized by Pictet and Crepieux in 1904. The structure of nicotine is 1-methyl-2-(3-pyridyl-pyrrolidine, $C_{10}H_{14}N_2$ (Fig. 1.8). It is clear liquid with a characteristic odor and it turns brown once is exposed to air and its boiling point is 274.5^{o}C (Schevelbein, 1962)

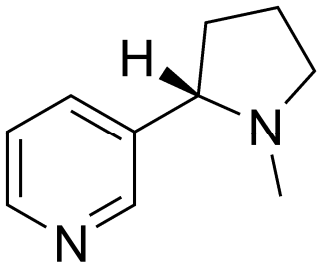


Fig.1.8. Structure of Nicotine (Yildiz, 2004)

Nicotine has been found to constitute 0.6-3% of dry weight of tobacco. It is synthesized in the roots of the plant and accumulates in the leaves. It functions as an antiherbivore chemical since it is a potent neurotoxin with particular specificity to insects (Weinzierl et al., 1991; Tomizawa and Casida, 2003). Nicotine was widely used as an insecticide in the past and currently nicotine derivatives such as imidacloprid continue to be used (Borlongan et al., 1998).

In low concentrations (an average cigarette yields 1mg of nicotine), the substance acts as stimulant in mammals and is one of the main factors responsible for the dependence forming properties of tobacco smoking. According to The US Department of Health and Human Services (1988), nicotine addiction have been one of the hardest to break. The pharmacological and behavioral characteristics that determine tobacco addiction is similar to those determine addiction to drugs such as heroin and cocaine (US Department of Health and Human Services 1988).

1.5.1. Absorption of nicotine

The absorption of nicotine can occur through the oral cavity, skin, lung, urinary bladder, and gastro-intestinal tract (Schevelbein, 1973). The absorption of nicotine through the oral mucosa has been shown to be the principal route of absorption for smokers who do not inhale and smokeless tobacco users. The plasma nicotine level is 2.5-8.0ng/ml for non inhaling smokers whereas the plasma nicotine levels in inhaling smokers' reaches 30-40ng/ml (Schevelbein et al., 1972). Nicotine is absorbed through the skin during harvesting and during the nicotine replacement therapy. It is also reabsorbed through the urinary bladder. The nicotine absorption through gastro-intestinal tract is poor because of the acidity of the stomach (Travel, 1940).

1.5.2 Pharmacology

When nicotine enters the body it is quickly distributed through the blood stream and can cross the blood–brain barrier. On average it takes 7 seconds for the substance to reach the brain when is inhaled. The half-life of nicotine in the body is around 2 hours (Benowitz,

1982; Feyerabend, 1985). The amount of nicotine inhaled with tobacco smoke is a fraction of the amount contained in the tobacco leaves. The amount of nicotine absorbed by the body from smoking depends on many factors, including the type of tobacco,

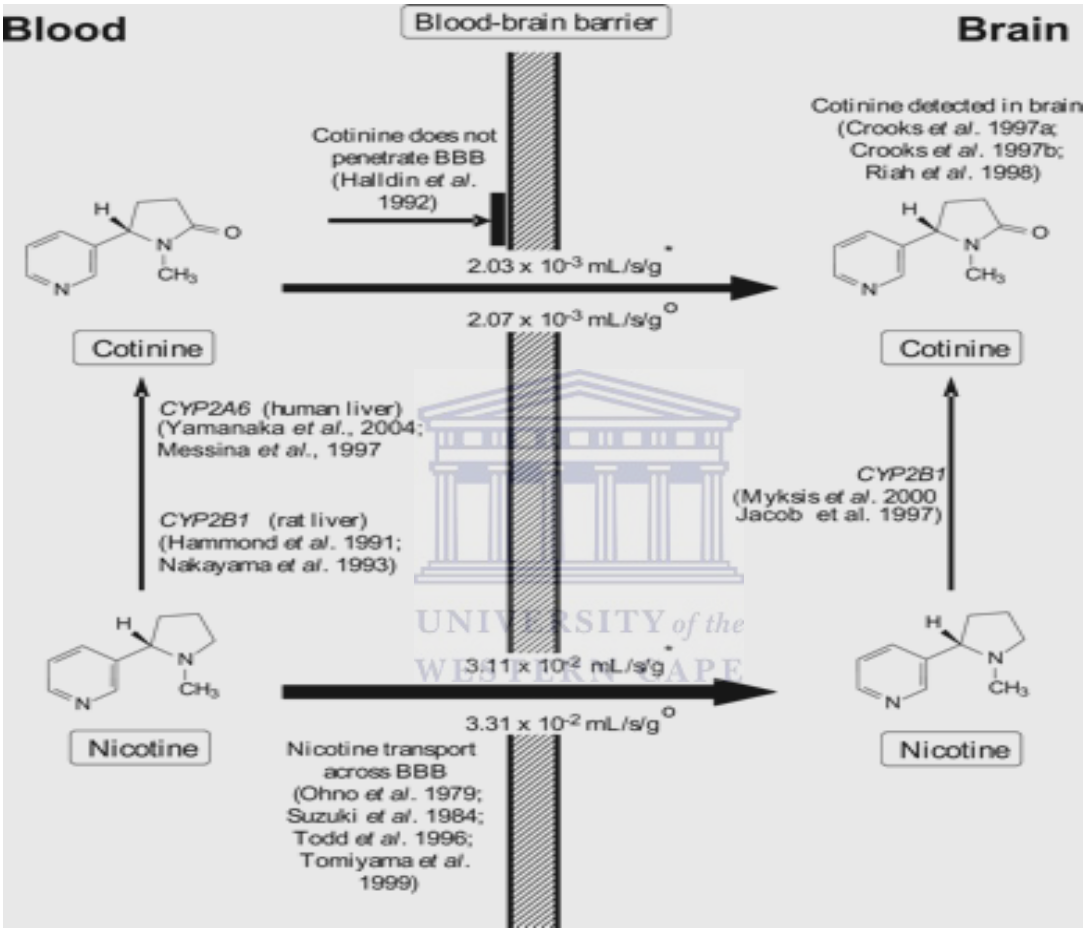


Fig. 1.9. A summary of the blood to brain transfer of nicotine and its major metabolite cotinine. Data are from previous literature and this current study. *, results of this study in naive rats; O, results in nicotine-exposed rats (Lockman et al., 2005).

whether the smoke is inhaled, and whether a filter is used. For chewing tobacco, dipping, and snuff, which are held in the mouth between the lip and the gum, or taken in the nose respectively, the amount released in the body tends to be much greater than via inhalation

of tobacco smoke (Benowitz et al., 1988). Nicotine is metabolised in the liver by cytochrome P450 2A6 enzymes, formerly known as coumarin 7-hydroxylase and also cyp2B6. A major metabolite is cotinine (Messina et al., 1997).

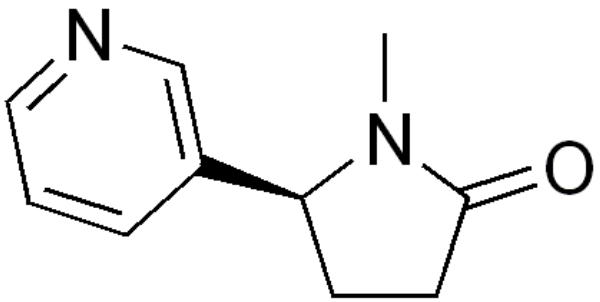


Fig.1.10. Structure of cotinine (Yildiz, 2004)

Nicotine is more efficient than cotinine at passing the blood-brain barrier (Fig. 1.7) in rats. It also have a longer half-life of 12 hours as oppose to the 2 hours of nicotine, and is active in a variety of animals such as mice, rats, cats and in humans (Riah et al.,1998).

1.5.3. Nicotine excretion.

Nicotine is excreted in urine, faeces, saliva, gastric juice, bile, sweat, and breast fluid (Seaton et al., 1993). Nicotine and cotinine have been determined in the urine of infants of mothers who smoked during pregnancy and lactation. Since nicotine readily crosses the placental barriers (Matta et al., 2007), it implies that it will also appear in the fetal blood and tissues. The nicotine is excreted by the lungs and kidneys into the amniotic fluid. Some of the nicotine enters the maternal blood again from which it is eliminated via the kidneys, saliva, breast milk and sweat (Luck and Nau, 1985).

1.5. 4. Nicotine and the lung.

Nicotine acts on nicotine acetylcholine receptors (nAChRs) in the placenta and the fetal lung. Consistent with this, it has previously been shown that α3, α5, and α7 nicotinic acetylcholine receptors (nAChR) are expressed in non-neuronal cells in monkey fetal lung, and that maternal nicotine exposure up-regulates nAChR expression in fetal lung (Sekhon et al.,1999). High affinity nAChRs are found in the membranes of normal lung cells and in lung cancer cells of all histological types (Maneckjee and Minna, 1990; Pontieri et al., 1996; Maus et al., 1998). These include α3, α5, α7, and β2 or β4 subunits (Maus et al, 1998), of which α7 may help to modulate cell shape and affect cell-to-cell contact (Wang et al., 2001). It has been demonstrated that nicotine promotes cell proliferation upon its interaction with nAChRs on the surface of rodent bronchial epithelium and may contribute to dysanaptic lung growth (Wongtrakool et al., 2007).

Recent hypotheses regarding the way in which maternal smoking affects fetal lung development have focused on the down regulation of the surface complexity of the parenchyma, increased collagen accumulation, up-regulation of the surfactant protein gene expression, and induction of neuro-endocrine cell hyperplasia in fetal lungs (Sekhon et al., 2001, 2002a). In addition Klapproth et al. (1997) and Proskocil et al. (2004) have demonstrated that non-neuronal cells in lung synthesize acetylcholine (ACh), and that a cholinergic autocrine loop exists in developing lung. Thus, prenatal nicotine exposure likely affects lung development by modifying the actions of this autocrine cholinergic loop. Much remains however to be determined about the mechanism by which nicotinic signaling alters lung development.

Nicotine also activates several pro-survival signals (Cattaneo et al, 1997; Minna, 2003). An example is the increase in the activity of protein kinase C (PKC) in various human and murine lung cancer cell lines when exposed to nicotine. Nicotine also elicits the activity of Raf-1 (Chu et al., 2005). The activation of these kinases has been shown to be responsible for the phosphorylation of Bcl-2 which antagonizes opioids-induced apoptotic signaling in lung cancer cells (Heeschen, 2001: Macklin et al., 1998; Maneckjee and Minna, 1994). An increase in the phosphorylation of Akt was detected *in vivo* in the lungs of nicotine-treated mice and in human lung cancer cells derived from smokers. The activation of this kinase is associated with tobacco-related carcinogenesis in the lung. Although the activation of these kinases was observed in cultured cells transiently exposed to nicotine, they suggest that nicotine directly or indirectly contributes to the process of lung carcinogenesis (Maneckjee and Minna, 1994, Cattaneo et al., 1997; Chu et al., 2005; West, 2003).

Furthermore, research has shown that long-term nicotine exposure results in a predisposition for the induction of genetic instability (Guo et al., 2005; Hartwell and Kastan, 1994; Vogelstein and Kinzler, 1992). Gene amplification is a hallmark of gene instability. Gene instability requires 2 critical elements, namely an inappropriate cell cycle progression, and DNA damage. Long-term nicotine exposure, through the activation of Ras pathways and up regulating cyclin D1, disrupts the G1 arrest. It also augments the production of ROS which may lead to DNA damage. This implies that exposure to nicotine via tobacco smoking or via NRT to quit smoking will make the lungs more prone to the development of cancer (Guo et al., 2005).

1.5.5. Nicotine and cell signaling: apoptosis and lung development:

Programmed cell death or apoptosis is an energy-dependent and genetically controlled process (White, 1996), that can be induced by a number of molecular tools (Wertz and Hanley, 1996). Apoptosis occurs in the mesenchyme as early as day 14 of gestation in fetal rat lung, the embryonic phase of lung development, during which time branching of conducting airways is the predominant feature. The percentage of cells undergoing apoptosis increased dramatically between 18 and 22 days of gestation and remains elevated in the first day of postnatal life.

This marked increase in apoptosis at birth may be initiated by a number of factors, such as breathing movements, hormonal changes due to labour and delivery, and/or expansion of the lungs with changes in cell shape and cell-cell relationships. Most cells require attachment to extracellular matrix for proper growth and function. Lung epithelial cell adhesion to the extracellular matrix is mediated by cell surface receptors known as integrins (Pilewski and Albelda., 1993) which trigger a number of intracellular signaling pathways. Some of these pathways that have been shown to be involved in apotosis include the Ras-Raf-MAP kinase pathway and the phosphatidylinositol 3-kinase pathway (Ichijo et al., 1997; Yao and Cooper., 1997).

During the phase of rapid alveolarisation between postnatal days 4 and 13 in rats, interstitial fibroblasts undergo rapid proliferation. Few alveoli are formed after this phase. Between postnatal days 13 and 21 the number of fibroblasts and type II cells decrease. This decrease in fibroblasts and type II cells occurs by means of programmed cell death or apoptosis, which peaks between postnatal days 17 and 19.

Apoptosis therefore plays a key role in the thinning of the alveolar septa that occurs after alveolarisation (Schnittny et al., 1998; Bruce et al, 1999). Although apoptosis is an ongoing process in the immature lung, the rate of apoptosis after alveolarisation increases owing to a decrease in the Bcl-2 mRNA and an increase in BAX mRNA in the fibroblasts on postnatal day 16. The gene products of Bcl-2 and BAX interact to form homodimers and heterodimers. Although Bcl-2 and BAX heterodimers are inactive, when BAX is in excess and BAX homodimers predominate, cells are likely to undergo apoptosis (Yang et al, 1995). This explains the decrease in the total numbers of type II epithelial cells and fibroblasts in rats during the third postnatal week (Randell et al., 1991).

The reduction, due to apoptosis, in the number of fibroblasts in the interstitium of the developing lung is likely to play a critical role in lung maturation, the final process of which is the transition of the alveolar wall from a double to a single capillary network (Bruce et al., 1999). Interference with the apoptotic process would be expected to have an adverse effect on lung maturation.

Cigarette smoke inhibits the proliferation and migration of human lung fibroblasts and fibroblast-mediated responses and therefore in this way also contributes to the development of emphysema (Nakamura et al., 1995). Nicotine and cotinine inhibit apoptosis in fibroblasts (Wright et al., 1993), but the mechanism by which they suppress apoptosis is not known. Nicotine is known to exert its effects on many cell types by binding to nicotinic cholinergic receptors. It has been suggested that paediatric, smoking-associated pulmonary diseases, and small cell lung carcinoma, may be caused by the

direct chronic stimulation of an α 7-nicotinic acetylcholine receptor-initiated autocrine loop by nicotine and 4-(methylnitrosoamino)-(3-pyridyl)-1-butanone (NNK), where NNK is formed from nicotine by nitrosation in the mammalian organism and during curing of tobacco (Fischer et al., 1990; Hecht and Hoffmann, 1990). It is also possible that certain effects of nicotine are not receptor mediated and may operate through unconventional nicotine receptors (Wright et al., 1993).

There is evidence that nicotine: a) activates the mitogen-activated protein kinase (MAP) signaling pathway and extracellular signal-regulated kinase (ERK-2), resulting in increased expression of the Bcl-2 protein and inhibition of apotosis, and b) blocks the inhibition of protein kinase C (PKC) activity in lung cells. Nicotine appears to have no effect on the activities of c-jun NH-2-terminal protein kinase (JNK), c-myc or p28 MAP kinases that are involved in apoptosis. While exposure to nicotine can result in the activation of two major signaling pathways (MAP-kinase and PKC) that are known to inhibit apoptosis, nicotine regulation of MAP and ERK kinase activity is not dependent on PKC. These effects of nicotine occur at concentrations that are generally found in the blood of smokers, and could lead to disruption of the critical balance between cell death and proliferation (Heusch and Maneckjee, 1998 a, b). The inhibition of apoptosis by nicotine may contribute to the slower thinning of the alveolar septa of the lungs of rat pups that were exposed to nicotine via the placenta and mother's milk (Maritz et al., 2000). It is also plausible that nicotine exposure of the fetus and newborn to nicotine during the phases of rapid cell division may render the lungs more susceptible to the development of cancer (Schuller et al., 2000).

