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

By

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KEYWORDS

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

ABSTRACT

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

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

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

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

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

DECLARATION

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

Keitumetse Mothibeli November 2013

Signed: 

DEDICATION

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

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

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

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

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

CHAPTER 1

Introduction

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

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

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

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

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

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

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

CHAPTER 2

Literature Review

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

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

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

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

2.1 Lung development

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

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

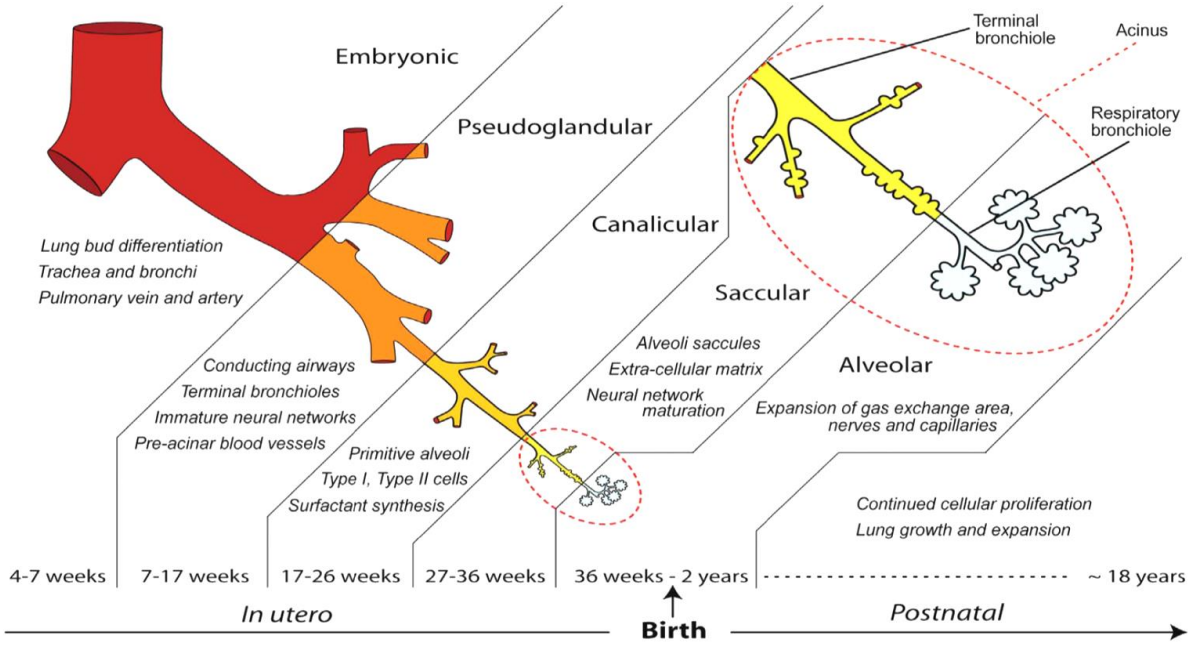


Figure 2.1. Stages of lung development in humans: diagrammatic representations of the timeline and developmental organization of trachea, primary bronchi, intrapulmonary bronchi, and acinus in the mammalian respiratory system. Reprinted from (Kajekar, 2007).

2.1.1 Embryonic phase

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

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

Ypn: Years postnatally Dnp: days postnatally

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

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

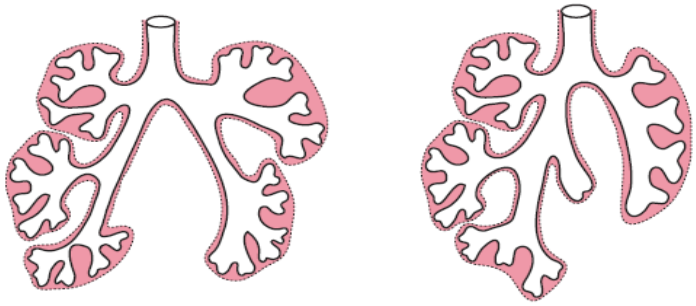
The embryonic phase (table 2.1) of rats start on day 11 and of mice on day 9.5 after mating from two endordermal buds sprouting from the ventral foregut. A trachea (containing two primary lung buds) and esophagus will then form at the same location from a single foregut tube. The two primary mouse lung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

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

2.1.2 Pseudoglandular phase

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

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



Human lung Mouse lung

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

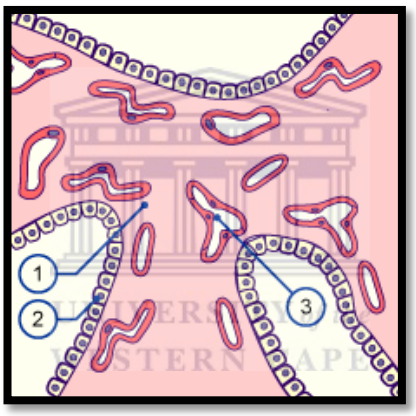


Figure 2.3. Lung tissue in the pseudoglandular phase: Lung has glandular appearance; Airways are lined with columnar epithelium and separated by poorly differentiated mesenchyme. 1) Lung mesenchyma; 2) Type II pneumocytes; 3) capillaries (Voigt et al. 1999).

