THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL RAT LUNG SEPTAL STATUS.

by

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DEDICATION

I dedicate this document to my friend Charlene, who died on 3 April 1995.

Having had you as my friend enriched my life, as it did countless others.

FRIENDS ALWAYS!

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ABSTRACT

The aim of this study was to determine the effect of maternal nicotine exposure on the status of rat lung alveolar septa of 1 to 21 day old offspring. Wistar dams were injected subcutaneously, using a dosage of 1 mg nicotine/kg body mass/day, and treatment commenced 7 days after conception, up to the third week after parturition. The data obtained showed an increase in septal cellularity, with a decrease in type I:type II cell ratio as a result of type I cell destruction and type II cell proliferation. The type I cells appear to be more sensitive to the effect of nicotine than type II cells. Data also illustrate swelling of type II and endothelial cell mitochondria, blebbing of both type I and endothelial cells and rupturing of the blood-air barrier in the nicotine exposed lungs of the rats of all the age groups. Lamellar body count are significantly higher in the type 11 cells of nicotine exposed lungs in all age groups compared to the control lungs. The number of capillaries per unit length of septum was also significantly less than that of the control lungs. Other morphological changes which were also observed in the nicotine exposed offspring in all the age groups are: loss of type II cell microvilli, swelling of type I and endothelial cells, and grouping of type II cells within the septal interstitium (an indication of proliferation). The results clearly indicate that maternal nicotine exposure interfered with the morphometric and morphologic characteristics of the alveolar septa of the lung tissue of the neonatal rats.

CHAPTER 1

AN OVERVIEW OF PRE- AND POSTNATAL LUNG DEVELOPMENT AND THE EFFECT OF MATERNAL NICOTINE ON THE DEVELOPMENTAL PROCESS.

1.1 INTRODUCTION

Development is the sum of a number of biologic processes, including regulation of gene expression, specification of cell fate, and formation of a morphologic pattern, which results in the formation of a tissue organ or organism. Each developmental event occurs at a specific time and in a characteristic location. In the lung, cellular differentiation, that is the acquisition of a specialized phenotype by a cell within a tissue, culminate in the formation of the most abundant epithelial cell types in the lung, namely the alveolar type I and type II cells (Brody and Williams, 1992). Therefore, the developmental events of the lung contribute in different ways to the transformation of an immature lung into a structurally and functionally competent organ (Vidic et al, 1989).

The design of the respiratory system features a series of specialized structures that functions co-operatively to serve a vital need of the mammalian organism, namely the exchange of oxygen and carbon dioxide. The respiratory system has a basic structure common to man and a number of other animals, including the rat. It consists of the nose, pharynx, larynx, trachea, bronchi and lungs (Tortora and Anagnostakos, 1990). Figure 1.1 shows the basic structure of the respiratory system

(Tortora and Anagnostakos, 1990). Conditioning of inspired air, by the removal of coarse particulate matter and gaseous impurities, while simultaneously warming and humidifying the air, occurs at different portions along the respiratory tract. The most important function of the respiratory system occurs in the lungs where the exchange of respiratory gases take place.

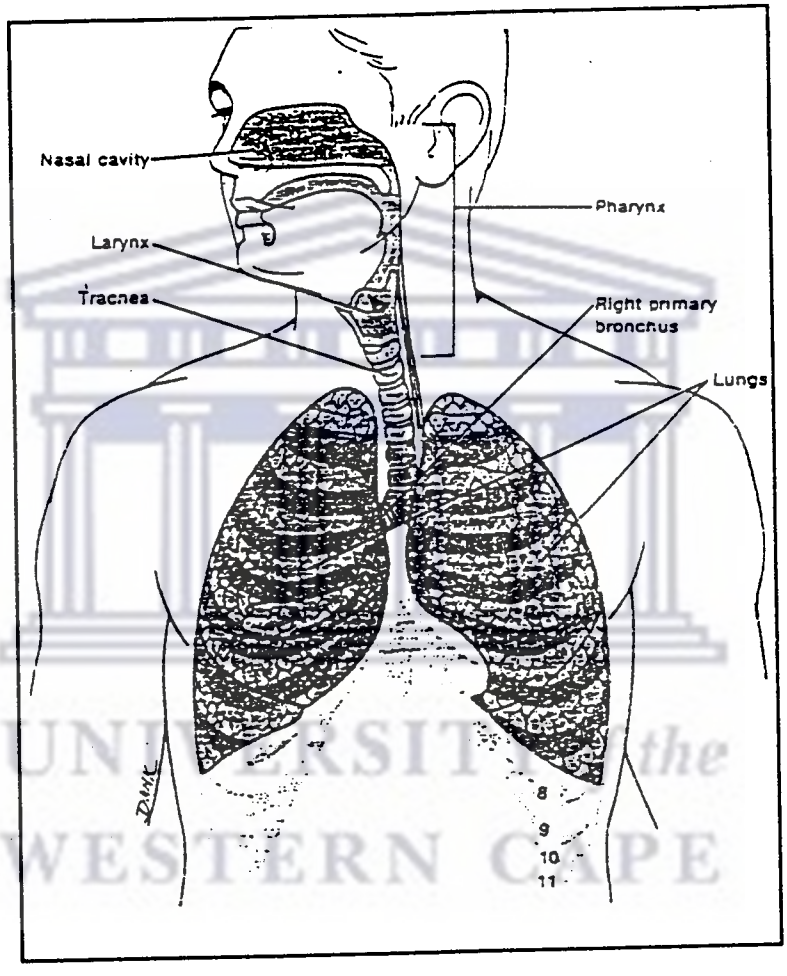

Figure 1.1 Diagram of the respiratory organs in relation to surrounding structures. Adapted from Principles of Anatomy and Physiology (Tortora and Anagnostakos, 1990).

Environmental factors during pregnancy can modify the process of lung

development (Lieberman et al, 1992), thereby leading to defective and insufficient functionality, thus decreasing its resistance to disease. Cigarette smoking by the pregnant mother, and fetal exposure to the cigarette smoke of others, are environmental factors that might affect lung maturation (Lieberman et al, 1992). Curet et al (1983) has shown that the risk of respiratory distress syndrome (RDS) for infants of smoking mothers who smoked during pregnancy, was half that of infants of nonsmoking mothers.

However, the literature is replete with studies confirming the adverse effects maternal smoking during pregnancy has on fetal development. These include fetal growth retardation (Collins et al, 1985; Hardy and Mellts, 1972; Miller and Hassanein, 1964), preterm birth (Meyer, 1977; Yerushelmy, 1971), consequential increased mortality (Meyer et al, 1977), increased incidence of pulmonary disorders (Saeta et al, 1985), and decreased body weight at birth (Rush, 1974). Lung cancer mortality in all cigarette smokers exceeds that of nonsmokers by 14-fold, with a clear dose-response relationship between amount smoked, depth of inhalation and mortality (Vial, 1986).

Knowledge about the pre- and postnatal growth and development of the lung provides important insight into pulmonary function in health and disease (Murray, 1986). Most of the information available on lung development, metabolism and functioning has been obtained using animal models, particularly that of the rat. Therefore a comparison needs to be drawn between the lung of the human and that of the rat in order to clarify developmental and morphological aspects within these

two systems. This will be addressed as follows:

1.2.1 Prenatal or fetal development

1.2.2 Postnatal or neonatal development.

Since this study deals specifically with the morphological aspect of the lung, the developmental processes to be addressed will deal with morphology only.

1.2 OVERVIEW OF NORMAL LUNG DEVELOPMENT

1.2.1 PRENATAL GROWTH AND DEVELOPMENT IN HUMANS

Intrauterine lung development is subdivided into 5 phases (Wigglesworth, 1987), namely the embryonic phase, the pseudoglandular phase, the canalicular phase, the terminal sac phase and the alveolar stage.

1.2.1.1 Embryonic phase

In man the embryonic phase includes events during the first 5 to 7 weeks after conception. The entire epithelial structure of the lung arises as a pouch from the primordial foregut and can be recognized about 22 to 26 days after fertilization (Engel, 1953). The single lung bud branches into primitive right and left lungs a few days later. By week 6 or 7, through a combination of monopodial and irregular dichotomous branching, ten principal branches can be discerned on the right and eight on the left (Murray, 1986).

1.2.1.2 Pseudoglandular phase

This period occurs from 7 to 17 weeks of gestation, during which time the lower

conducting airways are formed (Farrell, 1982). This phase is characterized by the presence of low columnar epithelial tubules surrounded by condensations of mesenchyme within a loose mesenchymal stroma (Wigglesworth, 1987). These tubules subsequently branch within the enclosing mesenchyme to form the gas-conducting portion of the lung by the end of the 16th postconceptional week (Bucher and Reid, 1961). Snyder and associates (1985) have reported that cells lining the terminal regions of the branching ducts consist of columnar epithelium, with a continuous prominent basement membrane to which a layer of fibroblasts is apposed. Collagen synthesis is a prerequisite for airway branching during lung development (Spooner and Faubian, 1980).