It has been suggested that *in utero* exposure of fetal pulmonary neuro-endocrine cells to nicotine or NNK may contribute to the development of paediatric lung disorders such as bronchitis and lower respiratory illnesses (Schuller et a., 2000), along with altered pulmonary mechanics in infants and children (Sekhon et al., 2001). The nicotine-induced alterations in lung function in monkeys parallel those observed in infants of mothers who smoke during pregnancy (Sekhon et al., 2001). These alterations in lung function could be induced via two mechanisms. The first is a direct effect of released 5-hydroxytryptamine (5-HT) in response to α_7 nicotinic receptor stimulation on bronchial and vascular smooth muscles and fibroblast growth; the second is an indirect effect of 5-HT on pulmonary neuro-endocrine cell numbers via activation of a Raf-1/MAP kinase pathway, resulting in yet more cells that can synthesize and release of 5-HT. Chronic exposure to nicotine and NNK of pregnant mothers may therefore up regulate the α_7 nicotinic receptor as well as components of its associated mitogenic signal transduction pathway, thereby increasing the vulnerability of infants to the development of paediatric lung disorders mentioned earlier (Sekhon et al., 2001).

1.5.6. Nicotine Replacement Therarpy (NRT).

Nicotine affects many neurotransmiters, but dopamine seems to be most responsible for the addictive properties of nicotine. The direct binding of nicotine to acetylcholine receptors on dopamine-containing neurons result in the overflow of dopamine in the reward centers of the brain. Several acetylcholine receptor subtypes, such as α4β2, are found in dopaminergic neurons. Unlike acetylcholine, which is rapidly degraded by acetylcholine-esterase, nicotine remains active at the α4β2 receptor sites for a prolonged

period of time. While prolonged stimulation by most entities usually causes receptor downregulation while nicotine stimulation of the acetylcholine receptors causes upregulation. This upregulation desensitizes acetylcholine receptors which again result in nicotine dependence and addiction (Potts and Garwood, 2007).

The use of NRT is widely promoted by health practitioners as a safe way to quit the smoking habit because it is suggested by some that nicotine is not harmful (Zwar et al., 2006). However, several studies showed that nicotine can damage fetal lungs, heart, and the central nervous system. Nicotine is genotoxic (Argentin and Cicchetti, 2004; Kleinsasser et al., 2005) and its toxic effect persists in the fetus after administration has stopped (Ruiz, 2006). Studies in monkeys (Sekhon et al., 1999) have clearly showed that nicotine exposure during pregnancy increases the development of α_7 nicotinic receptors in cells implicated in lung development. Pulmonary hypoplasia and other abnormalities in pulmonary and bronchial development were found in the offspring of experimental animals after exposure to nicotine during gestation by Sekhon et al, (1999) and Maritz and co-workers (1987, 1997, 2003a,b, 2005). It is also suggested that maternal nicotine exposure suppress lysyl oxidase activity and in this way contribute to the gradual deterioration of the lung parenchyma of the offspring. Furthermore, nicotine induces peroxidation of membrane lipids (Kalpana and Menon, 2004) which change the oxidant/anti-oxidant status of the lungs of the offspring. This is supported by the decrease in the vitamin C and E content of the lungs of the offspring (Balakrishnan and Menon, 2007). They also clearly show a decrease in the levels of the enzymes that catalyze the removal of antioxidants from the lung. Studies in our laboratories also show that the level

of superoxide dismutase in the lungs of rats that were exposed to nicotine via the placenta and mother's blood remains significantly lower than that of rats not exposed to nicotine (unpublished data). This implies that apart from its immediate effect in the lungs of those who use NRT, nicotine intake during pregnancy and lactation will have a long-term effect on the maintenance of lung integrity and respiratory health of the offspring (Maritz, 2001).

In addition, α4β2 nicotinic acetylcholine receptor is upregulated by nicotine in the central nervous system (CNS) and the neurepithelial bodies (NEB) in the airways where the latter is considered to play a role as an airway chemoreceptor possibly involved in the control of breathing (Cutz and Jackson 1999; Fu et al., 1999; Youngson et al., 1993). Chronic nicotine exposure increases the number of pulmonary type II cells as well as NEB cells. Epidemiological studies have identified a close relationship between smoking and Sudden Infant Death Syndrome (SIDS) (Maus et al., 1998). Although the exact mechanism is not known, nicotine, a major component of tobacco smoke, may increase the vulnerability of infants to SIDS via its action on the peripheral chemoreceptors in NEB. An increase in the size and number of NEB has been reported in the lungs of SIDS victims born to smoking mothers (Cutz et al., 1996). Studies by Holgert et al (1995) showed that nicotine from smoking may also interfere with the postnatal resetting of the oxygen sensitivity of the peripheral arterial chemoreceptors by increasing carotid body TH mRNA, as well as DA release in this period. Collectively these effects of nicotine on the peripheral arterial chemoreceptors may increase the vulnerability to hypoxic episodes and attenuate the protective chemoreflex response. These mechanisms may underlie the

well-known relation between maternal smoking and sudden infant death syndrome. It is therefore plausible that the responses of the hyperplastic NEB to acute hypoxia may be blunted making the infants of smoking mothers (Fu et al., 2003), or mothers using NRT, more susceptible to NRT.

Lynx1 is co-expressed in neurons that express α4β2 and α7 nicotinic acetylcholine receptors. It is also expressed in the lung airway epithelial cells as well as type II alveolar epithelial cells (Sekhon et al., 2005). As such it plays a role in cellular proliferation and differentiation as well as in the control of surfactant synthesis. This implies that lynx1 may have the capacity to modulate the effects of prenatal nicotine and fetal development. It is therefore plausible that varenicline (a new nicotine replacement therapy) binding to α4β2 and other nicotinic acetylcholine receptors during fetal development may interfere with lung growth and development in the offspring. It is therefore advisable not to smoke or to use NRT to quit smoking during pregnancy and lactation.

1.6. Oxidant and antioxidant

1.6.1. What is an oxidant?

Oxidants or free radicals are species capable of independent existence that contains one or more unpaired electrons; they are unstable and easily initiate oxidation (Halliwell, 1991, 1994). These free radicals species may be produced endogenously by metabolic reactions (e.g. from mitochondrial electron transport during respiration or during

activation of phagocytes) or exogenously, such as air pollutants or cigarette smoke (Rahman et al., 2006).

1.6.1.1. Oxidants sources in the lungs

1.6.1.1.1. Cell-derived oxidants

The lung epithelium is continuously exposed to oxidants generated internally as part of normal metabolism as well as to oxidants from in the ambient air such as ozone, nitrogen dioxide, diesel exhaust and cigarette smoke (Rahman et al., 2006). The most important reactive oxygen species of physiological significance are the superoxide anion, hydroxyl radical, nitric oxide and hydrogen peroxide. The primary reactive oxygen species in vivo are superoxide and hydrogen peroxide (Halliwell and Gauteridge, 1989). Hydrogen peroxide is generated through non-enzymatic or enzymatic dismutation of superoxide. The most reactive and harmful reactive oxygen species is the hydroxyl radical which can be formed from hydrogen peroxide and superoxide but also via the reaction of superoxide with nitric oxide to form peroxynitrite (Halliwell, 1991).

The main cellular sources of reactive oxygen species in the lungs are not only neutrophils, eosinophils, and the alveolar macrophages but also alveolar epithelial cells, bronchial epithelial cells and endothelial cells (Kinnula et al., 1995; Holland et al., 1990). The formation of the reactive oxygen species is enhanced in the lungs after exposure to various exogenous chemical and physical agents such as mineral dusts, ozone, nitrogen oxides and tobacco smoke (Church and Pryor, 1985). Oxidative stress can lead to peroxidation of membrane lipids, depletion of nicotinamide nucleotides, and the increase

of intracellular calcium ions, cytoskeleton disruption and DNA damage (Halliwell and Aruoma, 1991).

1.6.1.1.2. Inhaled oxidants and tobacco smoke.

The direct consequence of cigarette smoke and the inhalation of the airborne pollutants such as oxidant gases (ozone, NO_2, SO_2) or particulate matter in the air, is lung damage and elevated inflammatory responses in the lungs. Tobacco smoke contains a complex mixture of over 4700 different chemicals distributed in the aqueous, gas, and the tar phase of the smoke (Church and Pryor, 1985). The gas phase of tobacco smoke contains a high concentration of oxidants of $>10^{15}$ molecules /puff, including short-lived oxidants such as $O_2^-$and NO_2. The O_2^-reacts with NO_2 to form high reactive peroxynitrite molecule (Church and Pryor, 1985).

The tar phase contains long-lived organic radicals such as semiquinone radicals that react with oxygen in redox dependent manner to form O^-_2, OH^- and hydrogen peroxide (Nakayama et al., 1985). The tar phase of tobacco smoke may undergo redox recycling for a considerable period of time in the epithelial lining fluid of smokers and is an effective metal chelator wherein iron is chelated to produce tar-semiquinone+tar+$Fe^{2+}$and this can produce hydrogen peroxide continuously. The side stream of cigarette smoke contains more than 10^{17} reactive organic compounds/puff such as CO_2, nicotine, ammonia, formaldehyde, acetaldehyde, crotonaldehyde, acrolein, N-nitrosamines, benzo(a)pyrene, isoprene, benzene, ethane, pentane and other genotoxic and carcinogenic compounds (Rahman et al., 2006).

Reactive oxygen species have also been suggested to play a role in smoking induced chronic obstructive pulmonary disease and human lung fibroblasts recruit inflammatory cells by chemotactic activities in response to smoke extract (Rahman and McNee, 1996).

1.6.2. Antioxidant

An antioxidant is a substance capable of neutralizing oxidants oxygen. Antioxidants occur naturally in the body and in certain foods and beverages; they may also be ingested in the form of supplements (Haliwell and Gautteridge, 1989). There are 2 types of antioxidants in the lungs, namely non-enzymatic antioxidants such as vitamins and enzymes with antioxidants functions such as superoxide dismutase.

1.6.2.1. Non-enzymatic lung antioxidant.

These are low molecular weight molecules such as certain vitamins, for example vitamins C, E, A, glutathione and others substances (Mac Fadden et al., 2005). One such molecule is lycopene. It belongs to the carotenoid family of molecules, and is a strong antioxidant (Stahl and Sies, 1996; DiMasco et al., 1989). Some of the vitamins with antioxidant properties are located in the membranes of the cells. These include fat soluble vitamins such as vitamins E and A. Others, such as vitamin C occur in the aqueous compartment of the body such as the extracellular matrix. All the vitamins work together to protect the body against the harmful effects of the oxidants that it is exposed to.

1.6.2.1.1. Vitamin E

Vitamin E is a major antioxidant in biological systems acting as a powerful chain breaking agent through the scavenging of peroxyl radicals (Beyer, 1994). It is well established that vitamin E functions in vivo as an antioxidant protecting lipids against peroxidative damage.

Vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins. Pathogenic dysfunction of tissues due to cell death via apoptosis is one of the important consequences of oxidative stress that could be diminished using antioxidant such as vitamin E (Shirpoor et al., 2008).

1.6.2.1.2. Vitamin C

Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic functioning of the body (Carr and Frei, 1999). Many biochemical, clinical, and epidemiological studies have indicated that vitamin C may be of benefit in chronic diseases such as cardiovascular disease, cancer, and cataract, probably through antioxidant mechanisms (Carr and Frei, 1999). Vitamin C has several important functions in the body. This includes synthesis of amino acids and collagen, wound healing, metabolism of iron, lipids and cholesterol and others. In particular, vitamin C is well known antioxidant that scavenges free radicals. Vitamin C prevents the inhibition of gap-junction intercellular communication (GJIC) induced by hydrogen peroxide. It also protects against oxidative DNA damage through its free-radical scavenging activity (Lee,

2002). Hydroxylation of aromatic drugs and carcinogens by hepatic cytochrome P450 is also enhanced by reducing agents such as vitamin C (Tsao, 1997).

L-Ascorbic acid substantially minimizes the toxic effects of some environmental pollutants including tobacco smoke (Preston et al., 2003).Vitamin C needs are higher in smokers and several studies suggest that vitamin C may protect against smoking-related damage. Vitamin C supplements may be helpful in restoring reduced plasma vitamin C concentrations in smokers (Lehr et al., 1997).Vitamin C acts together with the other antioxidants such as vitamin E and betacarotene in many body processes. High levels of vitamin C appear to increase blood levels of the other antioxidants and therapeutic effects appear to be greater when combinations of antioxidants are used. Vitamin C improves the stability and use of vitamin E (Bőhm et al., 1997). However, it may interfere with selenium absorption and supplements should be taken at different times (Mutanen and Mykkanen, 1985).

Vitamin C may protect against the harmful effects of beta carotene supplements in smokers. Smokers tend to have low levels of vitamin C and this may allow a build-up of a harmful form of beta-carotene called the carotene free radical which is formed when beta-carotene acts to regenerate vitamin E. Smokers who take beta-carotene supplements should also take vitamin C. It helps to protect against the toxic effect of cadmium, copper, vanadium, cobalt, mercury, and selenium (Shibata et al., 1992).

Vitamin C supplementation during gestation and lactation can also behave as pro-oxidant and results in lung parenchymal deterioration in the offsprings (Podmore et al., 1998, Rayise and Maritz, 2009; Halliwell, 1990).

This is also supported by the finding that the vitamin C supplementation increases the malonaldehyde content of the lungs due to the peroxidation of the membrane lipids and the cellular injury (Baltalarli et al., 2006).

1.6.2.1.3. Vitamin A

Vitamin A is a fat–soluble vitamin. Vitamin A is essential in the developing fetus especially for the development of vision, the growth of eyes, ears, heart and lung (Groff et al., 1995). Sources of the vitamin A include eggs yolk, liver, fish oil, whole milk and butter. It also plays a role in development of gap junctions between cells and aid in glycoproteins synthesis (Ross et al., 1999). Reproductive processes, the immune system, and bone development are dependent on different isoforms of vitamin A (Groff et al., 1995). Vitamin A, like other carotenoids, may counter-act free radical damage and thus protect the membrane lipids against oxidative damage (Groff et al., 1995) and in this way maintain integrity of cells and prolong the lifespan of cells.

Like vitamin C, vitamin A also has pro-oxidant properties (Ross et al., 1999). the beta carotenes precurseurs of the vitamin A may undergo oxidation, leaving byproducts in the lungs and arterial blood. This result in oxidative damage and tumor growth especially in smokers and those exposed to second hand smoke or automobiles fumes (Ross et al., 1999).

1.6.2.1.4. Lycopene

Lycopene is a carotenoid, with molecular formula $C_{40}H_{56}$ and molar mass of 536.87 g mol^{-1}. It is an acyclic isomer of b-carotene, and has no vitamin A activity (Agarwal and Rao, 1999). Lycopene is a fat soluble antioxidant and a free radical scavenger and

protects DNA, low density lipoproteins and proteins against damage. The lycopene concentration in lungs is 0.22-0.57 nm/g wet weight (Zhang et al., 1991). It also participates in modulation of intercellular gap junction communication (Levy et al., 1995), as well as the hormonal and immune systems. As a polyene it undergoes *cis-trans* isomerization induced by light, thermal energy or chemical reactions (Nguyen et al., 1999). Lycopene from natural plant sources exists predominantly in *trans* configuration, the most thermodynamically stable form (Kobayashi et al., 1996). In human plasma, lycopene is an isomeric mixture containing 50% of the total lycopene as *Cis* isomers.

All trans, 5-*cis*, 9-*cis*, 13-*cis* and 15-*cis* are most commonly identified isomeric forms of lycopene (Clinton et al., 1996).

Lycopene, ingested in its natural *trans* form found in tomatoes, is poorly absorbed. Recent studies have shown that heat processing of tomatoes and tomato products induces isomerization of lycopene to the *cis* form which in turn increases its bioavailability (Stahl and Sies, 1992).

1.6.2.1.4.1. Bioavailability of lycopene

Lycopene is mainly available in red fruits and vegetables (Table 1.1). The lycopene intake in North America diet is estimated to 25mg/day with the processed tomato products accounting for 50% of the total intake (Rao et al., 1999).

Fruit/vegetable	Lycopene content (µg/g wet weight)	Reference
Gac (Vietnamese fruit)	2300	Agarwal & Rao, 1999
Raw tomatoes	8.2-42	Agarwal & Rao, 1999
Watermelons	23	Agarwal & Rao, 1999
Pink grapefruit	3.6-34	Agarwal & Rao, 1999
Apricots	0.1	Agarwal & Rao, 1999
Pink guavas	54	Agarwal & Rao, 1999
Tomato juice	86-100	Agarwal&Rao, 1999
Ketchup	124	Agarwal&Rao, 1999
Paste, sauce, soup	63-131	Agarwal&Rao, 1999

Table 1.1: Bioavailability of lycopene.

Lycopene from processed tomato products appears to be more bioavailable than from raw tomatoes (Stahl and Sies, 1992). Lycopene from tomato paste was shown to be more bioavailable than from fresh tomatoes (Gartner et al., 1997). Release of lycopene from the food matrix due to processing, presence of dietary lipids and heat induced isomerization from all *trans* to *cis* conformation enhance lycopene bioavailability (Clinton, 1998).