2.1.3 Canalicular phase

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

5.6.2(a) Number of proliferating cell/100µm

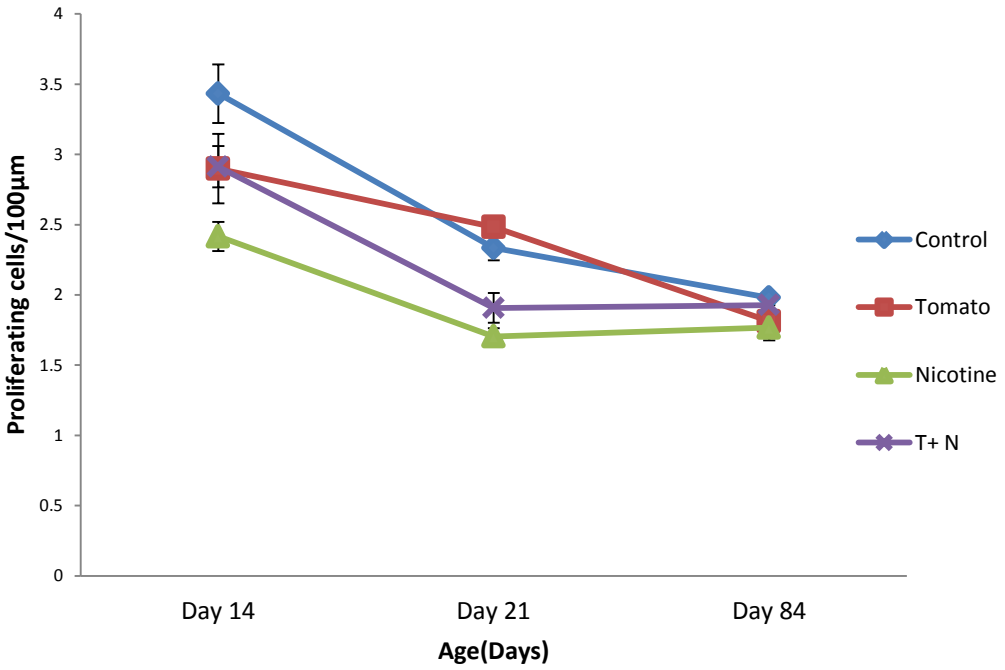


Figure 5.14. The effects of Maternal Nicotine and Tomato Juice exposure on Proliferating cells/100µm alveolar wall.

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

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

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

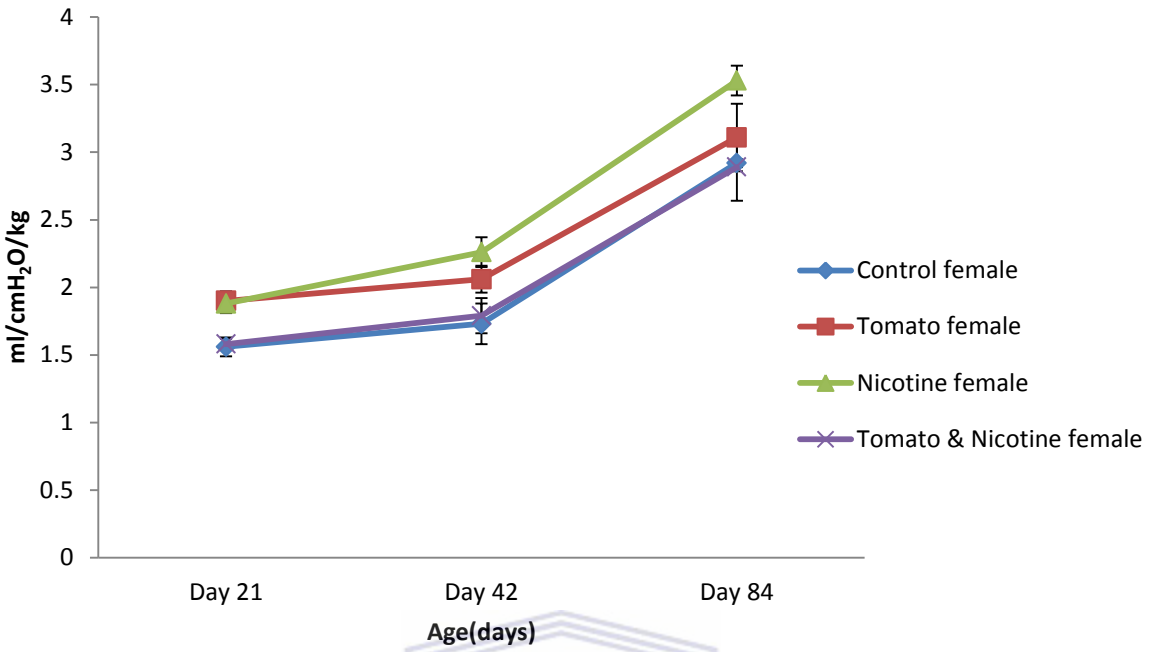


Figure 5.15(b). The effects of Maternal Nicotine and Tomato Juice exposure on male lung compliance.

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

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