1.2.1.3 Canalicular phase

In the human this period occurs from 17 to 24 weeks of gestation (Wigglesworth, 1987). The tubules become dilated and the peripheral epithelium thins out to a cuboidal form (Wigglesworth, 1987). There is a reduction in the relative amount of connective tissue by an increase in the number of capillaries which become intimately associated with the epithelial layer of the tubules or ducts (Snyder et al, 1985). A prominent developmental process during this period is the formation of structures which will ultimately serve as respiratory bronchioles. Elastin first appears within the airways (Wigglesworth, 1987).

1.2.1.4 Terminal Sac or Saccular phase

This period proceeds from 24 weeks to 32 weeks (Wigglesworth, 1987). This stage is characterized by the differentiation of the lining epithelium into type I and type

II pneumocytes, with consequent appearance of surfactant within the airways (Wigglesworth, 1987). There is a progressive development of the blood-air barriers together with an increase in elastic tissue content within the airways and at the tips of the developing alveolar septa (Emery, 1969). The early presence of elastin at the tip of the septal ridges suggests that it may play a role in septal formation (Burri, 1974). Alveoli can be recognized from 32 weeks on and up to one-third of the adult alveolar number may be present by term, i.e. 40 weeks (Hislop et al, 1986; Langston et al, 1984).

1.2.1.5 Alveolar stage

This period occurs from 32 weeks to term (Wigglesworth, 1987). During this phase immature alveoli forms with a double capillary network (Dornan and Meban, 1985). At birth up to 65% of the alveoli are formed (Dornan and Meban, 1985). In addition to acini formation there is a marked reduction in the amount of interstitial tissue (Murray, 1986). Beginning about 2 days before birth, the rate of tracheal fluid production progressively decreases (Kitterman, 1984) and apparently ceases by the time of birth. There is evidence that lung fluid secretion is important for fetal lung growth (Alcorn et al, 1977).

1.2.2 POSTNATAL GROWTH AND DEVELOPMENT IN HUMANS

For years the postnatal growth of the lung has been a matter of controversy. Various studies have proven that development of the mammalian lung is not yet completed at birth and that new alveoli are formed postnatally (Emery and Wicock, 1966; Reid, 1967). In man, approximately 20 million alveoli are present at birth and

increases more than 10-fold (300 x 10^6) over the first 6 to 10 years of life (Brody and Vaccaro, 1979). There is, however, no change in the number of conducting airways (Dunnill, 1962), although some studies have found evidence of the transformation of conducting airways into respiratory bronchioles (Burri, 1974).

The neonatal lung undergoes remarkable immediate functional transformations so that by the end of the first few minutes of life, it is serving as an adequate organ of gas exchange. At birth, the basic formation of cartilaginous airways is complete, though the number of generations of conducting airways decrease through the conversion of alveolarization of a few non-respiratory bronchioles into respiratory bronchioles (Murray, 1986).

Both arteries and veins increase enormously in number within the lobules during the first decade of life, and their development accompanies the formation of new respiratory bronchioles, alveolar ducts and alveoli (Murray, 1986). The multiplication of both alveoli and arteries slows down after 5 years (Murray, 1986), but Thurlbeck (1978) and Dunnill (1962) have found a doubling in the number of alveoli present in humans at birth during the first 8 to 10 years of life, with little or no alveolarization occurring after this time. Alveolar size continues to increase until growth of the chest wall ceases within attainment of adult thoracic size (Farrell, 1982).

1.3 PRENATAL GROWTH AND DEVELOPMENT IN ANIMAL MODELS

Morphological studies in humans and animals have led to the recognition of 3 stages of prenatal pulmonary development (Fukuda et al, 1983), unlike the 5 phases mentioned by Wigglesworth (1987). These stages are: the pseudoglandular stage, the canalicular stage, and the saccular (alveolar) stage. The following table, adapted from Meyrick and Reid (1977), and Boyden (1976), presents the approximate timing of the abovementioned stages in various species.

STAGE	RAT	RABBIT	SHEEP	MAN
PSEUDO-GLANDULAR	0-18 days	0-24 days	0-14 weeks	0-16 weeks
CANALICULAR	19-20 days	24-27 days	14-17 weeks	16-24 weeks
SACCULAR	21 →	27 →	17 →	24 →
TERM GESTATION	22 days	31 days	21 weeks (148 days)	40 weeks

Table 1.1 Comparison of gestation periods of various species

The growth and development of the fetal lung of the rat occurs over a much shorter time than that of humans, with profound changes occurring in its morphology over periods as short as 24 hours. Although the short gestational period simplifies

developmental studies, it also brings about a possible loss of vital developmental detail which may occur within these short time periods.

The developmental processes which occur in the abovementioned stages for the human can be compared to that of the rat, although the time may differ (Farrell, 1982). A noticeable difference between these two species, is the fact that the appearance of canaliculi and saccules occur at 90 to 95% of gestation time in the rat, while the human shows respiratory saccules at 60% of term (Meyrick and Reid, 1977).

1.3.1 PSEUDOGLANDULAR STAGE

The epithelial cells are simple columnar during early gestation and show few signs of organelle differentiation. The period of highest cellular proliferation is during the pseudoglandular stage (O'Hare and Townes, 1970). In rats the bronchial buds continue to divide and subdivide by asymmetric dichotomy, until the bronchial development is complete by 18 days gestation (Bucher, 1961).

1.3.2 CANALICULAR STAGE

During this stage proliferation of the mesenchyme occurs together with the development of a rich blood supply within it (Thurlbeck, 1978). Flattening of the epithelium lining the airways also occurs by it becoming irregularly thinned with cellular continuity only being maintained at their bases (Thurlbeck, 1978). Differentiation of the epithelium into type I and type II pneumocytes also occur, together with the appearance of lamellar inclusions within the type II pneumocytes

(Campiche et al, 1963). Synthesis of pulmonary surfactant occurs in the type II pneumocyte and the lamellar inclusions are the intracellular deposits of surfactant (Chevalier and Collet, 1972). The invasion of capillaries into the alveolar zones of the developing lung is the critical change in the canalicular stage (Burri and Weibel, 1977).

1.3.3 TERMINAL SAC STAGE

During this stage, surfactant production continues with further differentiation of the alveolar lining epithelium (Thurlbeck, 1978). The rate of cell proliferation decreases towards the end of gestation and lasts until the second day after birth in rats, with a subsequent increase by day 3 (O'Hare and Townes, 1970). At birth, the neonatal rat has $\pm 70 \times 10^6$ cells per lung, with the number increasing with age until maturity (Thurlbeck, 1978). Lung growth is mainly due to an increase in cell number (Brumley et al, 1967). At this stage in fetal sheep, alveolar septa are well developed with elastin occurring at the tips of the alveolar septa (Fukuda et al, 1983). In the rat and mouse, however, there are no alveoli present in the walls of the terminal sacs (Thurlbeck, 1978). In the rat capillary development precedes alveolar septa formation (Burri, 1974), while in the fetal lungs of sheep, capillarization follows the formation of primordia of alveolar septa (Fukuda et al, 1983). In the rat lung, saccules with thick septa become more prominent, thus increasing the lung's internal surface area (Farrell, 1982). In sheep, 'fetal pulmonary fluid' production decrease in the last month of gestation, and ceases just before birth (Kitterman, 1984). 'Fetal pulmonary fluid' also fills the potential airspaces in fetal rat lungs and is expelled with the onset of labour (Bland et al,

1978) and by the process of vaginal delivery (Karlberg et al, 1962).

A number of variables may influence the morphometric nature and period of lung maturation. In studies done on the rabbit (Kotas and Avery, 1980), fetal gender play a role in the airspace index. Relative dimensions of lung tissue and potential air space tend to increase more in females than in male fetuses. This is in accord with clinical data which implies a higher incidence of severe hyaline membrane disease in male infants (Farrell, 1982). Hyaline membrane disease is characterized by congested lungs with collapsed peripheral air- spaces and dilated respiratory bronchioles, in which the respiratory epithelium is replaced by an amorphous lining membrane (Wigglesworth, 1978). Studies of rabbits (Kikkawa et al, 1971) and sheep (Howatt et al, 1965) have shown that lobar differences are present during fetal lung development. It appears that the upper lobes mature at a faster rate than the lower lobes. There is also a great deal of evidence that fetal lung growth is markedly influenced by a variety of physical factors (Liggins and Kitterman, 1981). Table 1.2 lists the physical factors necessary for normal fetal lung growth, which had been determined experimentally (Kitterman, 1984).

- Adequate intra-thoracic space
- Adequate intra-uterine space (i.e. sufficient amniotic fluid)
- Fetal breathing movements of normal incidence and intensity
- 'Normal balance' of fluid volume and pressure within the trachea and potential airspaces

Table 1.2 Physical factors necessary for normal lung growth.

All the abovementioned variables need to be taken into account when studying lung development during the prenatal period.