1.6.2.2. Enzymes in the lungs with antioxidant function.

SOD is present in every cell in the body and plays important role in protecting cells against oxidative stress. There are 3 types of SOD, namely;

Cu-ZnSOD is located in the cytosol (Crapo et al., 1992) and is homodimeric protein (MWt, 32.5kDa) and requires both Cu and Zn at its active site (Fridovich and Freeman 1986). Copper is essential for the activity of the enzyme and zinc is for the stability to the protein structure (Fridovich 1975). Cu-ZnSOD is inducible and expressed in the type II alveolar and bronchial epithelial cells, fibroblasts, alveolar macrophages and capillary endothelium of the lungs (Ghang et al 1995). In the bronchial epithelium Cu-ZnSOD is highly expressed in ciliated epithelial cells. The Cu-ZnSOD mRNA expression decreases during hypoxia in alveolar type II epithelial cells and lungs fibroblasts in vivo. It has been reported to possess both superoxide reductase and superoxide oxidase activity (Jackson et al., 1996).

Guinea pigs treated with recombinant SOD showed protection against cigarette smoke-induced NF-κB-mediated cytokine release and subsequent leukocyte inflammation. SOD also suppresses redox cycling, and free radical generation of polycyclic aromatic hydrocarbons and o-quinones (Nishikawa et al., 1999).

1.6.2.2.1. Manganese SOD (Mn-SOD).

Manganese superoxide dismutase is considered to be one of the most important antioxidant components of a cell. It is a homotetrameric enzyme (MWt, 88 kDa) and requires manganese at its active center (Fridovich, 1975). Mn-SOD constitutes of about 10–15% of the total SODs and is localized in the mitochondria of type II pneumocytes,

alveolar macrophages and bronchial epithelium in rats and at least in the bronchial epithelial cells of human lungs (Tsan 2001). Mn-SOD mRNA is prominently expressed in cells in airway walls, the septal tips of alveolar ducts and in arteriolar walls located adjacent to airways. Altered cellular redox states, inflammatory cytokines such as interleukin-1 and -6, interferon-α, tumor necrosis factor-α, cigarette smoke and hyperoxia induce MnSOD gene expression (Rahman et al., 2006).

Experimental knockout models have also shown that MnSOD plays an essential role in protecting lung tissue against exogenous oxidants (Kinnula et al., 2004). Previous studies have indicated that the main cell population responsible for induction of MnSOD is especially type II pneumocytes (Coursin et al 1996). These findings suggest the potential importance of alveolar macrophages and type II pneumocytes in the protection of lung against oxidants during the development of interstitial lung diseases (Rhaman et al., 2006).

Mn-SOD expression was found to be elevated in central bronchial and alveolar epithelium of smokers with chronic obstructive pulmonary disease than in normal subjects, probably due to the increased oxidant burden in smokers' lungs.

Overexpression of SODs and catalase can protect against cigarette smoke-induced lung damage (Harju et al., 2004).

1.6.2.2.2. Extracellular superoxide dismutase

Extracellular superoxide dismutase (EC-SOD) is the major extracellular SOD of the pulmonary fluids and interstitial spaces of the lungs in both rats and humans. ECSOD is abundantly present in blood vessels and airways. It is a secretory, tetrameric glycoprotein (MWt, 135 kDa) and requires Cu and Zn for activity (Marklund, 1984). The expression of EC-SOD is induced by interferon-γ and depressed by tumor necrosis factor-α, transforming growth factor-β and interleukin-1α in cultured fibroblasts (Oury et al., 1996). Alveolar macrophages and neutrophils express high amounts of ECSOD on antigenic stimulation by lipopolysaccharide (Loenders et al., 1998). In human lungs, EC-SOD has also been found to be expressed by bronchial epithelial cells, type II alveolar cells, alveolar macrophages, chondrocytes and pulmonary endothelial cells (Su et al., 1997). Higher localization of the enzyme has been found in the extracellular matrix, predominantly around the larger blood vessels, airways, and around the alveolar and the capillary regions. EC-SOD in conjunction with glutathione peroxidase constitutes a major first line defence against the inhaled oxidants (Rahman et al., 2006).

1.6.2.2.3. Catalase

This antioxidant enzyme is a homotetrameric protein (MWt, 240 kDa) (Fridovich and Freeman, 1986). Catalase is ubiquitous to most aerobic cells in animals and is especially concentrated in the liver and erythrocytes. The brain, heart and skeletal muscle contain only low amounts. Catalase is found in peroxisomes and in the cytoplasm and is specially localized in the alveolar type II pneumocytes and macrophages. It is considered to be the most important antioxidant enzyme consuming exogenous hydrogen peroxide in rat type

II pneumocyte (Kinnula et al 1995). Catalase is the only antioxidant enzyme increased both at the mRNA and at the activity levels during human lung morphogenesis (Asikainen et al.,1998).

1.6.2.2.4. Glutathione peroxidase

Glutathione peroxidases are a family of selenium dependent and independent antioxidant enzymes and can be divided into two groups, namely, cellular and extracellular.

In general glutathione peroxidase is a tetrameric protein (MWt, 85 kDa). It requires 4 atoms of selenium bound as seleno-cysteine moieties that confer the catalytic activity (Kinnula et al., 1995). Three genetically distinct, selenium requiring glutathione peroxidase and one selenium independent, classical glutathione peroxidase have been identified in a wide variety of cells (Mullenbach et al., 1988). These enzymes are ubiquitously present in the cytosol of most cells. Other varieties include phospholipid hydroperoxide glutathione peroxidase, and gastrointestinal glutathione peroxidase (Ursini et al., 1985). An extracellular form of selenium dependent glutathione peroxidase has also been reported in epithelial lining fluid and other lung cells (Avissar et al., 1985). Some glutathione peroxidase activity has also been found in mitochondria (Mbemba et al., 1985).

The levels of both classical glutathione peroxidase and extracellular glutathione peroxidase are decreased after exposure to ozone (Avissar et al., 2000).

1.6.2.3 Other anti-oxidants

Several other substances have antioxidant properties. These include heme oxygenase, thioredoxins, peroxyredoxins, glutaredoxins, and others. These antioxidants are present in the lung where it plays a role in protecting the lungs against inhaled oxidants and oxidants that are present in the blood that is circulating through the lungs. It is conceivable that the various types of antioxidants work together to protect the lungs against oxidant damage. This implies that lack of one of the antioxidants will compromise the total protection of the lungs. For example, although vitamins E and C are fat and water soluble respectively, vitamin C is required to regenerate vitamin E and thus to maintain its levels in cell membranes (Chan, 1993). This means that a low level of vitamin C will result in a less effective level of protection by vitamin E. It is therefore important to maintain the levels within a normal range to ensure proper protection of the lung against oxidant damage.

1.7. Motivation of this study

For the lungs to function properly as a gaseous exchanger they must be structurally and metabolically mature. Normal alveolarisation and maintenance of lung structural integrity is therefore essential to ensure that the internal surface area available for gas exchange is adequate and maintained at a level that will support the oxygen demands of the body. Interference with alveolar formation and thus the creation of an adequate surface area for gas exchange, or inadequate protection of the alveoli, will result in a smaller internal surface area and may compromise lung function. Smoking is associated with damage to the alveolar walls and consequently with emphysema (Shapiro, 1995; 2005). Nicotine is implicated in some of the adverse effects of smoking and studies by Maritz and

Woolward 1992a, showed maternal nicotine exposure causes emphysema, increases alveolar thickness and decreases the number of alveoli (Maritz and Thomas 1994). Despite these findings, many pregnant and nursing mothers smoke and in this way expose the developing fetus and neonate to the ingredients of tobacco smoke. In some instances they use Nicotine Replacement Therapy (NRT) to quit smoking. Since nicotine readily crosses the placental membranes, the developing fetus is exposed to nicotine and its harmful effects. It was suggested that nicotine compromise the antioxidant capacity of the lungs and in this way induce changes in the "program" that controls development, lung maintenance and aging. This may explain why maternal nicotine exposure during gestation and lactation results in the development of emphysema in the offspring later in life.

1.8. Objectives of this study

The objectives of the study were

1. To assess the effects of maternal nicotine exposure on lung development and maturation of the offspring.

2. To determine whether supplementation with lycopene containing tomato juice the lungs can be protected against the deleterious effects of nicotine.

3. To determine whether the total antioxidant capacity of the nicotine exposed offspring was different from that of the controls on postnatal day 84.

4. To define a possible role of tomato juice supplementation in promoting public health.

CHAPTER 2

MATERIALS AND METHODS

2.0. Ethical clearance

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

2.1. Experimental animals

The animals used in this study were virgin white rats from the Wistar strain that were bred in the animal laboratory of the Department of Medical Biosciences of the University of Western Cape. Only animals with no signs of diseases were used in this study. All animals were fed with Epol rats' cubes and fresh water. Animals were kept in a controlled rooms, with no noise and unnecessary handling and room temperature was maintained at 22 ± 1^{0}C with a 12 hour light cycle.

Animals were mated for one week, after which the males were removed and the females' randomely divided into 4 different groups: nicotine group, tomato juice group, combination of tomato juice and nicotine and control group. Each group contains 6 rats. The body weight of each female was recorded weekly and an increase in mass over this time indicated that the mating was successful. Pregnant rats were placed in individual straw lined cages for the duration of the study.

From gestational day 7 up to postnatal day 21, the rats were given treatment as designated by the experimental group. Nicotine treated animals received 1 mg nicotine /kg body weight/day subcutaneously. Animals that were exposed to tomato juice had free access to a solution of diluted all gold tomato juice. The lycopene content of the solution was 6 mg/ 100ml. Control animals received a daily placebo of sterile saline to the dose of 1mg/kg body weight/day. The last experimental group was subjected to the following procedure: an injection of 1mg of nicotine/kg body weight /day.

These animals were injected between 9h00 and 10h00 with a 1ml syringes and needles. Noise in the animal room was kept to minimum. The treatment interventions started on the day of randomization and were continued during gestation, the 21st postnatal day.

2.2. Excision of lung tissue

The lung tissue of each of the experimental groups was obtained from the pups at postnatal days 21, 42, 63, and 84. In each of the age groups a total of between 15 and 20 lung samples were obtained.

Body weight was determined by weighing pups on top loader laboratory balance (Sartorious 1475 A). Animals were then euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (0.3ml for 21 days old, 0.6ml for 42 days old and 0.9ml for 63 and 84 days old).The thorax was then opened and the trachea intubated and infused with 10 % formaldehyde, and fixed at pressure gradient of 25mmHg for 30 minutes . The trachea was then ligated and the lung was removed en bloc.

The other thoracic organs were removed and lungs were fixed in 10% buffered formalin until processing. All lungs remained in fixative for a minimum of one week before processing.

2.3. Embedding and processing

After fixation in 10 % buffered formaldehyde, the middle right lobe of each sample was placed in a plastic tissue processing cassette. Tissue was processed in a tissue processor, using fresh reagents for each processing cycle. The processing procedure involved was:

70% alcohol for 2hours

80% alcohol for 2hours

90% alcohol for 2hours

100% alcohol for 2 hours

100% alcohol for 2 hours

Xylene 1 for 2 hours

Xylene 2 for 2 hours

Wax 1 for 2 hours

Wax 2 for 1hour and half

Samples were embedded in wax blocks which were then sectioned to make microscope slides.

2.4. Microscope slides preparation.

Waxes were cooled in a standard refrigerator for an hour prior to sectioning. A sliding microtome was used to make 5 μm sections of these blocks. Sections were floated on a water from where it was picked up on clean, marked microscope slides. Sections were fixed onto the glass slides in an incubator at 37 ^{0}C for an hour after which it was stained with haematoxylin and eosin.

2.5. Mayer's haematoxylin and eosin staining technique.

Haematin, the oxidation product of haematoxylin, is the natural dye that will cause staining of the tissue. This oxidation process is aided by sodium iodate. The poor affinity of haematoxylin for tissue is remedied by adding a mordant (aluminium salt) to the preparation. The nuclei of the tissue are stained a red colour which is blued in Scott's Tap water to a dark blue shade. The cytoplasm is stained a reddish pink with eosin, the counter stain (Bancroft and Stevens, 1990).

2.5.1. Reagents

All chemicals were of analytical grade and supplied by sigma (USA) and Merck (Germany).

Xylene: used as supplied by manufacturer.

Ethanol: diluted to desired concentrations using distilled water.

Mayer's alum haematoxylin: 1g of haematoxylin and 50g of potassium ammonium was dissolved in small volume of water. 0.2g of sodium iodate was added, made up to a volume of 1litre, and left overnight. 50g of choral hydrate and 1g of citric acid was added and it was boiled for 5 minutes.

1% acid alcohol: 750ml of 96% ethanol, 240ml of distilled water and 10 ml of concentrated hydrochloric acid were mixed together.

Scott's tap water: 2g of potassium bicarbonate and 20g of magnesium sulphate was dissolved in 1litre of distilled water.

Eosin: 3g of eosin and 2g of phloxine was dissolved in 1litre distilled water.

2.5.2. Procedure:

1. Sections were fixed onto glass slides for 3 minutes in a hot air oven at 80^0C
2. Glass slides with sections on were agitated in xylene bath for 5 minutes. Dewaxing was completed in second xylene bath.
3. Slides were agitated for 5 minutes in absolute ethanol
4. Hydration of sections was continued by agitating slides for 5 minutes each in 90%, 80% ethanol.
5. Hydration was completed by rinsing slides under tap water.
6. Sections were stained in haematoxylin for 15 minutes.
7. Excess stain was rinsed under tap water.
8. Sections were differentiated in 1% acid alcohol during 2 minutes, and rinsed under tap water.
9. Sections were then blued in Scott's tap water for 2 minutes, and rinsed under tap water.
10. Counterstaining in eosin took place for 3 minutes, and excess stain was rinsed under tap water.
11. Sections were hydrated for 2 minutes each in 80%, 90% and absolute alcohol.

12. Slides were cleared into 2 successive xylene baths, 2 minutes each.

13. After the slides were dry, they were covered with a slide cover and xylene and left overnight. After that the slides were ready to be observed in microscopy for histology purposes.

2.6. Morphometric techniques

Various morphometric techniques were used to assess abnormal lung development. Five of these techniques were used in this study to elucidate the effects of nicotine, tomato juice, and nicotine + tomato juice.

Haematoxylin and eosin stained slides free of cutting artefacts were used for these assessments. Three microscopic slides of each animal sample were used. A minimum of 5 fields per slide were counted.

2.6.1. Lung volume (L v)

2.6.1.1. Principle

The lung volumes of the animals were determined by the fluid displacement technique described by Scherle (1970). The method is based on the principle that, on submersion in a liquid, a body will lose weight quantitatively equal to the weight of the fluid that is displaced by the organ.

2.6.1.2 Method

After fixation of the lungs with 10% buffered formaldehyde, lungs were carefully excised en bloc. Excess fluid on the lung surface was removed by gently dabbing with disposable

tissue towels. Attached to a laboratory clamp by a length of cotton string, the lung was lowered into a beaker filled with buffered formaldehyde that was placed on a laboratory balance. A weight was hooked onto the lung to ensure complete submersion of the lung. The reading on the balance was taken. This reading on the balance in grams corresponds to the volume of the organ in millilitres (Scherle, 1970). The mass displaced by the weight was determined, and the net weight displaced by the lung, ie the lung volume (Lv) was calculated. This procedure was repeated five times and the mean reading taken.

2.6.2 Volume density (Vt).

A graticule with 100 points was used to count the number of points falling on tissue intercepts on a microscope slide. The percentage of tissue space (Vt %) was then calculated as follows: $Vt = \frac{\text{no. of intercepts}}{100} \times Lv$

The percentage of air spaces (Va %) was extrapolated from this. The actual volume of tissue (Vt) and volume of air space (Va) in millilitres was determined using the results obtained for lung volume (Lv), determined by means of fluid displacement (Scherle, 1970).

2.6.3. Mean linear intercept (Lm)

2.6.3.1. Principle

The mean linear intercept is the average distance between alveolar walls. Therefore, the bigger alveoli found in the lung, the greater is the mean linear intercept (Dunnill, 1962).

2.6.3.2. Method

The mean linear intercept was determined according to the method of Thurlbeck (1967) with these modifications:

Lungs were fixed at transpulmonary pressure of 25 mmHg formaldehyde

A×10 objective and ×10 eyepiece were used

5 fields per slide preparation were counted

A hairline cross eyepiece micrometer (OC-M Olympus 10/100×19m/m) was fitted into the 10×eyepiece to count the intercepts. The length of the hairline was 2.04mm.

The following structures were accepted as intercepts:

All alveolar walls crossing the cross hairs

Alveolar walls touching the upper side of the horizontal line

Alveolar walls touching left side of vertical line

The mean linear intercept was calculated according to the equation (Dunnill, 1962a):

$$Lm = \frac{N \times L}{M}$$

Where: N = the number of counted fields

L = the combined length of the cross hairs

M = the total number of intercepts counted

2.6.4. Internal Surface Area (Sa)

2.6.4.1. Principle

The internal surface area (Sa) of the lung is a measure of the potential area available for gas exchange (Butler, 1976).

2.6.4.2. Method

The internal surface area was determined according to the method of Butler (1976):

$$Sa = \frac{4 \times Lv}{Lm}$$

Where: Lv = the lung volume, and Lm = the mean linear intercept

2.6.5. Determination of alveolar thickness (Tsept):

2.6.5.1. Principle

The Tsept is determined by using the Weibel no 2 graticule (Weibel, 1963).

2.6.5.2. Method

We calculate the alveolar thickness by using the formula described by Bolender et al, (1993).

$$Tsept = \frac{Z \times Pse}{2 \times Ise}$$

Where: Z= 54 at 400 ×magnification

Pse = the intercepts on the alveolar walls

Ise = the intercepts which cross the alveolar

2.7. Determination of the total antioxidant capacity of the lungs

2. 7.1. Principle

The total antioxidant capacity of the lungs reflects the ability of the lungs to defend itself against the multiple oxidants found in the lungs from normal metabolism, cigarette smoking or air pollution (Gutteridge and Halliwell, 2000). Imbalance between the oxidants and antioxidants results in oxidative stress which is a cause of different diseases such as emphysema.

2.7.2. Materials

Buffer: 1 bottle, 100ml, of phosphate buffered saline

Chromogen: 5 vials, 10ml each, metmyoglobin and ABTS^{TM}

Substrate: 2 vials, 5ml each stabilized H_2O_2

Standard: 5 vials, 1 ml each, 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid.

Spectrophotometer.

Cuvettes with 1 cm path length.

2.7.3. Method

After removal of the lungs, they were blotted dry to remove excess fluid on the surface. Lung tissue samples were taken and deposited in test tubes on ice. Lung tissues were homogenized in water (1g lung tissue/ 10 ml distilled water) for 30 seconds using a Polytron Kinematica (GmbH) homogenizer. The homogenate was the centrifuged at 4000 rpm for 20 minutes to remove all tissue using a Beckman TJ6 centrifuge. The resulting supernatant was kept on ice and used for the determination of the total antioxidant capacity of the lungs according to the suppliers instructions (EMD Biosciences, Inc; Miller et al., 1993).

2.8. Statistical analysis

In this study 4 experimental groups were compared as well as different age groups within each experimental group. For each experimental group, we took 6 offsprings means 24 for each age group and 96 offsprings as the sample size of the whole experiment. Data was analysed using the one way ANOVA test. The GraphPad Instat program was used.

Data was expressed as Average ± SEM and a P-value of < 0.05 or = 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1. The fluid and lycopene intake of control rats and rats exposed to nicotine, tomato juice or a combination of nicotine and tomato juice.

From fig 3.1, 3.2 and tables 3.1, 3.2, it is clear that for all experimental groups the total fluid intake per week (ml/week) remained constant during weeks 1 and 2. However, at week 3, the total fluid intake increased drastically ($P<0.001$), whereafter (week 4) it again decreased to the levels less than weeks 1 and 2 (370.1±13.1 ml vs 264.7±22.98 ml for control; 516.2±32.4 ml vs 298.2±9.4 ml for combination of tomato juice and nicotine; 518.9 ± 41.5 ml vs 330.9 ± 47.9 ml for tomato juice and 394.0 ± 18.3 ml vs 224.2 ± 11.8 ml for nicotine). The total fluid intake of those animals that received tomato juice or a combination of nicotine and tomato juice was also significantly higher ($P<0.001$) than that of the control group and the animals that were exposed to nicotine only.

As expected the lycopene intake follows the same pattern as the total fluid intake. There was no difference in the amount of lycopene taken in by the animals that received only tomato juice when compared with those receiving both nicotine and tomato juice.

Table 3.0: Water and tomato juice intake per week (ml/week).

	Week 1	Week 2	Week 3	Week 4	Increase week 1 vs week 3
Control (c)	370.1±13.1	362.1±30.4	700.3±40.67	264.7±22.98	1.9 fold
Tomato + nicotine (T+ N)	516.2±32.4	499.3±52.9	1055.8±64.4	298.2±9.4	2.1 fold
Tomato (T)	518.9±41.5	527.6±31.4	1128.7±69.9	330.9±47.9	2.1 fold
Nicotine(N)	394. ±18.3	387.7±42.5	640±38.3	224.2±11.8	1.6 fold
C vs T	0.014	0.0053	0.01	0.01	
C vs N	0.036	0.53	0.002	0.002	
C vs T+ N	0.036	0.016	0.004	0.004	

The control animals and those exposed to nicotine intraperitoneally (IP) only, received water to drink at random. The tomato juice group and the rats receiving both nicotine (IP) and tomatoe juice, received diluted tomato juice only. The week 1 starts with the first day after mating.

Table 3.1: Lycopene intake per 100 g body weight per week between rats receiving tomato juice only and the rats receiving both tomato juice and nicotine (mg/g BW).

	Tomato(T)	Tomato + nicotine(T +N)	T vs T+N
Week 1	20.03±1.34	22.5±1.77	0.28
Week 2	15.92±0.94	15.75±1.04	0.9
Week 3	37.62±2.3	39.61±2.06	0.54
Week 4	10.67±1.4	10.72±1.02	0.98

Table 3.2: The body weight of the rats pups at birth

Experiment	Bw at birth mean ± sem
Control	9.07±0.32gr
T+n	9.09±0.8gr
T only	10.2±0.32gr
Nicotine	11.18±1.0gr

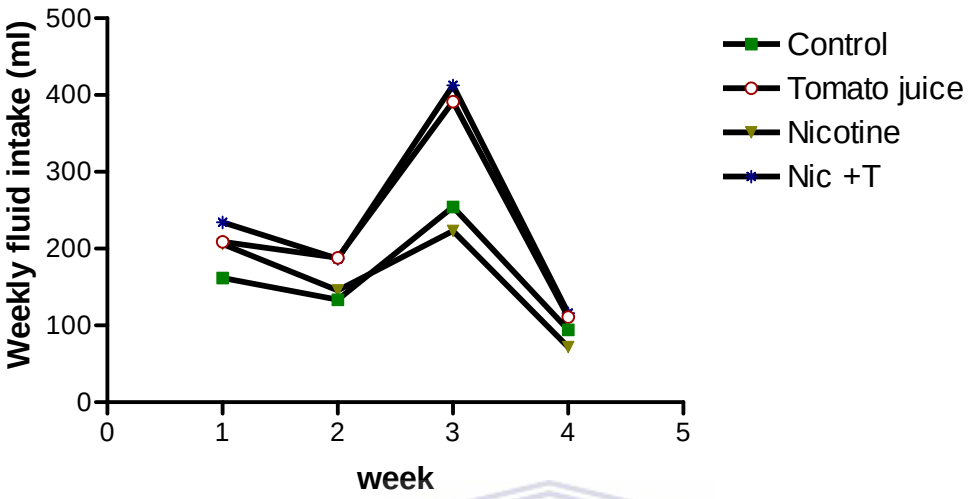


Fig. 3.1. The total fluid intake of the control animals as well as animals exposed to nicotine and tomato juice during gestation and lactation

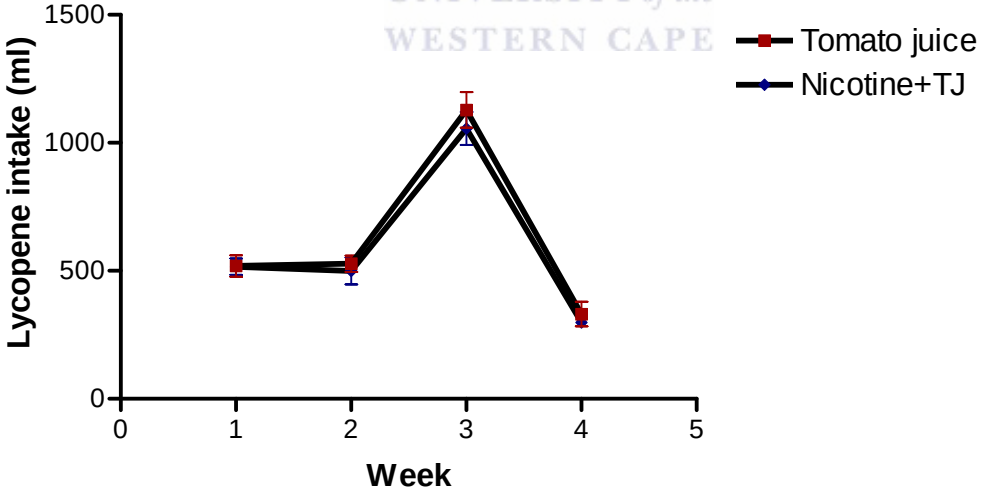


Fig. 3.2. The lycopene intake per week by the animals that were exposed to tomato juice during gestation and lactation

3.2. Effect of nicotine, tomato juice, and combination of tomato juice and nicotine on the growth of the offspring.

The data in table 3.3, illustrated in the figure 3.3, show that the body weight (BW) of the rats pups increased between day 21 and day 84 ($p<0.001$). Although the BW of the males and the females was the same on the postnatal day 21, the BW of the males were higher on postnatal days 63 and 84 ($p<0.001$) than that of the females. Neither nicotine exposure during gestation and lactation nor intake of tomato juice had an effect on the BW of the rats pups ($p>0.05$). The increase in body weight up to postnatal day 42 was therefore the same for males and females for control and experimental groups. Between postnatal days 42 and 84 the increase in body weight increase of the males and females control was 4.4g and 2.39g respectively. The increase of the BW of the animals exposed to nicotine was 4.20g for males and 2.16g for females, tomato juice, 5.18g for males and 2.99g for females, and combination of nicotine and tomato juice via placental and mother's milk was 4.38g for males and 2.46g/day for females. This means that from postnatal day 42 the growth rate of the females were about 50% slower than that of the males. Although the increase of the BW of the animals that were receiving tomato juice tended to be higher than that of the other experimental groups, it was not significantly higher than that of the other groups.

Table 3.3: Effect of nicotine, tomato juice, and of both tomato juice and nicotine on the body weight (BW) (g) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + Nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	40.1±0.8	40.1±0.8	36.8±1.2	36.8±1.2	44.8±1.3	44.8±1.3	36.4±1.6	36.4±1. 6
C VS T			0.003*	0.003*				
C VS N					0.005*	0.005*		
C VS T+N							0.004*	0.004*
42	138.9±5.9	129.8±3.4	122.0±3. 7	111.9±3.9	139.8±5.4	128.9±6.1	141±5.6	126.8±4.0
C VS T			0.03*	0.04*				
C VS N					0.5	0.9		
C VS T+N							0.4	0.6
63	273.2±5.7	197.3±4.7	251.7±5.1	184.8±5.1	268.8±8.0	213.1±6.7	249.9±2.7	188.4±6.7
C VS T			0.02*	0.08				
C VS N					0.7	0.07		
C VS T+N							0.004*	0.3
84	323.5±7.2	230.0±6.9	339.5±7.7	237.3±6.3	316.0±16.3	219.5±4.2	325.0±12.3	230.1±4.3
C VS T			0.16	0.5				
C VS N					0.7	0.23		
C VS T+N							0.9	0.99

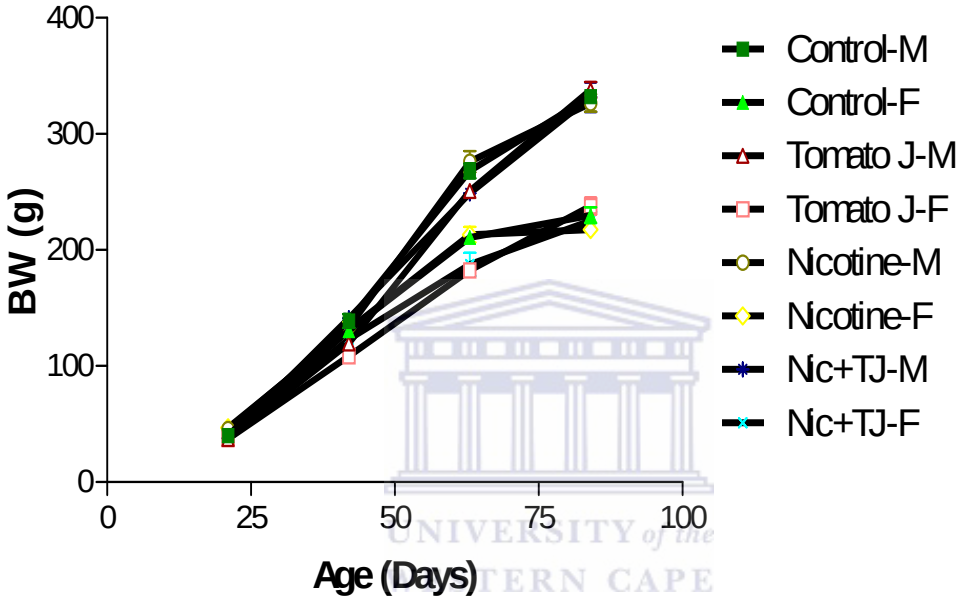


Fig 3.3 : Effect of maternal nicotine and tomato juice exposure during pregnancy and lactation on the body weight of the male and female offspring. (Contol-M= control male; control-F= control female; Tomato J-M= males receiving only tomato juice only; Tomato J-F= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; Nic+Tj-M= males receiving both nicotine and tomato juice; Nic+TJ-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

The other growth parameters such as chest circumference (CC) (Figure 3.4 and Table 3.4) changed with the change in body weight but were proportionally the same. A difference ($p>0.05$) between control and experimental groups were observed. The crown-rump distance (CR) (Figure 3.5 and Table3.5) was also not affected by tomato juice or by a combination of nicotine and tomato juice. However, contrary to expectations the CR length of the male rats that were exposed to nicotine via the placenta and mother's milk was bigger ($p<0.05$) on postnatal day 42 (control: 144.5± 1.77 mm vs nicotine: 150.67± 2.00 mm). No difference was observed on postnatal day 84.

3.3. The effect of maternal exposure to nicotine, tomato juice, or combination of nicotine and tomato juice on lung growth and development.

The lung volume (Lv) (Figure 3.6 and Table 3.6) of the animals exposed to nicotine, tomato juice, and combination of nicotine and tomato juice were the same at day 21($p>0.05$). From day 42 to day 63 the rate of increase in Lv was higher ($p<0.05$) in females exposed to nicotine (3.22 ml of difference) than in control (2.3 ml) and other groups, but at day 84 the Lv of females rats exposed to nicotine (10.7±0.6 ml) was not different from the females controls of the same age (10.4±0.6 ml) ($p>0.05$). The Lv of the males exposed to nicotine was larger than the control males at day 63 (11±0.5 ml vs. 9.6±0.4 ml) ($p<0.05$) and the difference in Lv between the 2 groups increased between day 63 and day 84 (13.1± 0.6 ml vs 11.3 ± 0.3 ml) ($p< 0.05$). There was no statistical difference between Lv of male nicotine exposed animals and the rest.

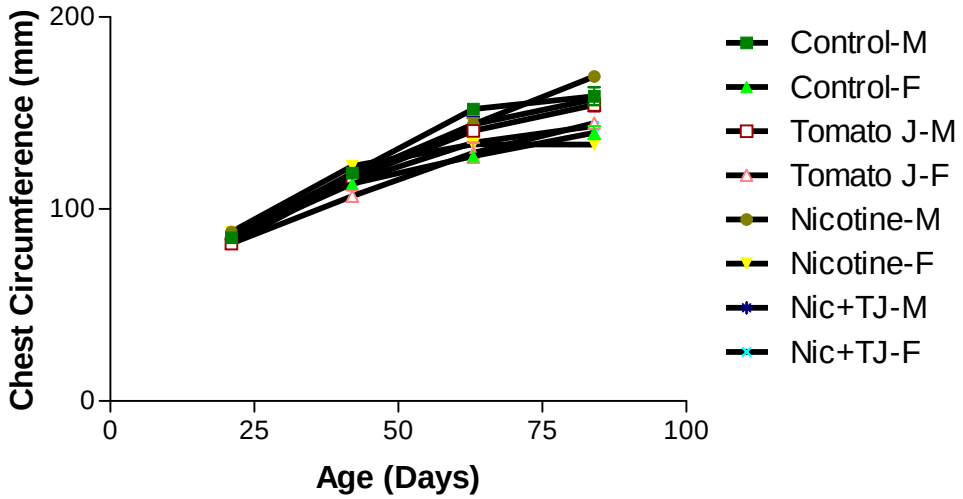


Figure 3.4: Effect of nicotine and tomato juice during pregnancy and lactation on chest circumference in females and males. . (Contol-M= control male; control-F= control female; Tomato J-M= males receiving only tomato juice only; Tomato J-F= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; Nic+Tj-M= males receiving both nicotine and tomato juice; Nic+TJ-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

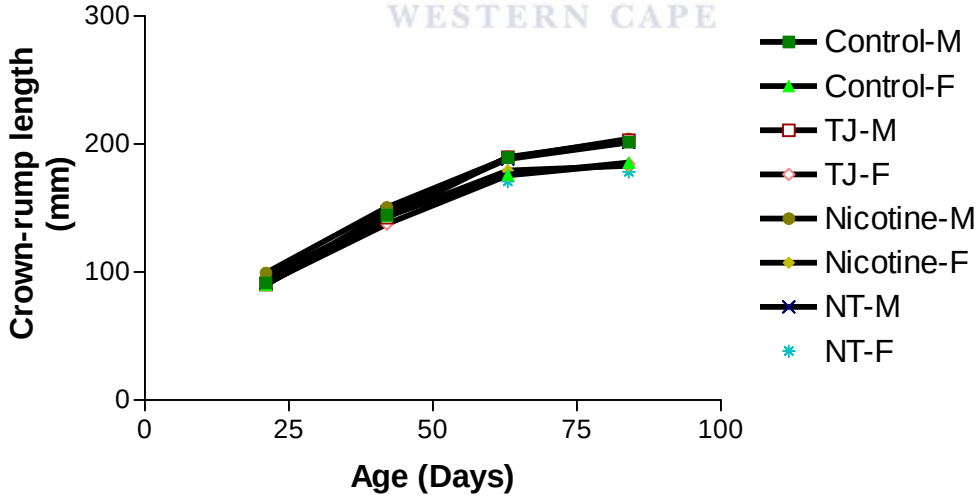


Fig 3.5: Effect of nicotine and tomato juice during pregnancy and lactation on the crown-rump length of females and males.