1.4 POSTNATAL GROWTH AND DEVELOPMENT IN RAT LUNGS

The lung of the newborn rat is an immature organ which undergoes major postnatal morphogenic changes during the first 21 days of life. This involves expansion of the airspaces, formation of alveolar septa, thinning of the interstitium and remodelling of the capillary network (Burri, 1974). These findings have solved the problem regarding the presence or absence of alveoli at birth. It has since been well documented that in most mammalian species, new alveoli are formed postnatally (Burri et al, 1974) and that the newborn lung can therefore not be seen as a miniature of the adult lung (Burri and Weibel, 1971; Davies and Reid, 1970).

The degree of development of the lung at birth varies widely. Rats and mice have lungs with no alveoli and no alveolar ducts at birth; kittens, calves and humans have few alveoli, whereas the lungs of lambs are quite well developed (Engel, 1953). In rats, gas exchange occurs in smooth walled channels and saccules, the prospective alveolar ducts and alveolar sacs (Burri, 1974).

The postnatal development of the rat lung occurs in 3 phases (Burri et al, 1974).

1.4.1 PHASE 1

During this period there is little lung tissue added (Burri et al, 1974). Lung enlargement is mostly due to an expansion of airspace volume by 87% (Kauffman et al, 1974). The rate of lung volume increases less than the body weight, so that the ratio of the lung volume to body weight decreases (Thurlbeck, 1978). The walls of the primary saccules present, have the characteristic feature of a double capillary network (Burri, 1974; Short, 1951), with thick cellular interstitium consisting of 3 to 4 loosely organized layers of cells (Thurlbeck, 1978). The primary saccules contain relatively large amounts of connective tissue, and are lined by type I and type II pneumocytes. The type II pneumocytes forms a high proportion of the alveolar surface in fetal lungs compared to adult lungs (Thurlbeck, 1978).

1.4.2 PHASE 2

This period of lung growth has been called the phase of 'tissue proliferation' characterized by the subdivision of the primary saccule by secondary crests, and the formation of definitive alveoli (Thurlbeck, 1978). The septa present at birth are primary septa, while those appearing after birth are secondary septa. Due to the process of septation, alveolar surface area rapidly enlarges (Weibel, 1967). The outgrowth of secondary alveolar septa is characterized by differential cell proliferation of 2 distinct interstitial cell populations, viz. myofibroblasts and lipofibroblasts (Brody and Kaplan, 1983), present in the septal buds (Kauffman et al, 1974). Also present in the septa are elastic fibres which apparently play a central role in alveolar development (Emery, 1969). There is a marked increase in the total number of endothelial cells which can be related to the proliferation of

new capillaries in the formation of secondary septa (Kauffman et al, 1974). Although capillary endothelial nuclei number reached a plateau after day 13, the capillary surface area continued to increase steadily. Proliferation of type II pneumocytes peaked on day 7, while type I pneumocytes showed its fastest increase in number between day 7 and day 10 after birth (Kauffman et al, 1974).

1.4.3 PHASE 3

This period is also termed the phase of 'equilibrated growth' (Thurlbeck, 1978). It is a period characterized by a slowing in the increase of lung volume and the continuing addition of new alveoli. Due to the decline in cellular proliferation and continued formation of alveoli, the interstitium undergoes thinning together with the thinning of the mean blood-air barrier thickness (Kauffman et al, 1974). The secondary septa lengthen and only a single capillary layer is present in the walls of the airspaces (Thurlbeck, 1978). Although there are only single layers of capillaries in the septa, capillary surface area continue to increase (Kauffman et al, 1974).

A fourth phase of simple expansion exists in most species, and occur when alveolar multiplication stops (Thurlbeck, 1978). It is not clear whether alveoli continue to multiply throughout the life of the rat, since continued somatic growth and growth of the lung is characteristic in this species (Short, 1952), or whether alveolar multiplication is complete by 10 weeks of age (Buhain, 1973). The lung has a simple appearance to that of the adult rat, with the alveolar walls being thin, less cellular, with few apparent interstitial cells.

In this study, specific attention was focused on the postnatal alveolar development in the rat lung, with emphasis on the process of alveolarization. Therefore it is deemed appropriate to take an indepth view into the postnatal formation of alveoli.

1.5 POSTNATAL FORMATION OF ALVEOLI

1.5.1 POSTULATIONS FOR POSTNATAL ALVEOLAR FORMATION

Various processes have been postulated for the postnatal formation of new alveoli in the mammalian lung. These include:

1. Outgrowth of tubular spouts at the end of the bronchiolar tree (Burri, 1974; Willson, 1922).
2. Centripetal partitioning of the airspaces, starting from the most peripheral parts (Burri, 1974).
3. Splitting of the alveolar walls by penetration of air (Ham and Baldwin, 1941).
4. Centripetal differentiation of new alveoli by outpouching on the walls of respiratory and terminal bronchioles (Boyden and Tompsett, 1961; 1965).

There has also been a debate about the period over which alveolarization occurs. In human lung, alveolar formation starts at 7½ months of gestation (Loosli and Potter, 1959) and lasts up to the age of 10 years (Thurlbeck, 1978), when 300 x 10^6 alveoli are present in the lung, which is similar to the amount of alveoli found in adult man. In the rat, the vast majority of alveoli form between the 4th and 13th day after birth (Burri et al, 1974).

The primary septa of newborn rat pups showed a difference in structure when compared to the interalveolar septa of adult lungs. The primary septa contain a double capillary network and relative vascularisation of the septa is lowest on day 4 after birth (Burri et al, 1974). The septa of a newborn rat lung are thick with little folding or branching, and contain a relatively large central layer of connective tissue. Lipofibroblasts are abundant within the interstitium and occur in groups at the septal junctions (Burri, 1974). Blood-air barriers which are present in the newborn lungs have the same structure as those found in the older animals, and are nearly as thin in diameter. The formation of a functional blood-air barrier involves the attenuation of the type I pneumocyte (Mercurio and Rhobin, 1976) and the progressive approximation of the parenchyma capillaries to the epithelial layer, by the reduction of interstitial tissue (Burri and Weibel, 1977).

1.5.2 PROCESS OF ALVEOLAR FORMATION IN RATS

The actual process of alveolar formation within the rat lung can be summarized under the following headings:

1.5.2.1 Formation and growth of secondary septa

1.5.2.2 Capillarisation of the secondary septa

1.5.2.3 Maturation of the immature septa and capillary remodelling

1.5.2.1 Formation and growth of secondary septa

This process starts peripherally on the primary septa in the form of humps bulging into the airspaces of the saccules and prospective alveolar ducts. These humps contain an interstitial skeleton of fibroblasts, collagen fibrils, a small bundle of

elastic tissue and a capillary loop. The thin epithelial sheet which covers these protrusions, are not primarily involved in secondary septal formation (Kauffman et al, 1974), where septal formation is due to the growth of the mesenchymal derivatives, namely endothelium and fibroblasts (Burri, 1974). The formation of secondary septa could be induced by the mechanical tension of the mature elastic network within the lung, which in turn affect the rate, amount and direction of fibrous development within the lung (Stearns, 1939).

By the 7th day after birth, the primary septa assume a zig-zag pattern, due to the formation of slender crests which arise from the primary septa. These crests are relatively wide at the base and become narrowed towards the edge, and corresponds with the humps which are observed on day 4 after birth. By the 13 day, septation had progressed due to the lengthening of the secondary septa, and the formation of additional secondary crests on the primary septa. Some of the primary septa have the single capillary system together with the connective tissue layer and the secondary crests (both young and older ones) on either side. The elastic fibres are well developed and located around the mouth of the developing alveolus. Expansion and thinning of the secondary septa are found on day 21, with the secondary septa having the same morphological structure as the primary septa. The stroma becomes reduced, with less tissue content at the septal junctions and the fibroblasts appearing smaller and less numerous (Brody and Kaplan, 1983).

No respiratory bronchioles are present at birth. Alveoli are also formed by the transformation of conducting airways into respiratory airways. Boyden and

Tompsett (1961; 1965) found a quantitative decrease in the volume density of total bronchial and bronchiolar laminar, thus supporting the fact that alveoli are formed by the transformation of conducting airways (Murray, 1986). In the distal parts of the conducting airways, the cuboidal epithelium become thinned out and capillary loops become apposed to the epithelium, thus forming a blood-air barrier, typical for gas exchange (Murray, 1986).

1.5.2.2 Capillarisation of the secondary septa

The secondary septa resemble the primary septa by possessing a double capillary system. The secondary crests contain a single apical capillary loop, with the capillaries on either side tapering towards the apex as the crests grow higher. Elastic tissue are present just below the capillary loops and therefore could affect the length of the capillaries along the secondary septa. Capillary formation within the secondary septa could be due to capillary loops being lifted up from the primary septa along with active septal growth by elongation. At the tip of the secondary septa, the capillaries may be narrowed or closed, with unidentified cells enwraping the capillaries with their cytoplasmic extensions. Due to the immature nature of the septa, as compared to the alveolar septa in the adult, these septa will have to undergo capillary remodelling (Burri et al, 1974).