Table 3.4: E ffect of nicotine, tomato juice, and the combination of tomato juice and nicotine on the chest circumference (CC) (mm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato+ nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	81.9± 1.0	81.9± 1.0	81.2± 1.0	81.2± 1.0	87.1± 1.0	87.1± 1.0	81.6± 1.8	81.6± 1.8
C VS T			0.62	0.62				
C VS N					0.001*	0.001*		
C VS T+N							0.87	0.87
42	116.6±2.0	113.4±0.7	115.4±1.1	108.7±2.2	120.5±2.3	121.9±2.4	115.6±1.3	114.2±1.4
C VS T			0.62	0.09				
C VS N					0.23	0.07		
C VS T+N							0.67	0.62
63	149.8±2.3	131.8±2.2	144.6±2.3	130.3±2.1	142.4±2.9	133.7±1.2	142.1±2.4	127.7±1.3
C VS T			0.15	0.23				
C VS N					0.07	0.44		
C VS T+N							0.4	0.12
84	156.7±2.2	139.8±3.5	157.2±3.1	144.6±2.1	164.8±1.7	140.5±2.7	154.7±3.3	142.8±1.3
C VS T			0.9	0.28				
C VS N					0.02	0.9		
C VS T+N							0.62	0.45

Table 3.5 : E ffect of nicotine, tomato juice, and the combination of tomato juice and nicotine on the distance crown- rump (CR) (mm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+N)	
Sex	M	F	M	F	M	F	M	F
21	91.7±1.2	91.7±1.2	90.3±1.6	90.3±1.6	99.3±1.1	99.3±1.1	91.4±1.9	91.4±1.9
C VS T			0.5	0.5				
C VS N					0.001*	0.001*		
C VS T+N							0.9	0.9
42	144.5±1.8	143.9±2.2	142.7±2.1	137.7±2.5	150.7±2.0	143±2.4	146.9±2.0	142±2.2
C VS T			0.5	0.08				
C VS N					0.04*	0.8		
C VS T+N							0.4	0.54
63	189.5±1.6	176.2±1.4	189.8±0.8	175.6±1.4	189.1±2.4	179.3±2.1	188.4±2.2	170.8±1.6
C VS T			0.9	0.8				
C VS N					0.9	0.22		
C VS T+N							0.7	0.02
84	201.6±2.5	185.5±1.3	203±0.7	184.6±2.5	204±2.9	183.2±3.5	201.3±1.0	178.3±1.9
C VS T			0.6	0.75				
C VS N					0.54	0.6		
C VS T+N							0.9	0.01

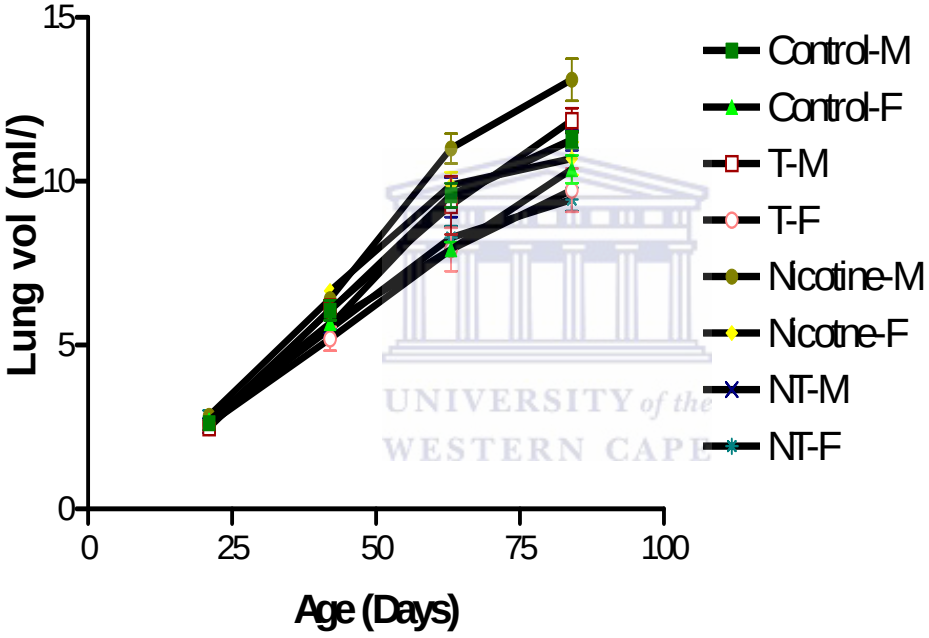


Fig. 3.6: Effect of maternal nicotine exposure during gestation and lactation and intake of tomato juice on the lung volumes of the offspring. (Contol-M= control male; control-F= control female; Tomato J-M= males receiving only tomato juice only; Tomato J-F= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; Nic+Tj-M= males receiving both nicotine and tomato juice; Nic+TJ-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

Table 3.6: E ffect of nicotine, tomato juice, and the combination of tomato juice and nicotine on the lung volume (Lv) (ml) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	2.6±0.1	2.6±0.1	2.5±0.1	2.5±0.1	2.8±0.1	2.8±0.1	2.5±0.2	2.5±0.2
C VS T			0.4	0.4				
C VS N					0.2	0.2		
C VS T+N							0.7	0.7
42	6.1±0.3	5.7±0.2	6.1±0.3	5.2±0.4	6.4±0.2	6.7±0.2	5.6±0.4	5.5±0.2
C VS T			0.96	0.28				
C VS N					0.4	0.002*		
C VS T+N							0.4	0.53
63	9.6±0.4	7.9±0.2	9.3±0.9	7.9±0.7	11±0.5	9.9±0.5	9.5±0.6	8.3±0.4
C VS T			0.8	0.07				
C VS N					0.03*	0.001*		
C VS T+N							0.92	0.4
84	11.3±0.3	10.4±0.4	11.9±0.4	9.7±0.7	13.1±0.6	10.7±0.6	11.3±0.3	9.5±0.4
C VS T			0.22	0.4				
C VS N					0.04	0.63		
C VS T+N							0.95	0.13

The ratio of lung volume to chest circumference (Lv/CC) was the same for all the experimental and control groups at day 21 after birth ($p>0.05$) (Figures 3.7 and tables 3.7). The ratio Lv/CC increases from day 21 to day 84 in control animals and experimental groups ($p<0.001$). A difference in Lv/CC between female control animals and the female nicotine exposed group was observed at postnatal day 42 (5 ± 0.2 vs 5.5± 0.2)($p< 0.05$) . At day 63 both males and females in nicotine exposed groups had higher Lv/CC ratios compared to control animals (6.4±0.3 vs 7.7±0.2)($p = 0.007$) for males and (6.1±0.2 vs 7.4±0.4)($p= 0.008$) for females). There was no difference in Lv/CC ratios between the control and experimental groups at postnatal day 84.

The ratio of lung volume to body weight Lv/BW on postnatal day 21 was also the same for control and all experimental groups (Table 3.8) ($p>0.05$). The Lv/BW ratio of tomato juice exposed male animals was higher than that of control animals at postnatal day 42 (4.4±0.2 ml/g vs 5±0.2 ml/g) ($p<0.05$) and the Lv/BW of the females in the nicotine exposed group was higher than the control animal of the same age (4.4±0.2 ml/g vs 5.2±0.2 ml/g) ($p<0.05$). Also the Lv/BW of both 63 day old male and female nicotine exposed animals was higher than the control animals of the same age and the same sex (3.5±0.1ml/g vs 4.1±0.12 ml/g ($p=0.006$) for males, 4.1±0.2 ml/g vs 4.6±0.2 ml/g ($p=0.03$) for females). No differences were observed between control and experimental group at postnatal day 84. The Lv/BW ratio decreased in all experimental and control group from postnatal day 21 up to day 84 ($p<0.001$).

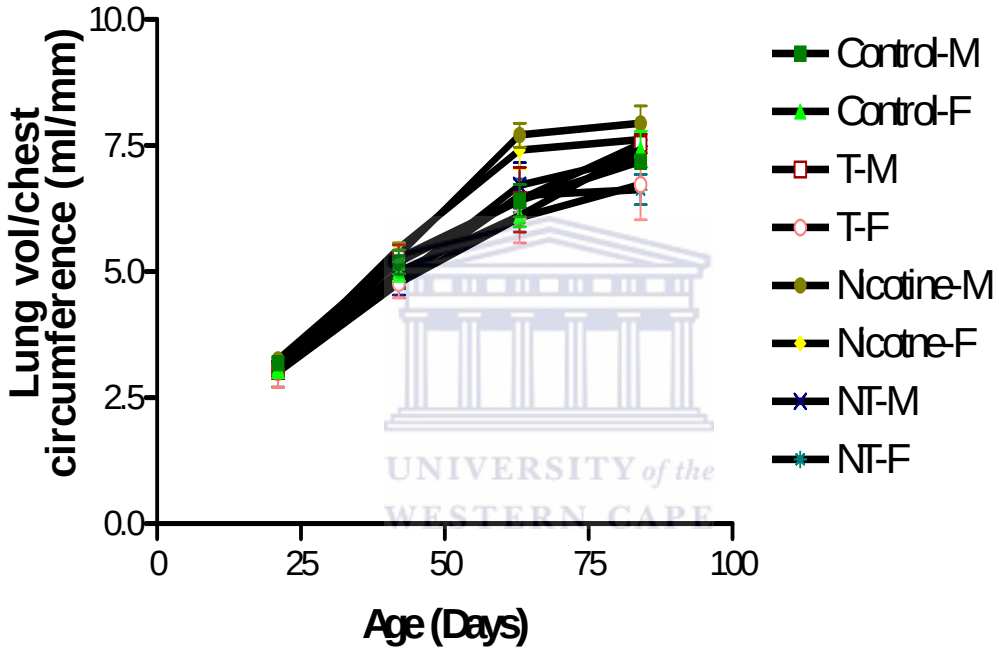


Fig. 3.7: The effect of maternal nicotine exposure and intake of tomato juice on the Lv/cc ratio of the offspring. . (Contol-M= control male; control-F= control female; TM= males receiving only tomato juice only; TF= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males receiving both nicotine and tomato juice; NT-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

Table 3.7: E ffect of nicotine, tomato juice, and the combination of tomato juice and nicotine on the ratio (Lv/CC) (ml/mm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	3.2±0.1	3.2±0.1	3.1±0.2	3.1±0.2	3.3±0.2	3.3±0.2	3.1±0.2	3.1±0.2
C VS T			0.5	0.5				
C VS N					0.7	0.7		
C VS T+N							0.6	0.6
42	5.2±0.2	5±0.2	5.3±0.3	4.8±0.3	5.3±0.2	5.5±0.1	4.9±0.3	4.8±0.2
C VS T			0.83	0.53				
C VS N					0.7	0.04*		
C VS T+N							0.4	0.42
63	6.4±0.3	6.1±0.2	6.4±0.6	6.1±0.5	7.7±0.2	7.4±0.4	6.7±0. 5	6.5±0.3
C VS T			0.98	0.95				
C VS N					0.007*	0.008*		
C VS T+N							0.61	0.34
84	7.2±0.1	7.4±0.4	7.5±0.2	6.7±0.7	8.0±0.3	7.6±0.3	7.3±0.2	6.6±0.3
C VS T			0.16	0.21				
C VS N					0.08	0.72		
C VS T+N							0.72	0.12

Both the chest circumference/crown-rump (CC/CR) ratios and the chest circumference/body weight (CC/BW) decrease as the rats pups get older from postnatal day 21 until postnatal day 84 ($P<0.001$) in both control animal and experimental groups (tables 3.9, and 3.10). The CC/CR of 42 day old females exposed to nicotine was higher compared to control animals (0.8 ± 0.01 vs. 0.9 ± 0.01) ($P<0.05$). There was no difference in CC/BW and CC/CR ratios of postnatal day 21, 63 and 84 day old animals observed between the controls animals and the nicotine group, tomato juice group, and the combination of tomato juice and nicotine in both males and females($P<0.05$) (tables 3.9 and 3.10).

Table 3.8: E ffect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the Lv/BW ratio of the offspring (ml/g). (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	6.6±0.3	6.6±0.3	6.7±0.3	6.7±0.3	6.4±0.2	6.4±0.2	6.9±0.3	6.9±0.3
C VS T			0.67	0.67				
C VS N					0.6	0.6		
C VS T+N							0.51	0.51
42	4.4±0.2	4.4±0.2	5.0±0.2	4.6±0.3	4.6±0.2	5.2±0.2	4.0±0.2	4.3±0.1
C VS T			0.03*	0.46				
C VS N					0.45	0.004*		
C VS T+N							0.2	0.82
63	3.5±0.1	4.1±0.2	3.7±0.3	4.3±0.4	4.1±0.1	4.6±0.2	3.8±0.3	4.4±0.3
C VS T			0.66	0.6				
C VS N					0.006*	0.03*		
C VS T+N							0.31	0.35
84	3.5±0.1	4.5±0.2	3.5±0.2	4.1±0.2	4.2±0.4	4.9±0.2	3.5±0.1	4.1±0.2
C VS T			0.97	0.23				
C VS N					0.1	0.24		
C VS T+N							0.87	0.16

Table 3.9: E ffect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the ratio (CC/BW) (mm/g) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato+ nicotine (T+N)	
Sex	M	F	M	F	M	F	M	F
21	2.1±0.0	2.1±0.0	2.3±0.1	2.3±0.1	2.0±0.1	2.0±0.1	2.3±0.1	2.3±0.1
C VS T			0.016*	0.016*				
C VS N					0.15	0.15		
C VS T+N							0.005*	0.005*
42	0.9±0.0	0.9±0.0	1.0±0.0	1.0±0.0	0.9±0.0	1.0±0.0	0.8±0.0	0.9±0.0
C VS T			0.03*	0.02*				
C VS N					0.75	0.07		
C VS T+N							0.66	0.43
63	0.6±0.0	0.7±0.0	0.6±0.0	0.7±0.0	0.5±0.0	0.6±0.0	0.9±0.0	0.7±0.0
C VS T			0.16	0.15				
C VS N					0.49	0.18		
C VS T+N							0.22	0.74
84	0.5±0.0	0.6±0.0	0.5±0.0	0.6±0.0	0.5±0.0	0.6±0.0	0.5±0.0	0.6±0.0
C VS T			0.96	0.32				
C VS N					0.23	0.16		
C VS T+N							0.59	0.63

Table 3.10: E ffect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the ratio (CC/CR) (mm/mm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	0.9±0.0	0.8±0.0	0.9±0.0	0.9±0.0	0.9±0.1	0.9±0.1	0.9±0.0	0.9±0.0
C VS T			0.99	0.99				
C VS N					0.16	0.16		
C VS T+N							0.96	0.96
42	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.9±0.0	0.8±0.0	0.8±0.0
C VS T			0.96	0.93				
C VS N					0.74	0.004*		
C VS T+N							0.23	0.39
63	0.8±0.0	0.7±0.0	0.8±0.0	0.7±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0
C VS T			0.19	0.87				
C VS N					0.05*	0.92		
C VS T+N							0.082	0.83
84	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0	0.8±0.0
C VS T			0.93	0.25				
C VS N					0.78	0.04*		
C VS T+N							0.16	0.52

3.4. The effect of maternal exposure to nicotine, tomato juice, and combination of tomato juice and nicotine on lung structure:

3.4.1. V_t and V_a

The tissue volume (V_t) (Fig. 3.8) of the lungs of all the groups increased significantly between postnatal days 21 and 84 ($P<0.001$). However, while the tissue volume of the control group as well as the groups that received tomato juice, as well as a combination of nicotine and tomato juice, increased linearly between postnatal days 21 and 84, that of the animals that were exposed via the placenta and mother's milk to nicotine only, plateaus between postnatal days 42 and 63. After postnatal day 63 the rate of the tissue volume of these animals increased faster than that of other groups. Further analysis of the data shows that the tissue volume of the male and female nicotine exposed rats between postnatal days 63 and 84 was at 45.1% and 34.4% respectively not different from the 56.2% and 31.8% respectively of the male and female rats of the control group of the same age. It is interesting to note that the increase in tissue volume up to postnatal day 42 was not affected by maternal nicotine exposure. It is only during the period between postnatal day 42 and 63 that the increase in tissue volume slowed down markedly in the lungs of the nicotine exposed offspring. Tomato juice intake by the mother prevented the effect of nicotine on tissue growth in the lungs of the offspring (4.4±0.2 ml vs 3.3± 0.1 ml for males and 3.6± 0.1 ml vs 2.6±0.1 ml for females) (table 3.11 and figures 3.8).

Fig 3.9 illustrates the impact of the exposure of the mother during gestation and lactation to nicotine only on the Vt of the 42- and 84-day-old offspring. It clearly shows that the Vt of both males and females decreased only after postnatal day 42. This is most likely due to late onset of parenchymal degradation.

Table 3.11: Effect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the tissue volume (Vt) (ml) of the offspring.(* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	0.7±0.0	0.7±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.1	0.6±0.1
C VS T			0.03*	0.03*				
C VS N					0.87	0.87		
C VS T+N							0.03*	0.03*
42	1.7±0.1	1.6±0.1	1.9±0.1	1.5±0.1	1.9±0.1	1.9±0.1	1.6±0.1	1.6±0.1
C VS T			0.1	0.93				
C VS N					0.06	0.02*		
C VS T+N							0.5	0.5
63	2.7±0.1	3.0±0.2	3.1±0.2	3.0±0.3	2.3±0.2	1.9±0.1	2.7±0.2	2.7±0.2
C VS T			0.1	0.95				
C VS N					0.07	0.005*		
C VS T+N							0.98	0.36
84	4.2±0.0	3.9±0.2	4.0±0.1	3.4±0.2	3.3±0.1	2.6±0.1	4.4±0.2	3.6±0.1
C VS T			0.22	0.11				
C VS N					0.002*	0.0003*		
C VS T+N							0.3	0.12

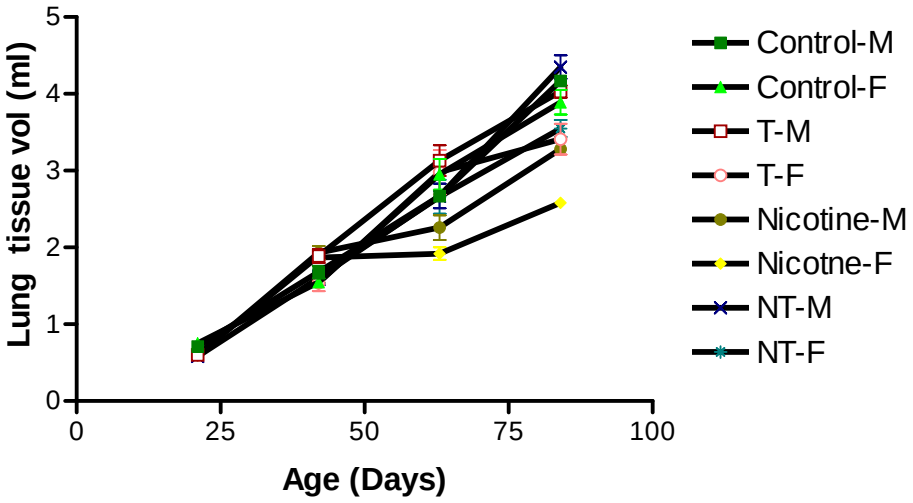


Fig. 3.8: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the lung tissue volume of the offspring. (Contol-M= control male; control-F= control female; TM= males receiving only tomato juice only; TF= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males receiving both nicotine and tomato juice; NT-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

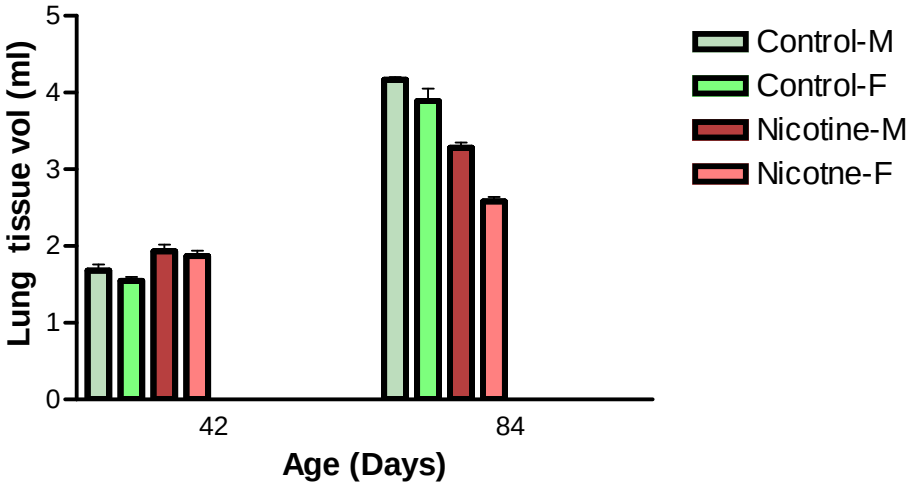


Fig. 3.9: Comparison of the tissue volumes of those animals that were exposed to nicotine via the placenta and mother's milk.

3.4.2. Mean linear intercept (Lm)

The Lm (table 3.12 and fig. 3. 10) of the control males and those exposed to tomato juice as well as those exposed to both nicotine and tomato juice decreased ($P<0.005$) between postnatal days 21 and 84. Although it appears that the Lm of the female offspring decreased between postnatak days 21 and 63, it was not significant ($P>0.05$). The Lm of the females, therefore, remained constant throughout this period of lung maturation. On the other hand, the Lm of the males and females exposed to nicotine via the placenta and mother's milk increased ($P<0.05$). Consequently the Lm of the nicotine exposed animals were bigger ($P<0.05$) than that of the other groups for all age groups and both male and female. The difference in Lm between control and nicotine exposed animals was on postnatal day 84 ($P<0.003$) more pronounced than on postnatal days 21 and 42.

Like for the other parameters tested so far, the intake of tomato juice by the pregnant and lactating rats prevented the increase in Lm as observed for those animals that were exposed to nicotine only during gestation and lactation.

Table 3.12: E ffect of nicotine, tomato juice, and the combination of tomato juice and nicotine on the mean linear intercept (Lm) (μm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+N)	
Sex	M	F	M	F	M	F	M	F
21	33.2±0.7	33.2±0.7	34.4±0.5	34.4±0.5	36.0±0.6	36.0±0.6	36.1±0.7	36.1±0.7
C VS T			0.13	0.13				
C VS N					0.05*	0.05*		
C VS T+N							0.006	0.006
42	32.0±0.5	33.0±0.8	30.8±0.7	32.7±1.2	38.4±1.1	36.4±1.2	31.5±1.4	30.5±0.9
C VS T			0.19	0.84				
C VS N					0.001*	0.04*		
C VS T+N							0.8	0.06
63	30.7±0.8	31.2±1.3	26.4±4.7	31.3±0.3	38.4±1.5	36.1±1.2	33.5±0.5	34.6±1.5
C VS T			0.73	0.94				
C VS N					0.006*	0.02*		
C VS T+N							0.03*	0.12
84	30.5±0.5	33.4±1.3	31.7±0.8	32.8±0.8	40.3±1.7	39.3±0.5	31.5±0.7	33.0±1.1
C VS T			0.23	0.69				
C VS N					0.003*	0.007*		
C VS T+N							0.28	0.78

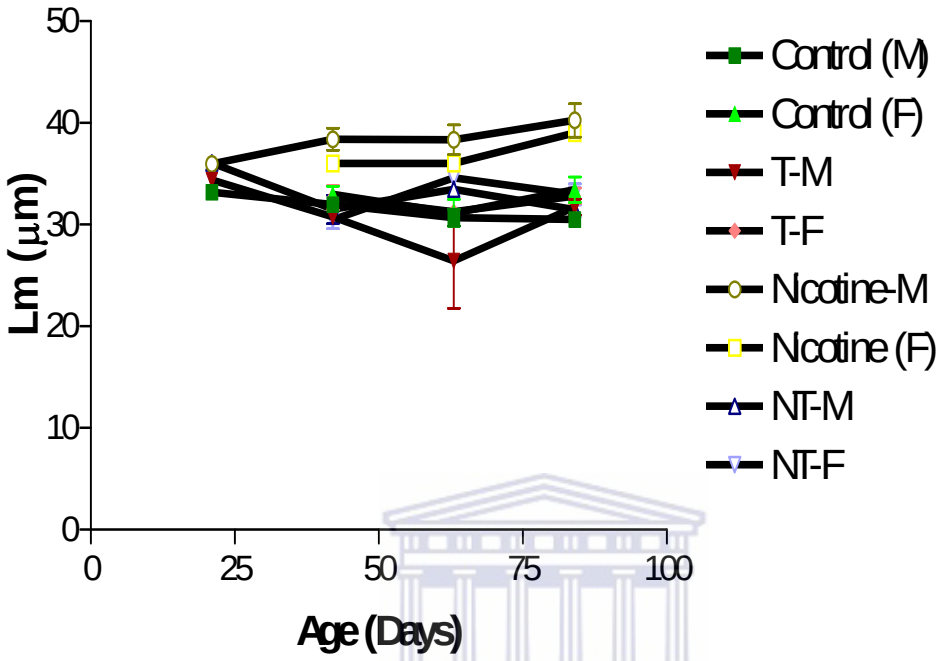


Fig. 3.10: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the Lm of the lungs of the offspring. (Contol-M= control male; control-F= control female; TM= males receiving only tomato juice only; TF= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males receiving both nicotine and tomato juice; NT-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

3.4.3. Alveolar wall thickness (Tsept)

The thickness of the walls between adjacent alveoli of the control rats, those that received tomato juice as a dietary supplement and those exposed to both nicotine and tomato juice, was the same. There was also no difference ($P>0.05$) in alveolar wall thickness between males and females (table 3.13 and Fig 3.11). On the other hand, the alveolar wall thickness of those animals that were exposed to nicotine only during gestation and lactation resembles that of the other groups up to postnatal day 63. Between postnatal day 63 and postnatal day 84 the alveolar wall thickness of the nicotine exposed rats increased by 68.1% ($P<0.001$) for the males and that of the females by 31.6% ($P<0.001$)

Table 3.13: Effect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the alveolar wall thickness (Tsept) (μm) of the offspring. (* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
Sex	M	F	M	F	M	F	M	F
21	9.3±1.2	9.3±1.2	7.2±0.7	7.2±0.7	8.1±0.9	8.1±0.9	6.9±1.0	6.9±1.0
C VS T			0.19	0.19				
C VS N					0.45	0.45		
C VS T+N							0.16	0.16
42	6.5±0.5	6.6±0.3	7.4±0.8	6.6±0.5	7.6±0.4	9.1±1.1	8.2±0.9	7.7±0.7
C VS T			0.36	0.96				
C VS N					0.1	0.09		
C VS T+N							0.15	0.24
63	7.4±0.8	9.0±1.5	8.5±1.0	9.9±1.8	9.8±1.5	10.3±1.3	7.4±0.8	9.6±2.0
C VS T			0.4	0.7				
C VS N					0.17	0.5		
C VS T+N							0.63	0.82
84	9.6±0.1	9.4±0.7	10.1±0.8	8.5±1.1	16.4±3.2	13.6±1.3	9.8±0.7	9.1±1.6
C VS T			0.66	0.5				
C VS N					0.04*	0.045*		
C VS T+N							0.8	0.9

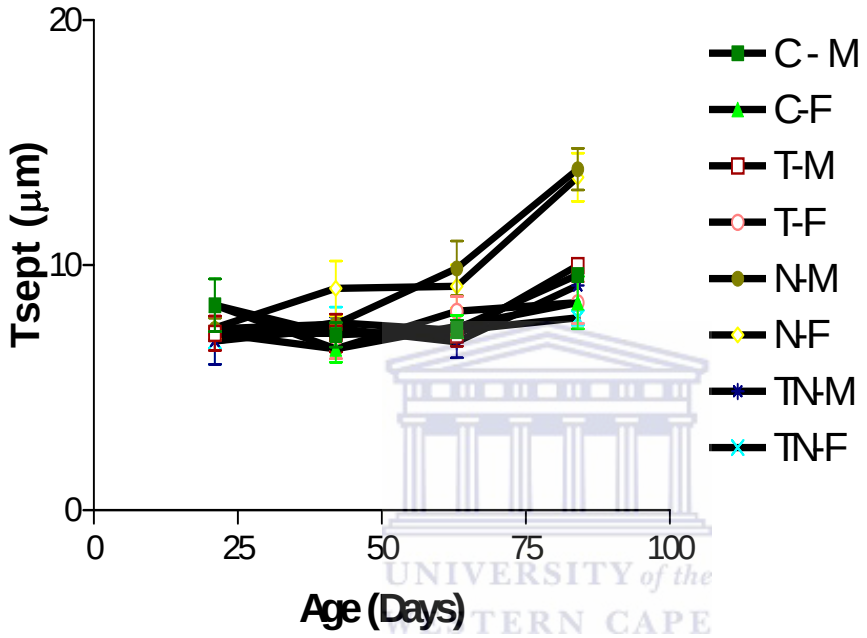


Fig.3.11: Effect of maternal nicotine exposure and intake of tomato juice during gestation and lactation on the alveolar wall thickness of the offspring. (Contol-M= control male; control-F= control female; TM= males receiving only tomato juice only; TF= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males receiving both nicotine and tomato juice; NT-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

3.4.4. Internal surface area (Sa)

The Sa of the lungs of all the animals gradually increased ($P<0.001$) as the animals matured (Fig 3.12). The data summarized in table 3. 14 and illustrated in fig 3.12, 3.13a and b shows that on postnatal day 21 the Sa of all the experimental animals, including those that were exposed to tomato juice only, were smaller than those of the control animals. From postnatal day 21 the Sa of the nicotine animals, as well as the rats exposed to both nicotine and tomato juice and tomato juice only, caught up with that of the control animals so that on postnatal day 63 there was no difference. The Sa on postnatal day 84 of the nicotine exposed animals was smaller in males ($1370.5\pm56.1mm^2$) and females ($1091\pm8.04mm^2$) than in control males ($1503.6\pm14.2\ mm^2$) and females ($1273.2\pm 63\ mm^2$) ($p<0.05$) table 3.14 and fig 3.13a &b.

It is interesting to note that tomato juice prevented the decrease in Sa of the 84-day old male, it was not protecting the lungs of female rats in tomato combined with nicotine group even if the decrease was not statistically significant ($p>0.05$)(fig 3.13 a and b, table 3.14).