1.5.2.3 Maturation of septa and capillary remodelling

Of the several concepts that have been postulated, the following 2 seem the most probable to occur:

1.5.2.3.1 Capillary fusion

In immature septa, the two lumina of capillaries which are situated on the left and right side of the septa, are only separated by a single endothelial cell. Since capillary fusion cannot easily be proven, embryonal morphogenesis of the lung makes this concept more plausible. As the lung develops, the capillary networks are progressively brought together, after being separated by mesenchymal tissue during the early postnatal stages. Due to expansion of connective tissue within the septa, newly formed capillaries within the capillary network are required to fuse in order to accommodate for the expansion (Burri, 1974).

1.5.2.3.2 Excavation principle

From day 4 to day 21, the cellularity of the interstitial compartment is reduced from 83,6% to 63%, while the extracellular compartments doubled in size (Brody and Kaplan, 1983). Reduction of the tissue occur in the central layer as well as between the capillaries, thus resulting in the thinning effect of the septa. The capillary segments also lengthen, thus causing a widening of the capillary network. The fraction of interstitium in the septal tissue stabilize at around 46% during the adult stage (Haies et al, 1981). Figure 1.2 gives a graphical representation of the volumetric composition of the parenchymal septa at day 4 and day 21.

During septal formation, the fibroblasts seen at birth undergo major structural and functional changes. The fibroblasts occurring at the tip of the developing septa appear to be involved in connective tissue synthesis and secretion (Gabbiani et al, 1976), as well as the biosynthesis of elastin. Elastogenesis has been associated with

two morphologic components (Greenlee and Ross, 1967). Microfibrils appear as aggregates within the cell and form an amorphous component which gradually encompasses the microfibril mesh, thus giving rise to the mature elastic fibre (Vaccaro and Brody, 1978). Collet and Des Biens (1974) observed elastogenesis in alveolar saccules of prenatal rat lungs which was associated with these fibroblasts. Elastin seem to be a prerequisite for secondary septal development (Brody and Vaccaro, 1979). Vaccaro and Brody (1978) observed the myofibroblast to be responsible for interstitial elastogenesis.

According to Vaccaro and Brody (1978), the general process of alveolar formation can be associated with two major features, namely the differentiation of the interstitial fibroblast, and the role that the interstitial fibroblast plays in septal elastogenesis.

Two distinct populations of fibroblasts, myofibroblasts or nonlipid interstitial cells and lipofibroblasts or lipid interstitial cells, have been associated with alveolar formation (Brody and Kaplan, 1983). The second type of fibroblast is found at the base of the developing septa. These contain neutral lipids which occur as droplets within the cell (Brody and Kaplan, 1983). The source, role and fate of the lipids are unclear, but they appear in great abundance during the period of alveolarization. Kauffman et al (1974) has shown that these lipofibroblasts increase their DNA synthesis during septal formation. The role of the lipofibroblast in lung development is not clear, but they do not serve as intermediary cells in the process of differentiation of mesenchymal cells to myofibroblasts, since these cells do not

contain abundant intracytoplasmic protein synthetic organelles.

1.6 CYTOLOGY OF THE ALVEOLAR EPITHELIUM

The lung is a complex, heterogenous organ, consisting of approximately 40 different cell types (Sorokin, 1967). Lung parenchyma of the adult rat contain predominantly five cell types, viz. type I and type II pneumocytes, endothelial cells, alveolar macrophages and interstitial cells. Type III epithelial cells are also found occasionally (Farrell, 1982).

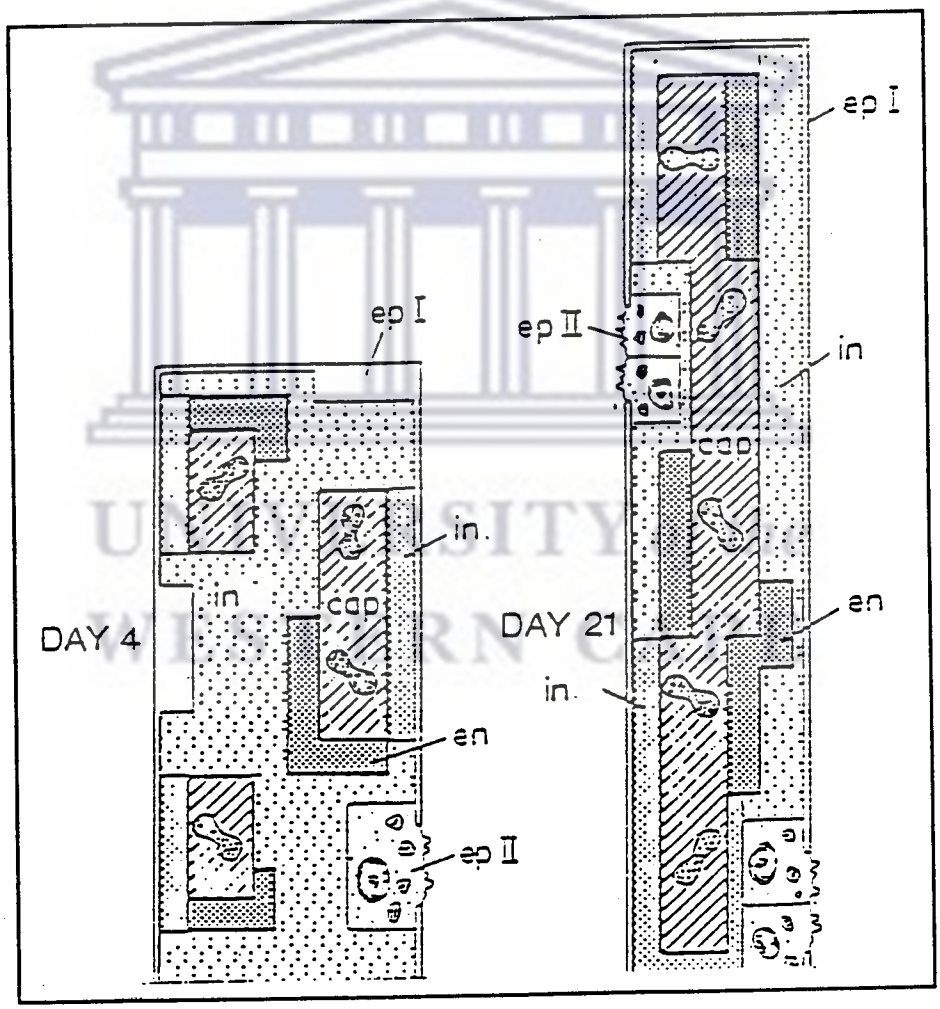

Figure 1.2 Graphical representation of the volumetric composition of day 4 and day 21 parenchymal septa. Adapted from Vidić and Burri (1983). Abbreviations: epI=type I; epII=type II; in=interstitium; en=endothlium; cap=capillary.

Brody and Vaccaro (1974) have distinguished two types of interstitial cells, viz. lipofibroblast and the myofibroblast (Meyrick and Reid, 1977). Table 1.3 depicts a relative distribution of these cells as determined by Weibel and Gil (1977) within the adult rat lung.

	% Cell Distribution
Type I epithelial cells	10
Type II epithelial cells	12
Endothelial cells	40
Alveolar macrophages	5
Interstitial cells	33

Table 1.3 Cell types in the lung parenchyma of the adult rat.

The alveolar epithelium, which covers most of the lung's internal surface area, consist mainly of two types of cells after the maturation process. These cells are the type I and type II pneumocytes (Vidic and Burri, 1983). Due to the nature of this study, more attention will be focused on the characteristics of the various cell types found in the alveolar septa.

1.6.1 TYPE I PNEUMOCYTES

In the lung of the adult rat these alveolar cells cover 97% of the alveolar surface

and are slightly less in number than the type II pneumocytes (Weibel et al, 1976). Gradual cellular differentiation leads to a change of columnar pseudostratified epithelium into mature type I and type II cells. This process involves a change in cell shape, first to cuboidal then highly flattened (Mercurio and Rhobin, 1976). The cytoplasm of these cells is attenuated with mitochondria and endoplasmic reticulum seen in the perinuclear area, with the nucleus being located centrally (Atwal and Sweeney, 1971). As the height of these cells decrease, the cellular junctions between adjacent cells become more convoluted. Due to the thin nature of these cells and their close proximity to the capillary endothelial cells (forming the blood-air barrier), they are ideally suited for gas exchange. Type I cells, because of their topologic properties, cannot divide (Weibel, 1974) and must therefore be compensated for by the rapid differentiation of the type II cells (Evans et al, 1978).