Table 3.14: E ffect of maternal exposure to nicotine, tomato juice, and the combination of tomato juice and nicotine on the internal surface area (Sa) (cm^2) of the offspring.(* = significant difference)

Age in days	Control (C)		Tomato juice (T)		Nicotine (N)		Tomato + nicotine (T+ N)	
sex	M	F	M	F	M	F	M	F
21	364.9±13.3	364.9±13.3	300.4±14.1	300.4±14.1	317.6±21.0	317.55±21.0	288.9±23.0	288.9±23.0
C VS T			0.002*	0.002*				
C VS N					0.07	0.07		
C VS T+ N							0.005*	0.005*
42	771.9±39.4	713.6±17.8	790.9±45.0	638.9±38.9	704.0±29.9	746.7±31.1	773.9±59.4	705.1±45.4
C VS T			0.78	0.07				
C VS N					0.34	0.35		
C VS T+ N							0.98	0.87
63	1198.3±83.8	1054±61	1310.7±67.9	1149.7±86.8	1122.9±58.2	1075.9±69.6	1058.9±92.7	951.3±27.8
C VS T			0.35	0.38				
C VS N					0.5	0.8		
C VS T+ N							0.3	0.2
84	1503.6±14.2	1273.2±63	1457.2±39.4	1283±76.2	1370.5±56.1	1091±8.04	1461.6±45.7	1092±34.7
C VS T			0.33	0.92				
C VS N					0.03*	0.05*		
C VS T+ N							0.52	0.52

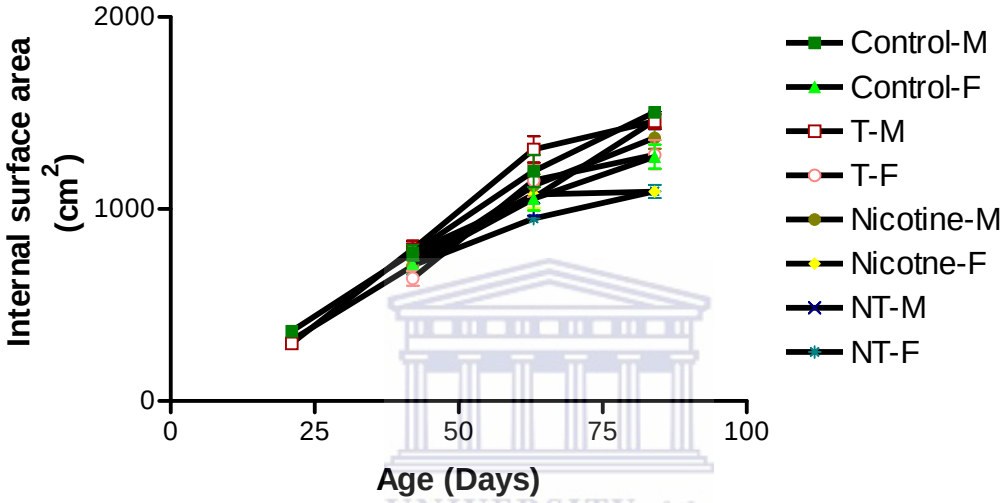


Fig. 3.12: Effect of maternal nicotine exposure during gestation and lactation and intake of tomato juice on the internal surface area of the lungs of the offspring. (Contol-M= control male; control-F= control female; TM= males receiving only tomato juice only; TF= Females receiving tomato juice only; Nicotin-M= males receiving nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males receiving both nicotine and tomato juice; NT-F= Females receiving both nicotine and tomato juice. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice so that the offspring receive these components only via the placenta and mothers' milk.)

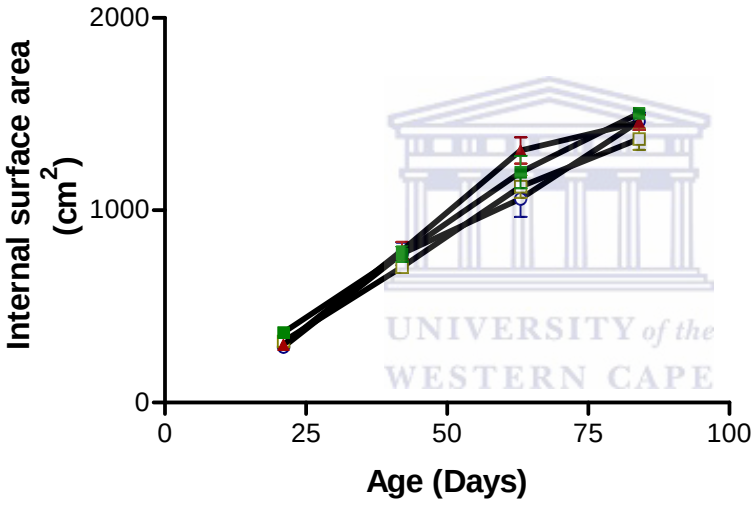


Fig 3.13a. Showing the changes in Sa between males in response to maternal nicotine exposure during gestation and lactation and the effect of maternal tomato juice supplementation.

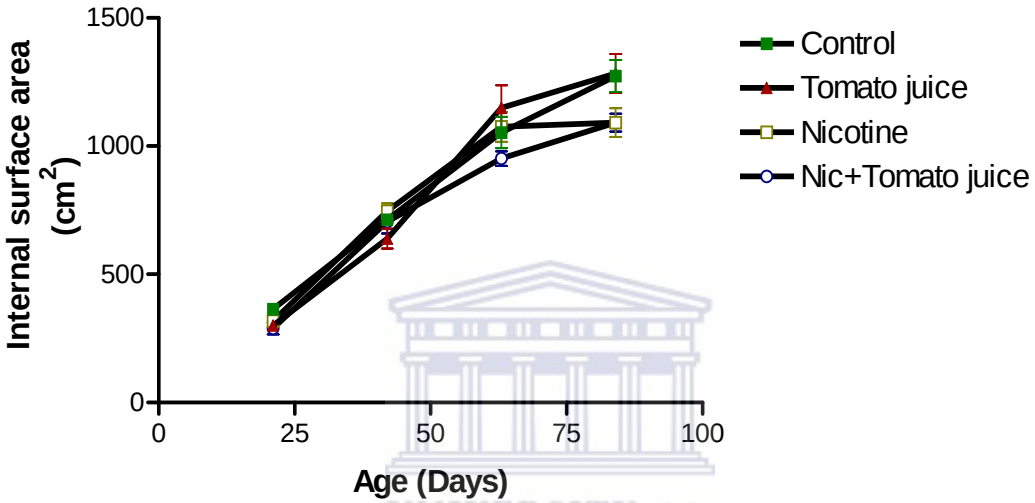


Fig 3.13 b. showing the changes in Sa between females in response to maternal nicotine exposure during gestation and lactation and the effect of maternal tomato juice supplementation .

3.5. Morphology

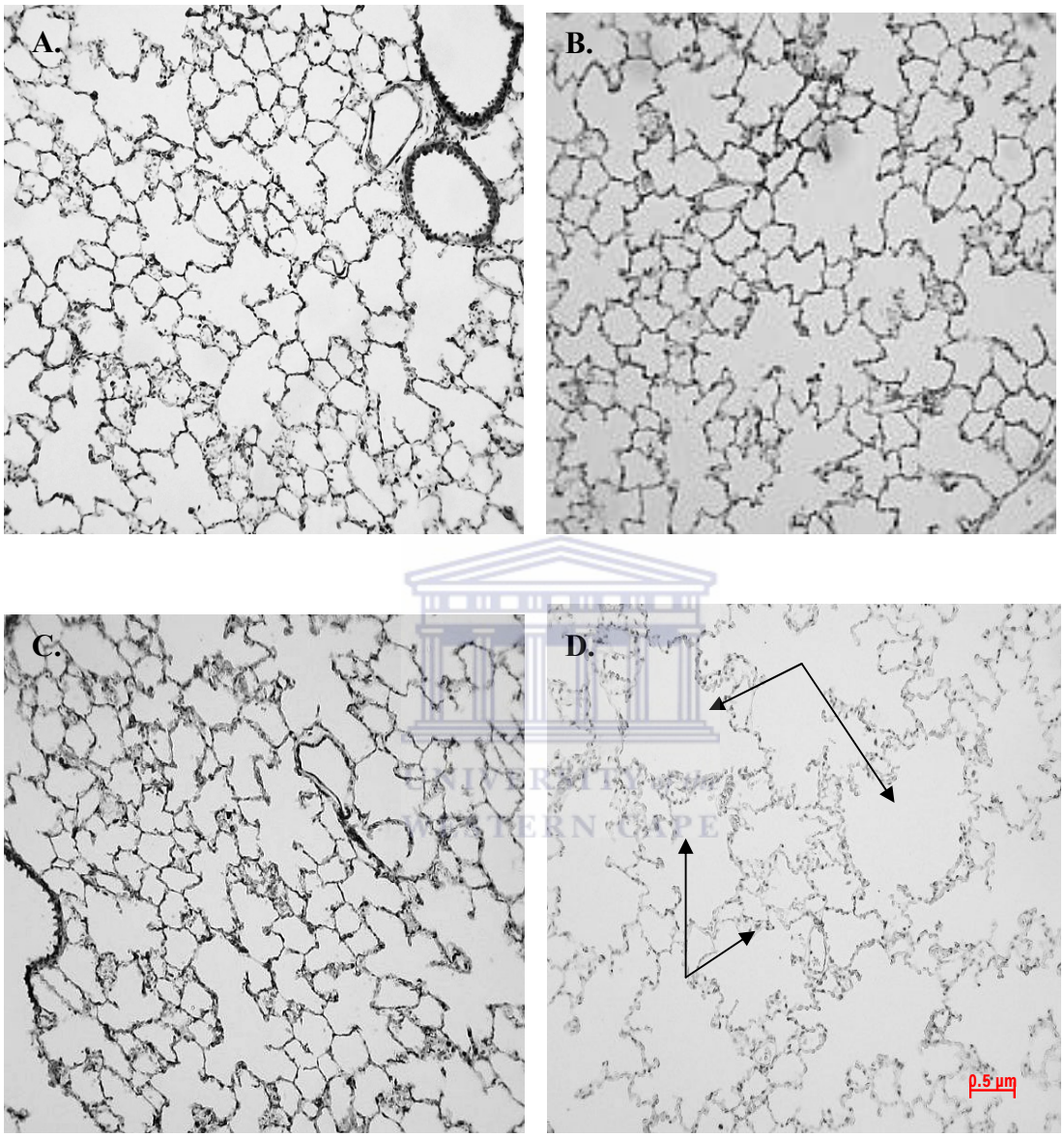


Fig. 3.14:. The influence of maternal exposure to nicotine, tomato juice and a combination of nicotine and tomato juice on lung parenchyma of the 84-day-old offspring. C. = Control; B.= Tomato juice; C. = Nicotine + tomato juice; D. = Nicotine. Arrows = Emphysema. (Bar = 0.5µm)

3.6. Effect of maternal nicotine exposure on the total antioxidant capacity of the 84 day old offspring.

The total antioxidant capacity of the lungs of the 84-day-old rats that were exposed to nicotine via the placenta and mother's milk was the same (Control vs Nicotine: 1.89±0.15 vs 1.92±0.16 μmol antioxidant/kg lung tissue; $P>0.05$) as that of the lung tissue of the control animals of the same age.

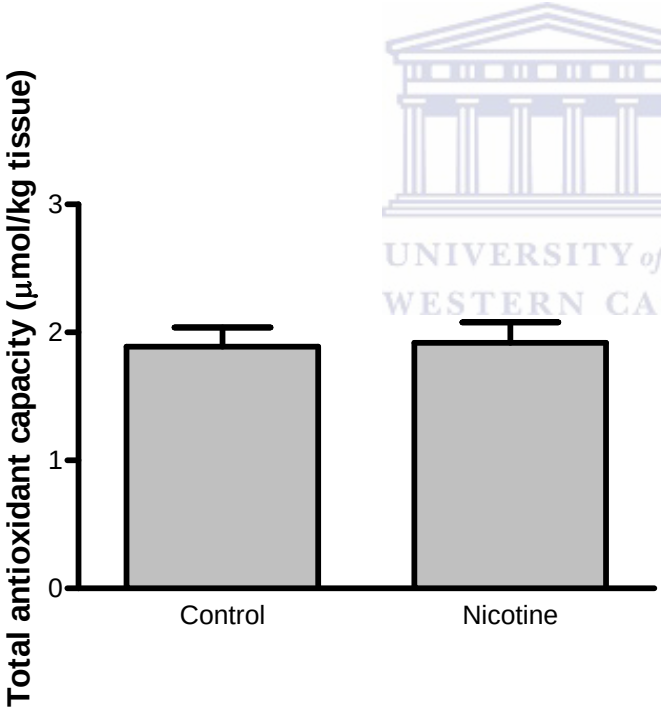


Fig. 3.15. The influence of maternal nicotine exposure during gestation and lactation on the total antioxidant capacity of the 84-day-old offspring.

CHAPTER 4

DISCUSSION

4.1. Effect of age and sex on growth and development.

It is known that adult female rats are smaller than adult male rats. In this study I compared the growth of the males and females from birth up to maturity to establish any differences and when such differences occur. The data in this study showed that growth of the male and female rats were the same from birth up to postnatal day 42. After postnatal day 42 the BW of the male rats continue to increase virtually linearly while that of the female rats started to plateau. This is also true for the chest circumference and crown-rump length of the males and females as a function of age. The slower increase in body weight, lung volume, and chest circumference and crown-rump length of the females after postnatal day 42 can only be attributed to male female differences and not to inadequate food and water supply as all animals received the same type and quantities of food and water. The living conditions were also exactly the same so that interference by any external environmental conditions can be ruled out as a possible cause of the growth differences after postnatal day 42. It is also important to note that nicotine exposure via the placenta and mother's milk had no effect on growth of either male or female offspring.

The increase in lung volumes of the male and female rats follows the same trend. A comparison of the Lv/CC ratio as well as the BW/Lv ratio showed that it was the same

for both males and females. This shows that the increase in body weight and lung volumes as well as growth in general was proportional. This means that any differences in growth between male and female rats exposed to nicotine, or tomato juice or a combination of nicotine and tomato juice can not be attributed to a specific growth parameter or to interference with the specific growth parameter by nicotine or tomato juice or a combination of nicotine and tomato juice via the placenta and mother's milk.

The total fluid intake of the pregnant rats was monitored during gestation and lactation up to weaning on postnatal day 21. It is interesting to note that while the fluid intake of the control and nicotine exposed rats were the same, it was higher in those rats that were exposed to tomato juice only or to nicotine and tomato juice simultaneously. Despite this, the pattern of growth of female and male animals was the same. The increase in the daily fluid intake from around postnatal day 14 can be ascribed to the increase in fluid intake of the offspring.

4. 2. The effect of maternal nicotine exposure during gestation and lactation on lung development of the offspring.

In this study, we injected nicotine to the pregnant rats from day 7 of gestation until weaning at postnatal day 21. This means that the rat pups were exposed to nicotine from the beginning of the formation of the lungs which starts at day 10 of gestation in embryonic phase in mice and rats (Warburton, 2006). Nicotine accumulates in mother's milk (Luck and Nau, 1984; Dahlstrom et al., 1990) and this alkaloid also freely crosses the placental barrier and occurs in amniotic fluid (Luck et al., 1985; Dempsey and

Benowitz, 2001). Therefore, the rat pups were exposed to nicotine via the placenta during gestation and via mother's milk during the whole period of lactation. The amount of nicotine in breast milk and in amniotic fluid is higher than in the mother's blood (Luck et al., 1985; Dempsey and Benowitz, 2001). This implies that the rat pups were exposed to high amounts of nicotine during gestation and lactation. Furthermore, since its excretion system is less developed than of the mother, the rat pup will be more affected by nicotine than the mother at every stage of lung development, particularly the phase of rapid cell proliferation, such as alveolarization during which most of alveoli of the lungs are formed. Because of exposure to higher concentrations of nicotine for longer periods of time, this means that all mechanisms and transformations which are necessary for lung maturation, will be affected and impact on lung growth, development and susceptibility to respiratory diseases in later life.

Lung development is divided into different stages: embryonic phase, pseudoglandular phase, cannalicular phase, saccular phase, and, in the rat, after birth, the phase of rapid alveolarisation, and, lastly, the phase of equilibrated lung growth. Some of these phases started and finished during the foetal life and others such as the phase of rapid alveolarization started after birth from postnatal day 4 up to postnatal day 13 in rats. During these phases rapid tissue proliferation occur so that this vital organ of respiration can be metabolically and structurally mature for efficient gaseous during its entire life (Burri et al., 1974).

The *in utero* environment affects the growth and development of the fetus and thus also of the lungs. A normal environment *in utero* and after birth will ensure normal development of the lungs of the offspring. This is also essential for lung maintenance and aging. It means that a change in this environment will have an impact on the development of the fetus and the lungs of the fetus. The impact may depend on the type of change as well as the magnitude of the change. *In utero* and after birth each phase contributes in a specific manner to the development of the lungs into efficient gas exchangers. Therefore, interference with any one of these phases of development, for example by; 1) impaired nutrition, particularly the lack of the glucose which is the source of the energy for cell proliferation (O'Neill and Tierney, 1974 ; Maritz, 1986), lung growth and function, or, 2) exposure to tobacco smoke (Davies and Albertiny, 1976; Bardy et al.,1993), or, 3) nicotine, or, 4) other pollutants during the critical periods of rapid cell proliferation, may change the "programme" that controls lung growth, development, maintenance and aging. As a consequence the susceptibility to respiratory diseases such as emphysema, and chronic bronchitis may increase (Maritz and Burger, 1992).

Previous studies proved that the maternal nicotine exposure result in low birth weight at normal gestational age (Kleinman and Madans, 1985). It was suggested that it might be due to the capacity of nicotine to induce vasoconstriction especially in placental microvasculature, and a decrease in the placental absorption of different nutrients resulting in under nutrition of the fetus (Economides and Braithwaite, 1994). Contrary to the above, other researchers showed that maternal nicotine exposure does not affect the placental absorption and body weight and the lung volume of the offspring (Bainbridge

and Smith, 2006; Maritz and Windvogel, 2003). This means that any changes in neonatal lung structure observed in this project will be due to other causes than a lack of nutrient intake by the fetus or neonate.

In my study I found that exposure of the pregnant and lactating rats to nicotine during the whole period of gestation and lactation had no affect on the body weight of the offspring at birth (9.07±0.32 g for control, 9.09±0.8 g for tomato juice + nicotine, 10.2±0.32 g for tomato juice and 11.2±1.0 g for nicotine)(table 3.2). This means that maternal nicotine exposure did not affect the nutrient supply from mother to foetus through the placenta or through mother's milk. Not only did maternal nicotine exposure not affect the growth of the rat pups, but it also had no effect on Lv and their Lv/BW ratio from birth up to postnatal day 84. The increase in Lv was therefore proportional to the increase in BW in all the age groups. However, this proportional increase in Lv does not reflect changes in the lung parenchyma of the nicotine exposed offspring.

Nicotine, an alkaloid found in tobacco smoke or in patches, lozenges, sprays and gums used during nicotine replacement therapy (NRT) (Benowitz and Dempsey, 2004) induces microscopic emphysema (Maritz, 1996, 1997a). Apart from nicotine, tobacco smoke contains more than 4700 different chemicals. Some of these chemicals are carcinogenic and others can be converted into carcinogenic compounds. Each puff of cigarette smoke contains more than 10^{14} oxidants which can pass to the blood from the alveoli in the lungs (Yamaguchi et al, 2007). The accumulation of all these oxidants in the lungs provoke the decrease in the amount of the natural antioxidants of the body in general and lungs in particular (Balakrishnan and Menon, 2007). This causes oxidative stress which

in turn causes lipid peroxidation (Kalpana and Menon, 2004) and DNA damage. The single strand DNA breaks can change the "program" that control the structure and integrity of the different organs of the body, including the lungs (Maritz, 2001).

It is important to note that, although the vitamin C content of the lungs of the rat pups were markedly lower than that of control animals while they were exposed to nicotine (Balakrishnan and Menon, 2007), the total antioxidant capacity after 8 weeks of nicotine withdrawal was not different from that of control animals. This means that an oxidant-antioxidant imbalance occurred during the phases of rapid cell proliferation and differentiation in the lungs of the offspring. This suggest that any changes that were induced in the "program" that controls lung development and aging by this imbalance could only happen in utero and/or while the rat pups were dependent on mother's milk because the changes in the lung parenchyma only occurs later in life when the oxidant-antioxidant capacity of the lungs were restored. This is supported by the fact that nicotine's half life is only 1.5 to 2 hours (Benowitz, 1982 and Feyerabend, 1985), and that after its elimination from the body the oxidant effect of nicotine will be removed and a return of the oxidant-antioxidant capacity to normal can be expected as shown in this study.

4. 2 a. Alveolar tissue volume and alveolar wall thickness

After birth the lung growth of the rat can be divided into three phases: lung expansion up to postnatal day 4, the phase of rapid alveolarisation from postnatal day 4 up to postnatal day 13, and equilibrated lung growth from postnatal day 13 up to postnatal day 21 (Burri

et al., 1974). During the first phase, the lung volume is increased by enlargement of the existing air spaces. The second phase from day 4 after birth up to day 13, the alveoli are formed by outgrowth of the secondary septa from the primary septa existing at birth (Burri et al., 1974).

The phase of rapid alveolarisation is characterized by rapid cell proliferation and interference with cell proliferation may result in stunted lung growth and development. The thinning of the septa and respiratory membranes also occur during this phase to improve better oxygen and carbon dioxide exchange (Weibel and Knight, 1964). At postnatal days 21 and 42, the tissue volume of the control animals as well as those that were exposed to tomato juice only, or nicotine only or to both nicotine and tomato juice, was the same. This means that at this stage of lung development and maturation, no change in lung cell proliferation and growth was evident in the lungs of the animals exposed to nicotine. However, as the lungs age, gradual thickening of the alveolar walls of the lungs of the nicotine exposed rats was observed after postnatal day 63. This is reflected in the higher tissue volume of the lungs of the animals that were exposed to nicotine via the placenta and mother's milk. This late appearance of the thicker alveolar walls of the nicotine exposed rats can not be ascribed to the presence of nicotine since nicotine was withheld from weaning on postnatal day 21. It is unlikely that any nicotine, or even products of nicotine metabolism, such as cotinine, was still present because the half life of nicotine is only 1.5 to 2 hours and that of cotinine 16 hours (Benowitz et al., 1999; 2002a). It is therefore plausible that the late appearance of thicker alveolar walls was due to nicotine – induced changes in the "program" that controls the maintenance of

the alveolar wall. Thickening of the walls can be due to: 1) increased cellularity of the alveolar walls, or, 2) an increased deposition of extracellular matrix, or, 3) both. The increase in cellularity might be due to and increase in the number of fibroblasts or due to a proliferation of type II pneumocytes. The latter is plausible because damage to type I pneumocyets is followed by type II pneumocyte proliferation (Crapo et al, 1980). Previous studies by Maritz and Thomas (1995) indeed illustrated type I pneumocyte damage in the lungs of nicotine exposed rats as well as an increase in type II pneumocyte number. If the thicker alveolar walls in the lungs of the nicotine exposed animals are due to an increased cellularity it might be due to the suppression of apoptosis (Heuch and Maneckjee, 1998). Studies to investigate this are presently in progress. It is interesting to note that alveolar wall thinning during the early phases of lung development is not affected by the exposure of the offspring to nicotine via the placenta and mothers'milk. Since alveolar wall thinning is dependent on apoptosis (Bruce et al, 1999), it implies that apoptosis at this early phase of lung development was not affected by nicotine. If this is indeed so it means that the maternal nicotine exposure induced changes in the "progam" that control the thinning of the alveolar walls by suppressing or slowing apoptosis of the interstitial fibroblasts in the aveolar walls. It is also plausible that the "program" that controls extracellular matrix synthesis and breakdown might be compromised.

4. 2 b. Internal surface area, alveolar number and volume.

The occurrence of the secondary septa during postnatal lung development, results in an increase in the alveolar number and thus also in an increase in the internal surface area available for gas exchange. As the animals grow and the body weight increase the

demand for oxygen also increase. To meet the increased demand for oxygen the lung volume increased as well as the alveolar number and consequently the internal surface area available for gas exchange. This means that the increase in internal surface area and the increase in body weight of the growing rats must be proportional (Massaro and Massaro, 2002). This will ensure that the lungs will supply all the oxygen that is required for growth and development of the body.

At postnatal days 21 and 42 the internal surface area of the animals that were exposed to nicotine via the placenta and mother's milk was the same as for the control animals. However, in this study it was shown that deterioration of the lung parenchyma occurred after postnatal day 42 so that on postnatal days 63 and 84, the internal surface area of the nicotine male and female exposed animals, were lower than in the controls animal of the same age. This decrease of the internal surface area was already present at postnatal day 21 at the stage when the rapid alveolarization was completed and during equilibrated lung growth, and cannot be ascribed to the direct effect of nicotine on the lung parenchyma after such a long time of nicotine withdrawal. Since the internal surface area and alveolar numbers and volume was not affected up to postnatal day 42, it means that nicotine exposure does not have any impact on the control of alveolar formation during the earlier phases of lung development. This decrease is perhaps due to the slower formation of the alveoli during the phase of the equilibrated growth of the lung or gradual damage of the lung parenchyma (Maritz, 1996; Maritz and Windvogel, 2003). They showed that maternal nicotine exposure during gestation and lactation result in the gradual destruction of the lung parenchyma. These gradual deterioration of the parenchyma give rise to the

appearance of microscopic emphysema and support the findings of my study. This is reflected in the increase in the linear intercept, and thus the larger alveolar volume of the day 84 animals.

Several researchers reported a number of genes that are candidates for determining the susceptibility of the individual to pulmonary emphysema (Finlay et al., 1997; Smith and Harrison, 1997; Hautamaki et al., 1997). Amongst those genes, the Klotho gene which, once disrupted, results in pulmonary emphysema in mice which are anatomically and physiologically similar to rats used in this study. It is plausible that a disruption of these genes may result in a change in the "programme" that controls lung growth, development, structural and functional maintenance, as well as aging. If this is indeed so, it explains why gradual deterioration commenced earlier in the life of the animals than normal, and consequently the appearance of microscopic emphysema later in the life of the offspring.

In summary, it is clear that maternal nicotine exposure during the gestation and lactation results in a) destruction of the lung parenchyma, b)smaller internal surface area for gas exchange, c) late onset of alveolar wall thickening and an increase in alveolar tissue volume. All these changes might be due to the change in the "program" that control lung development and maturation. It also appears that these changes, there are not reversible.

4. 3. Protection of the lungs by tomato juice.

It has recently been shown that diets rich in tomatoes and tomato products have potential health benefits (Rao and Agarwal, 1998; Rao and Agarwal, 1999). Lycopene is a carotenoid without provitamin A activity. It is present in tomatoes and tomato products and is considered responsible for the beneficial effects of tomatoes (Stahl and Sies, 1996; Gerster, 1997; Clinton, 1998, Rao and Agarwal, 1998). It is a most powerful antioxidant and it is thought that it is responsible for protecting cells against oxidative damage and so decreases the risk of chronic diseases (DiMasco et al., 1989; Rao and Agarwal, 1999). The oxygen singlet quenching ability of lycopene is twice higher than that of the β-carotene and 10-times higher than that of α-tocopherol (Rao and Agarwal, 2000; Stahl and Sies, 1996).

In addition to its antioxidant properties it has also been shown that lycopene induce cell-to-cell interaction (Zhang et al, 1991). It also modulate hormonal and immune systems as well as other metabolic pathways (Fuhram et al, 1997; Astorg et al, 1997) which may also contribute to its beneficial effects on health (Rao and Agarwal, 1999).

Lycopene is only optimally active in the presence of other phytonutrients (Rao and Agrwal, 1999). Furthermore, it is only synthesized by plants and micro organisms but not by animals. That is why animals and humans are dependent on its presence in their daily diet. This antioxidant is present in different organs in different quantities, for example, in the human lung it is 0.22-0.57 nml/g wet weight while in rats lung it is 0.115 nml/ g wet weight.

Like antioxidants such as vitamin C, the level of lycopene in the body decreases when exposed to oxidants such as during smoking because it is actively contributing to neutralizing the oxidants. Apart from inducing the production of oxidants, it also have oxidant action which will therefore also reduce the antioxidant capacity of the lungs (Kasagi et al., 2005). Lycopene is a powerful antioxidant and is therefore important in protecting the lung against these oxidants, as well as cell-to-cell communication. This implies that lycopene will play a role in the communication between parenchymal fibroblsts and the alveolar epitheial cells that is important for matrix metabolism and thus maintenance of lung structure and thus function (Rao and Agarwal, 2000). Since lycopene plays a role in cell-cell interaction (Zhang et al, 1991), it may play an active role in lung growth and maturation. If this is so, it is conceivable that a lowering of the lung lycopene content during lung growth and development may compromise this process and increase the propensity for respiratory disease later in life.

In this study, the animals which were exposed to a combination of the tomato juice and the nicotine and animals exposed to tomato juice only, have the same tissue volume, the same alveolar volume, the same alveolar wall thickness, the same mean linear intercept and the same internal surface area as the control animals. This shows the powerful protection role of the tomato juice against the effects of the nicotine during different phases of lung development and maturation since foetal life up to postnatal day 84.

Since the Lm is an indicator of alveolar size, it means that the alveolar integrity of the nicotine exposed rats were protected by lycopene in tomato juice and thus also the alveolar numbers and consequently the internal surface area available for gas exchange. This also means that the reserve capacity of the lungs of thoase rats that were exposed to nicotine during gestation and lactation was normal.

Previous studies showed that maternal nicotine exposure drastically reduces the vitamin C content of the lungs of the offspring (Balakrishnan and Menon, 2007). It also suppresses the total SOD activity of the lungs of the offspring (Maritz, 1993; Hussain et al., 2001). This simultaneous lowering of the antioxidant capacity of the developing lung, and the increase in the oxidant levels associated with the presence of nicotine, result in an imbalance in the oxidant/antioxidant status of these lungs. It is therefore conceivable that this imbalance will adversely impact on cell function and integrity in the developing lung. It was indeed illustrated by Schins et al (2002) that certain oxidants, such as H_2O_2 is endogenously produced by alveolar epithelial cells and in this way cause DNA damage in these cells. They showed that DNA damage already occurred at levels where cytotoxicity is not evident. Apart from H_2O_2 and other oxidants, nicotine is also genotoxic (Kleinsasser et al, 2005). It has been shown that damage at molecular level by oxidants leads to a number of age associated diseases (Ames et al, 1993; Hussain et al, 2003). It is, therefore, conceivable that the integrity of the Klotho gene, which was mentioned earlier, was compromised by nicotine and other oxidants. If this is so, it may contribute to the slow deterioration of the lung parenchyma as seen in this study. Strong support for the role of oxidants in promoting aging comes from studies that showed that increasing levels

of protective enzyme as well as increased concentrations of exogenous and endogenous antioxidants can increase the lifespan of different species (Squier, 2001; Miquel, 2001; Miquel et al, 1995; Dela Fuente et al 1993). It is therefore plausible that together with the accumulation of nicotine in the fetal lung (Brewer et al., 2004), the high nicotine content of the amniotic fluid (Luck et al., 1985; Benowitz and Dempsey, 2001), and that the level of other oxidants in utero and in the fetus of the nicotine exposed group of animals will be higher than in that of the control animals. Since the nicotine levels remain high in the amniotic fluid and the fetal lung over an extended period, it is possible that DNA repair will also not be adequate. If this is so, it implies that it may result in a change in the "program" that directs lung growth, development and aging. This notion is supported by previous studies which showed that; 1) vitamin C supplementation partially prevented the adverse effects of maternal nicotine exposure during gestation and lactation (Rayise, 2009), and, 2) as illustrated in this study tomato juice completely prevented the effect of maternal nicotine exposure on lung development and aging in the lungs of the offspring. The protective effect of tomato juice can be ascribed to the presence of lycopene, a powerful antioxidant (Di Masco et al., 1989). It is, therefore, likely that supplementing the diet of the pregnant animals with tomato juice maintained the oxidant-antioxidant status of the mothers and offspring during pregnancy and lactation and so prevented changes in the "program" that control lung growth, development and aging. It is therefore conceivable that intake of tomato juice during pregnancy by smoking mothers or those using nicotine replacement therapy will protect the offspring against the harmful effects of smoking and in this way reduce the incidence of respiratory disease in the offspring.

5. Conclusion and future perspectives.

In conclusion, this study showed that maternal nicotine exposure during gestation and lactation adversely affected maintenance of lung structure as the offspring aged. This is most likely due to premature aging of the lungs due to changes to the "program" that controls aging of the lung. This premature aging is likely due to an imbalance in the oxidant-antioxidant status of the lung during early lung development induced by nicotine. This notion is strongly supported by the fact that all these changes are prevented by supplementing the mother's diet with tomato juice containing the powerful antioxidant lycopene.

Based on these studies it is suggested that nicotine replacement therapy not be prescribed to pregnant and/or lactating mothers unless steps are taken to maintain the oxidant-antioxidant status of the mother and the *in utero* environment within which the fetus develops.

It is reasonable to believe that nicotine is genotoxic and changes the "program" that controls lung development and aging that these changes will be transferred to future generations. Studies to establish whether these changes will be inherited by future generations are in the planning stage.

Apart from investigating the impact of maternal smoking on respiratory health of the offspring in the long term, it is essential to also educate the public as to the long-term consequences of lifestyle on the health of the offspring. In addition, new strategies must be developed to ensure that, for example, the right diet be followed by the mother during gestation and lactation to ensure no changes in the "program" that directs growth, development and maintenance of health is induced during fetal development *in utero*.

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Appendix:

Product	**supplier/ manufacturer**
Automatic tissue processor	Shandon histokinette
Coverslips	Marienfield
Disodiumhydrogen phosphate	Merck
DPX	Merck
Ethanol	Merck
Formaldehyde	Merck
Graticules	Graticules Ltd
Hydrochloric acid	Sigma
Hypodermic needles	Promex
Laboratory balance	Sartorius 1475A
Magnesium sulphate	Sigma
Microsope	Zeiss research type
Microsope slides	Chance Proper
Microtome knives	Leica
Nicotine	Merck
Pentobarbitol	Rhone-Poulenc
Plastic cassettes	Anglia scientific
Potassium bicarbonate	Merck
Rats'	Medical research council

Refrigerator	Flucksware domestic type
Sliding microtome	Reichert-Jung
Sodium chloride	Merck
Sodiumdihydrogen phosphate	Merck
Syringes	Promex
Xylene	Merck