1.6.2 TYPE II PNEUMOCYTES

These cells are also referred to as granular pneumocytes, septal or great alveolar cells (Macklin, 1954). They are recognized by their characteristic lamellar inclusion bodies. They also contain numerous mitochondria, endoplasmic reticulum, polyribosomes and Golgi apparatus, thus suggesting the capability of high metabolic activity (Farrell, 1982). The type II cells serve many important functions within the lung, namely:

1. the synthesis and secretion of pulmonary surface-active material, surfactant (Chevalier and Collet, 1972).
2. Serve as progenitor cells of type I cells and proliferate in order to re-establish a continuous epithelium after damage to type I cells (Adamson and

Bowden, 1974; Evans et al, 1975).

3. Hydrogen peroxide production (Kinnula et al, 1991).
4. Synthesis of extracellular matrix (Lee et al, 1994).
5. Transport of Glutathione, an antioxidant that protects the lung against oxidative injury (Bai et al, 1994).
6. Uptake and secretion of surfactant lipids (dipalmitoylphosphatidylcholine) as a means of maintaining a balance between rate of surfactant production and rate of removal (Griese et al, 1991).
7. Intracellular protein transport (Massaro and Massaro, 1972).
8. Responds to some lung injuries by becoming more resistant to subsequent injury (Mason et al, 1977).
9. Secretion of substances other than surface-active material, for example lysosomal enzymes (Mason et al, 1977).
10. Control of the electrolyte composition of the alveolar subphase, that is the thin aqueous layer between the alveolar epithelial cells and the surface film (Mason et al, 1977).

The lamellar bodies contain a variety of lysosomal enzymes which may participate in the clearance and degradation of pulmonary secretions, cellular debris or inhaled material (Mason and Williams, 1977). Phosphatidic acid phosphohydrolase, an indicator of lung maturity, has also been found in the lamellar bodies (Spitzer et al, 1975). This specific enzyme tends to increase in the fetal lung during normal lung maturation (Schultz et al, 1974). Alkaline phosphatase, an enzyme which can be used as an indicator for type II cell rupture, has also been found in these cells (Henderson, 1984).

1.6.3 INTERSTITIAL CELLS

During the period of alveolar proliferation, the interstitial fibroblasts seem to differentiate into two main cell types, viz. myofibroblasts and lipofibroblasts (Brody and Vaccaro, 1979).

The myofibroblast are located at the tips of the developing septa. Each myofibroblast has a nucleus which appears contracted, with the cytoplasm containing rough endoplasmic reticulum, many mitochondria, Golgi apparatus and vacuoles. These are all ultrastructural features which is characteristic of a cell which is engaged in protein synthesis and secretion. The cell extends long cytoplasmic arms which contain fine filaments associated with connective tissue synthesis and secretion (Gabbiani et al, 1972). This cell also contain elastin which is intimately associated with alveolar formation (Brody and Vaccaro, 1979).

The lipofibroblast is found at the bases of developing septa. The functions of these cells are unclear. They contain few organelles and have an extensive accumulation of intercellular lipid. Scattered intracellular droplets appear at birth, but occur in great abundance during the period of alveolarization (Vaccaro and Brody, 1978). These cells do not appear in adult lungs, and must therefore either differentiate into another cell type or degenerate after the process of alveolarization (Vaccaro and Brody, 1978). The other possibility may be that there are substantial numbers of genotypically distinct lipofibroblasts present in the interstitium of the adult lung, even though their lipid-filled phenotype vanishes as the high lipid content of the neonatal rat serum disappears (Maksvytis et al, 1982). The activity of the

lipoprotein lipase found in these cells tend to increase as their lipid content dissipates (Hamosh et al, 1982).

1.7 THE BLOOD-AIR BARRIER

The blood-air barrier of the lung alveolus is the site of gas exchange. The blood-air barrier forms part of the alveolar septum consists of 3 main components, from the direction of air in the alveolus to the blood in the capillary:

1. the type I epithelial cell
2. the fused basal laminas of the closely apposed epithelial and endothelial cells
3. the endothelial cell.

The total thickness of these layers varies from 0,1 to 1,5 μm, thus optimizing the exchange of oxygen and carbon dioxide between the alveoli and the blood, respectively. The pulmonary diffusion capacity of the lung maybe adversely affected should there be any thickening of any of the components constituting the blood-air barrier, thus increasing the distance across which effective oxygen diffusion can occur, namely 2μm (Despopoulos and Silbernagl, 1986).

1.8 THE EFFECTS OF SMOKING AND NICOTINE ON LUNG DEVELOPMENT

By virtue of its function, the lung is continuously being exposed to the environment which often contains pulmonary irritants. Exposure of the lung to factors which interfere with its developmental process may subsequently adversely affect the metabolic, structural and functional aspects of the lung, resulting in a decreased resistance to disease.

Of the many compounds (over 1 000) obtained from cigarette smoke, the alkaloid nicotine has been implicated as the one which produces adverse effects on fetal growth (Murrin et al, 1987). Animal experiments using nicotine instead of cigarette tobacco smoke show similar effects to those resulting from smoke, therefore implicating nicotine as a major causative factor (Becker et al, 1968). Cigarette smoking has been recognized as the most important risk factor for developing chronic obstructive pulmonary disease (COPD), and has also been associated with increased incidence of pulmonary disorders (Saeta et al, 1985). Habitual cigarette smoking is probably the single most important etiologic co-factor in the production of emphysema (Karlinsky and Snider, 1978), a disease characterized by the destruction of alveolar walls (Janoff, 1983). Since nicotine interferes with elastogenesis (Maritz and Woolward, 1992), and other metabolic pathways (Maritz, 1988) as well as with lung cellular development (Maritz and Woolward, 1994), a possibility exists that maternal nicotine exposure could induce a sequence of events which may result in emphysema. Hernandez et al (1966) has described a degree of parenchymal damage in dogs breathing cigarette smoke, an effect which closely resembles the emphysematous lesions of humans suffering from smoke-associated bronchitis. Table 1.4 shows potential mechanisms of smoking-induced emphysema, all of which have some experimental support (Vial, 1986).

Increase elastolytic burden in the lung
Recruitment of elastase-bearing inflammatory cells
Enhanced release of elastase by inflammatory cells
Chemotactic factor-induced elastase release
Cigarette smoke-induced elastase release
Cytotoxicity of cigarette smoke for inflammatory cells
Decreased antiprotease activity in the lung
Direct oxidation of α-1-PI by free radicals in smoke
Oxidation of α-1-PI by oxygen radicals from activated inflammatory cells
Other
Direct interference with elastin synthesis

Table 1.4 Potential mechanisms of smoking-induced emphysema.

Collins et al (1985) has demonstrated that maternal smoking adversely modifies fetal lung growth. Luck and Nau (1984) has shown that maternal smoking results in the accumulation of considerable amounts of nicotine in the fetal blood and the mother's milk. Nicotine is rapidly absorbed by the infant (Greenberg et al, 1984) and accumulates in the respiratory tract after absorption (Szüts et al, 1978), and may therefore have an adverse effect on the lung. Studies associated with maternal tobacco smoking during pregnancy, have shown severe damage to the vessel wall of the umbilical artery, the umbilical vein and vessels of the placental villi (Asmussen, 1979). Studies also clearly demonstrate that pregnant women who smoke have a higher risk of abortions in the first trimester. The children who do

not abort have a higher risk of premature birth, still birth, low birth weight and neonatal death (Asmussen, 1979). Cigarette smoking has also been found to impair placental blood flow in humans, most likely due to the vasoconstrictive effect of nicotine caused by elevated levels of circulating catecholamines (Phillip et al, 1984). It also decreases the plasma membrane fluidity of the alveolar macrophages in rats (Hannan et al, 1989), while increasing alveolar permeability in rabbits (Witten et al, 1985). Nicotine administration to pregnant rats has been shown to reduce birth weight (Weathersbee and Lodge, 1979) and prolong their gestational period by 2 to 4 days (Weathersbee and Lodge, 1979). It has also been shown that nicotine increase corticosteroid levels in pregnant animals, thus affecting cellular division in the fetus, and resulting in the alteration of the development of diverse bodily systems, including the respiratory system (Cryer et al, 1976).

1.9 AIMS OF PROPOSED STUDY

Despite a plethora of research efforts, the physiological mechanisms by which nicotine exerts its effects on the lung's developmental process, is still not fully understood. Most studies pertaining to maternal smoking or nicotine exposure is approached from either a biochemical, structural or functional level. This study deals with the structural aspect of lung development, specifically investigating the morphology of the alveolar septum. Between days 4 and 21 more alveoli formed by the formation and growth of secondary septa (Burri, 1974). Recent studies illustrated that maternal nicotine exposure suppress alveolar formation (Motoyama, 1988). In addition, damage to alveolar septa also occur. The aim of this study is therefore to investigate the effect of maternal nicotine exposure on neonatal rat

lung septal status by:

1. investigating the cellular characteristics of the alveolar septa. This include studies of the type I and type II epithelial cells, endothelial cells and total cellularity. Emphasis will be on the type II cell because of its very important role in maintaining alveolar epithelial integrity and lung modelling (Evans et al, 1975).

2. investigating the capillary density of the septa because it is known that nicotine adversly influence blood vessels (Asmussen, 1979).

This may give more information as to the site in the septa which is most sensitive to the effect of nicotine and thus result in the observed damage to alveolar septa in the lungs of rat pups exposed to nicotine via the placenta during gestation, and mother's milk during lactation.

1.10 TECHNIQUES

In order to investigate the abovementioned aspects, the following techniques were employed:

1. Light microscopy: This technique served to give an overview of the structural changes which had occurred in the control and experimental neonatal rat lungs.
2. Morphometric studies on the various cell types mentioned above.
3. Electron Microscopy: This technique was used to obtain ultrastructural

information pertaining to the various cells as well as the cell organelles.

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CHAPTER 2

THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON THE MORPHOLOGY OF THE INTERALVEOLAR SEPTA AS OBSERVED USING LIGHT MICROSCOPY.

2.1 INTRODUCTION

Cigarette smoking has, and is still being associated with many medical problems, linking it to cardiovascular disease, chronic obstructive pulmonary disease and bronchogenic carcinoma (Hannan et al, 1989). Disease, after all, is the condition in which the normal function of some part or organ of the body is disturbed and react to that disturbance or injury. This therefore includes situations in which cells, tissues or organs are acted upon unfavourably, either by injurious agents in conjunction with environmental circumstances, or by inborn errors acting alone. The sequence of events which follows may be dominated by the direct effects of the injurious agents upon the cell (such as alveolar epithelial cells after exposure to chronic cigarette smoke), or may be a combination of direct effects and the local and general cell and tissue reactions which may be elicited (Yarnell and St. Leger, 1979).

The functional disturbances produced by injury to cells are often reflected by structural changes. Structural changes or damage incurred after exposure to toxic agents may subsequently be followed by alteration or even loss of some normal function. However, disordered function may not necessarily be accompanied by

significant structural changes. Figure 2.1 shows a schematic diagram depicting the events which generally follow after injury to the cell, tissue or organ.

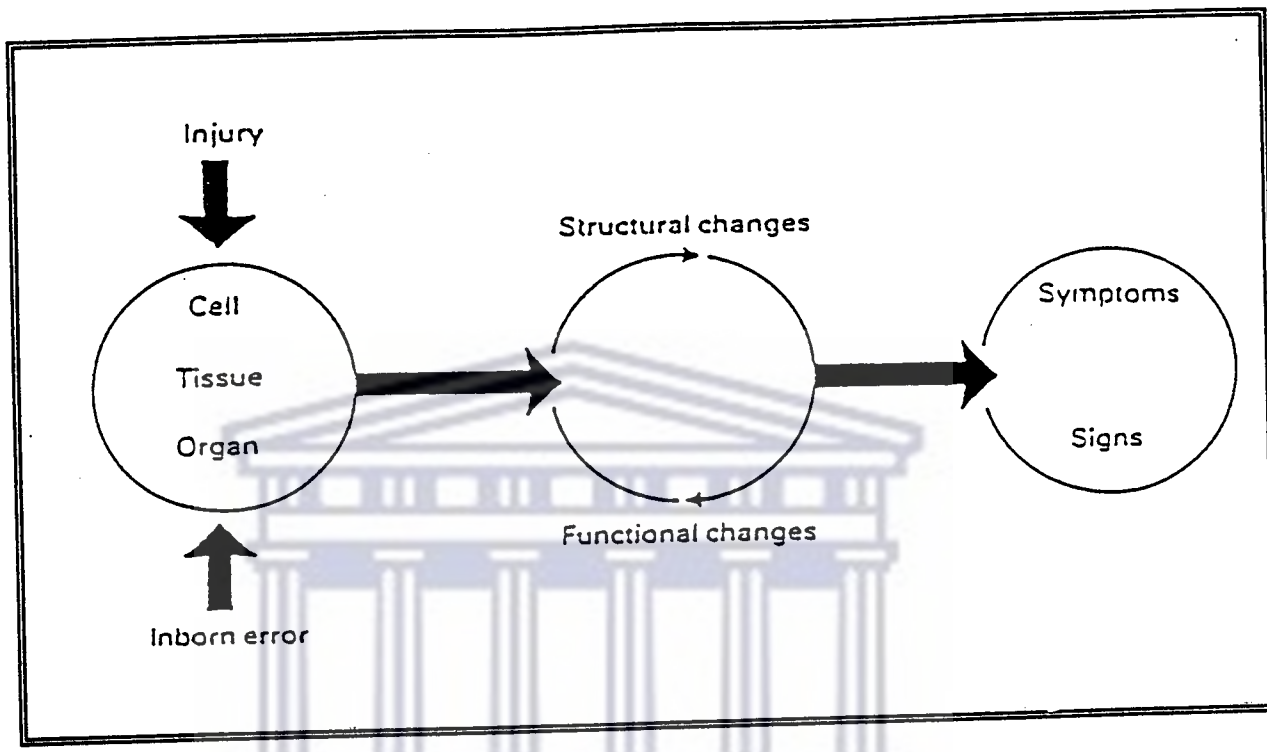

Figure 2.1 Injury or inborn error may lead to functional and then structural disturbances within cells and tissue. Adapted from Woolf, N. (1977).

The multiplication of the functional gas exchange units, namely the alveoli of the rat lung, occurs between day 4 and day 13 after birth (Burri, 1974). Thereafter lung growth involves the enlargement rather than the multiplication of alveoli, increasing the lung gas exchange surface by a factor of 5 (Maksvytis et al, 1981). In a study done by Collins et al (1985), it was shown that maternal cigarette smoking causes fetal lung hypoplasia. The rate of postnatal lung growth is also reduced in children of women who smoke (Tager et al, 1983). Due to decreased growth and development of both the fetal and postnatal lung, the neonate may subsequently be

more susceptible to respiratory disease.

In this chapter, results will be presented of general structural changes which has been observed in the lungs of the group which had been exposed to maternal nicotine during gestation and lactation via the placenta and mother's milk, respectively, compared to the group in which normal lung development occurred.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

White virgin female Wistar rats, weighing between 200 and 250 grams, are used in this study. A stock diet of Epol rat cubes are used to feed the rats throughout the experiment. The animals are fed on a daily basis and supplied with tap water as required. The temperature within the animal room is kept constant at 22°C and a 12-hour day-night cycle is maintained. We maintained our own breeding programme for both control and experimental animals.

Animals are mated overnight and afterwards randomly assigned to control and experimental groups. Those rats assigned to the control group are allowed to develop normally without being given any nicotine treatment, thus creating a measure against which the experimental group could be compared. The rats assigned to the experimental groups are treated with nicotine on a dosage of 1 mg nicotine/kg body wt/day as described by Maritz (1987). Nicotine is administered via subcutaneous injection until the birth of the pups, thereafter the dams are injected intraperitoneally, thus ensuring that nicotine reaches the fetus and suckling

rats only after having been absorbed into the bloodstream. The average length of the gestational period is 22,5 days. Treatment commenced on day 7 of gestation, thus avoiding nicotine interference with blastocyte implantation and initial embryonic growth, and continued throughout gestational period, until the pups are weaned three weeks after birth.

Ten rat pups are randomly selected from ten different litters belonging to the control and experimental groups, respectively. The rat pups are killed by decapitation, 24 hours after the last nicotine injection had been administered to the mother. The various age groups which are examined in this study are 1, 7, 14 and 21 days after birth. The lung tissue is then expeditiously removed from the thoracic cavity and tissue preparation then commences as follows:

2.2.2 LUNG TISSUE EXCISION AND TISSUE PREPARATION

The medial lobe of the right lung is removed and fixed in formalin. The primary fixation process occurs over a period of 6 days. The tissue is then rinsed in running water for 30 minutes before being placed into a labelled plastic cassette. Secondary fixation, dehydration and wax impregnation is done using a Histokinette Type E7326 Tissue Processor and using the following program:

Step no.	Reagent - % composition	Time
1	Buffered formalin	1 hour
2	70 % Alcohol	1,5 hours
3	80 % Alcohol	1,5 hours
4	96 % Alcohol	2 hours
5	96 % Alcohol	2 hours
6	100 % Alcohol	1,5 hours
7	100 % Alcohol	2 hours
8	100 % Alcohol	1,5 hours
9	Xylol/Xylene	1 hour
10	Xylol	2 hours
11	Paraffin Wax	2 hours
12	Paraffin Wax	2 hours

Since it is a 20 hour program, the processing of the tissue is usually done overnight. The tissue is removed the next morning and embedded in paraffin wax at 60 degrees Celcius using a Tissue-Tek II tissue embedding centre. The embedded samples are then placed in an embedding dish and refrigerated at 4 degrees Celcius for 1 hour before cutting.

2.2.3 MICROTOMY

A rotary microtome is used for sectioning the lung tissue. Before sectioning, the angle of the blade is adjusted according to the wax block present in the microtome chuck. Thick sections of 10 μm are first cut to remove excess wax from the block and thus cut down to the tissue sample. On attaining the correct depth, the setting is readjusted to 5 μm, and ribbons of 3 to 4 sections are then cut and floated onto a waterbath of ± 45 degrees Celcius. To enhance the transfer of the ribbon onto a clean, labelled microscopic slide, some alcohol is squirted onto the water, thus straightening out any folds present in the wax sections. The slides are then placed into a 70 degrees Celcius incubator for 1,5 hours in order to remove the wax prior to staining.

2.2.4 STAINING

Mayer's (progressive) formulation, that is the Haematoxylin and Eosin (H & E) stain is utilized as a staining technique for light microscopy (Bancroft and Stevens, 1982).

2.2.4.1 Principle

The oxidative product of haematoxylin, haematein, is used as a natural dye in this staining process. In Mayer's formulation, chemical oxidation by sodium iodate produces the oxidant haematein. In order to increase its affinity for tissue, metallic salts (for example aluminium, potassium, tungsten and iron) are used as mordants to enhance affinity. The stain then interacts with the acidic nuclei by means of van der Waal's forces and forms covalent bonds. Staining of the tissue occurs

progressively, and counterstaining with Eosin is used to stain the cytoplasm. The conversion of the red colour of the nucleus, to its characteristic blue-black colour, is done with an alkaline called Scott's tap water.

2.2.4.2 Reagents

1. Buffered neutral formalin. Dissolve 4 grams sodium dihydrogen phosphate and 6,5 grams disodium hydrogen phosphate in a solution containing 100 ml 40 % formaldehyde and 800 ml distilled water. Using a 1L volumetric flask, make this solution up to 1L using distilled water.
2. Absolute (100 %) alcohol. Use to dilute to various concentrations needed for processing and staining purposes. Distilled water is used for dilution purposes.
3. Mayer's haematoxylin. Dissolve 1 gram of haematoxylin in 1L of distilled water. Add 50 grams ammonia alum and 0,2 grams sodium iodate, heat and stir to dissolve fully. Then add 1 gram citric acid and 50 grams chloral hydrate.
4. Scott's tap water. Dissolve 4 grams potassium bicarbonate and 40 grams magnesium sulphate in 2L distilled water.
5. Eosin. Dissolve 5,0 grams eosin in 1L distilled water.
6. Acid alcohol. Add 1 ml concentrated hydrochloric acid to 99 ml 70 % alcohol.
7. Xylol. Used as supplied by manufacturer.
8. Canada Balsam. Used as supplied by manufacturer.

2.2.4.3 Program for standard H and E staining

The following program is implemented for H and E staining:

Reagent	Time
Xylol	2 minutes
Xylol	2 minutes
100 % alcohol	30 seconds
90 % alcohol	30 seconds
80 % alcohol	30 seconds
70 % alcohol	30 seconds
Running tap water	1 minute
Haematoxylin	8 minutes
Running tap water	30 seconds
Acid alcohol	immerse twice
Running tap water	30 seconds
Scott's tap water	1 minute
Running tap water	30 seconds
Eosin	1 minute
Running tap water	30 seconds
70 % alcohol	10 seconds
80 % alcohol	30 seconds
90 % alcohol	30 seconds
100 % alcohol	30 seconds
100 % alcohol	30 seconds
Xylol	15 seconds
Xylol	15 seconds

Mount in Canada Balsam and allow to set and dry. Canada Balsam has a refractive index of 1.54 when dry.

2.2.4.4 INTERPRETATION OF HAEMATOXYLIN AND EOSIN STAIN

The nuclei in the section stain blue-black, whilst the cytoplasm stains a reddish-pink. For black and white prints used in this section, the nuclei stains black and the cytoplasm stains shades of grey.

Since the prints taken of the neonatal rat lung tissue after H & E staining does not show the connective tissue component clearly, permission was granted by my promoter to utilize prints which had been taken of neonatal rat lungs which had been stained for connective tissue using the PTAH Staining technique. Collagen, cartilage and elastic fibres stains deep brownish red; cytoplasm stains pale pinkish brown and nuclei stains blue (Bancroft and Stevens, 1982).

2.3 RESULTS

2.3.1 MORPHOLOGY OF CONTROL NEONATAL RAT LUNGS

Day 7 lungs (Figure 2.2) show expansion of airspace and formation of secondary crests (alveolar buds) growing into the airspaces. Connective tissue, as depicted in Figure 2.3 can be seen at the growing tips of the secondary septa. A concentration of connective tissue can also be seen around the blood vessels. Connective tissues can be seen near the tips of the secondary septa. The type I cells can be detected by the flattened nuclei which occurs on the alveolar surfaces, as pointed out by the arrow in Figure 2.2.

Day 14 lungs (Figure 2.4) shows a similar pattern of growth as that seen in day 7

neonatal lung. The alveolar space appears more enlarged, with more secondary septa protruding into the expanding alveolar space.

Day 21 neonatal lungs (Figure 2.6) contain very thin septa with large alveolar spaces for gaseous exchange. The type I cells are thinly attenuated across the surface of alveoli, with the capillary lying in close proximity to the epithelial cell, Figure 2.7, thus forming the blood-air barrier across which gaseous exchange occurs. The maturation process appears to be continuing since secondary septa are still visible in the alveolar spaces.

2.3.3 MORPHOLOGY OF THE NICOTINE EXPOSED NEONATAL RAT LUNGS

Day 7 lungs (Figure 2.8) appears to have a larger airspace than those found in the control lungs of the same age group. The degree of growth within these lungs appears slower than that of the control group since the septa persist to maintain its convoluted and thickened appearance. The secondary septa (alveolar buds) are also present, but they appear tapered and stunted in growth. The number of secondary septa present also seem less than those observed in the day 7 control lungs. Small amounts of connective tissue can be seen around the blood vessels, with differences being observed in the quantity present (Figure 2.9). Staining for connective tissue is less intense than in the control lung of rat pups of the same age group (Figure 2.3). Type I cells can again be distinguished by the flattened nuclei which occur along the alveolar surfaces. Breaks in alveolar septa can also be observed, as depicted in Figure 2.8.

Day 14 lungs (Figure 2.10) appear are more expanded and the discrepancy between these lungs and that of control lungs also appears to become smaller. The secondary septa are more numerous than those found in day 7 lungs, with breaks also visible in the septa. The areas where the breaks occur has abnormally large alveolar spaces. The connective tissue content appears similar to that found in day 14 control lungs (Figure 2.11). Figure 2.12 shows the predominant presence of connective tissue at the tips of the secondary septa.

Abnormally shaped alveoli, due to breaks occurring in the septa, are present in the day 21 lungs (Figure 2.13). The septa appear more stretched and thinner than those of the previous age group. Connective tissue can be observed in the septa and around the blood vessels (Figure 2.14). The alveoli appear collapsed (Figure 2.14) in certain areas. On comparing the control and experimental lungs on a gross morphological level, the experimental lungs appear less developed than the control lungs due to the decreased air space and stunted growth of the septa, therefore depriving the experimental lungs from that well defined mature appearance of the adult lung as found in the control lungs.

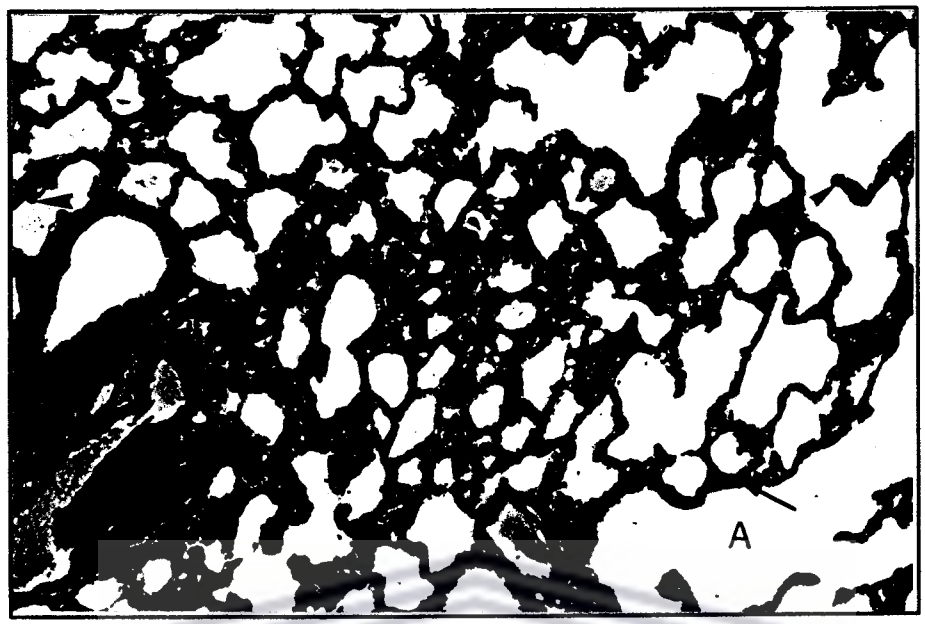

Figure 2.2 An illustration of day 7 control neonatal rat lung tissue showing type II cell (arrow); alveolar bud (small arrowhead); Type I cell (larger arrowhead); A=Alveolus; b=blood vessel. (140X magnification)

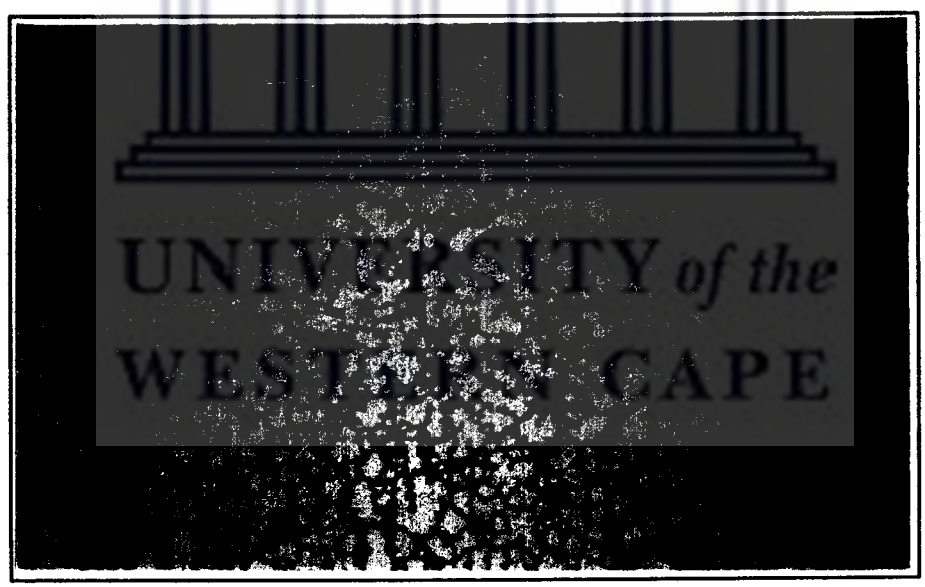

Figure 2.3 An illustration of day 7 control neonatal rat lung tissue. Note the connective tissue at the alveolar septal tips (arrowheads). Type I nucleus (arrow); b=blood vessel; a=alveolus. (45X magnification)

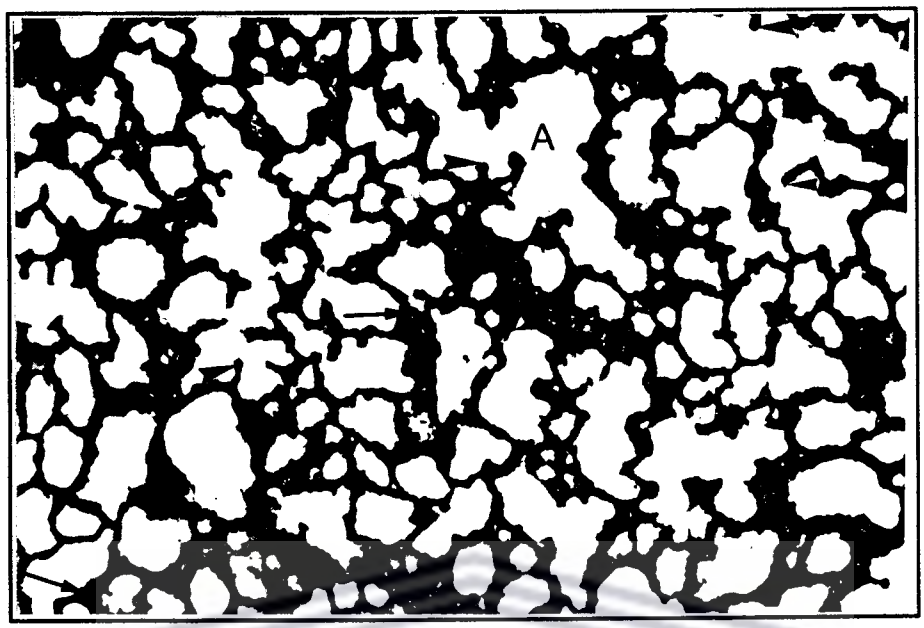

Figure 2.4 An illustration of day 14 control neonatal rat lung tissue showing expanded alveoli (A) and numerous secondary septa (arrowheads). Type II (arrows) found at septal junction. (140X magnification)

Figure 2.5 An illustration of day 14 control neonatal rat lung tissue showing connective tissue at alveolar septal tips (arrowheads). a=alveolus; b= blood vessel. (45X magnification)

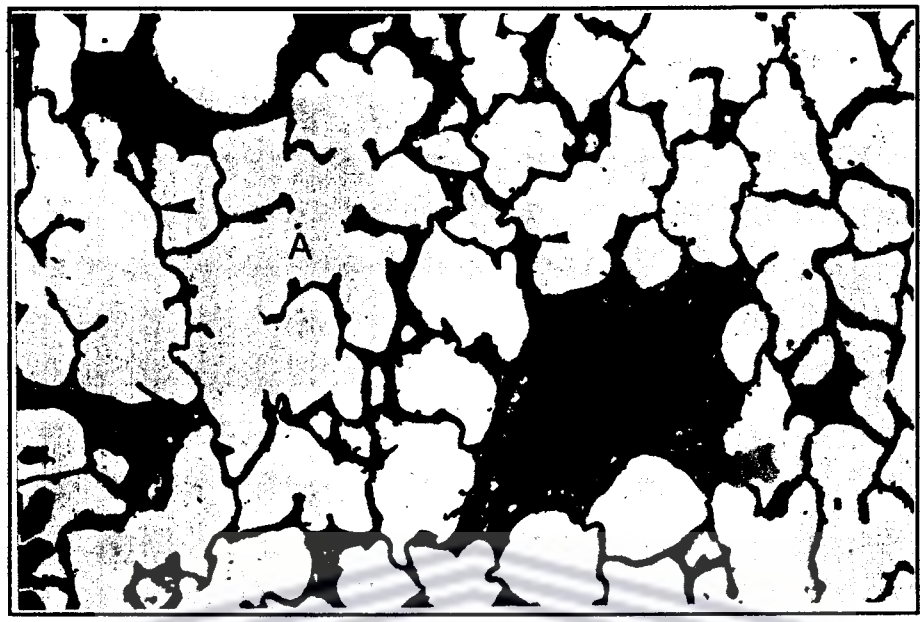

Figure 2.6 Thin septa and large alveoli (A) are visible in the day 21 control neonatal rat lung. Type I nucleus (arrowhead); b=blood vessel. (140X magnification)

Figure 2.7 An illustration of day 21 control neonatal rat lung tissue showing connective tissue (arrowheads) and the expanded alveoli (a). b=blood vessel (450X magnification)

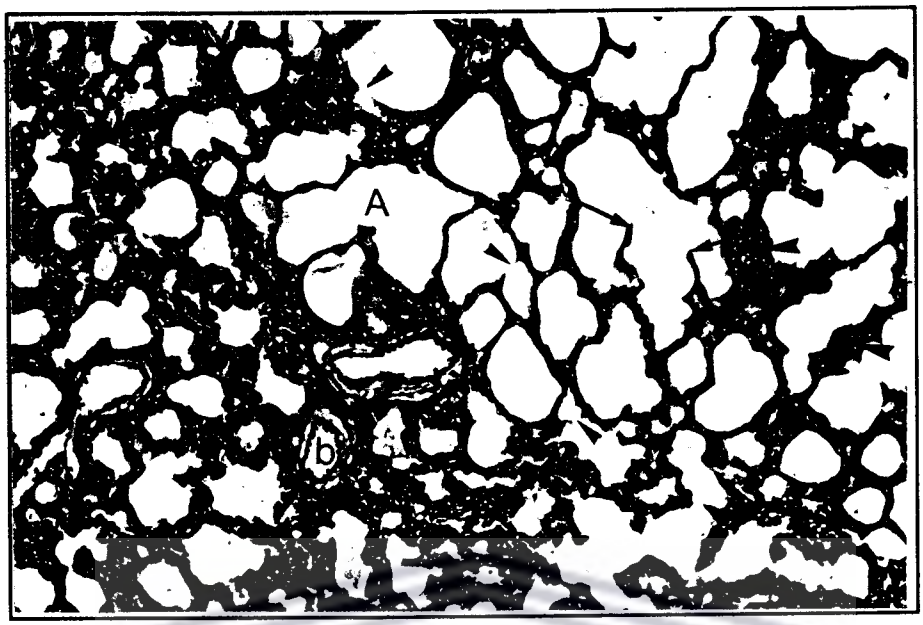

Figure 2.8 Alveolar buds appear tapered (arrows) and septa are thickened (large arrowheads) in day 7 nicotine exposed lung. Breaks (small arrowheads) are visible in the septa. a=alveolus; b=blood vessel. (140X magnification)

Figure 2.9 An illustration of day 7 nicotine exposed neonatal rat lung tissue showing connective tissue in the septa. a=alveolus; b=blood vessel. (40X magnification)

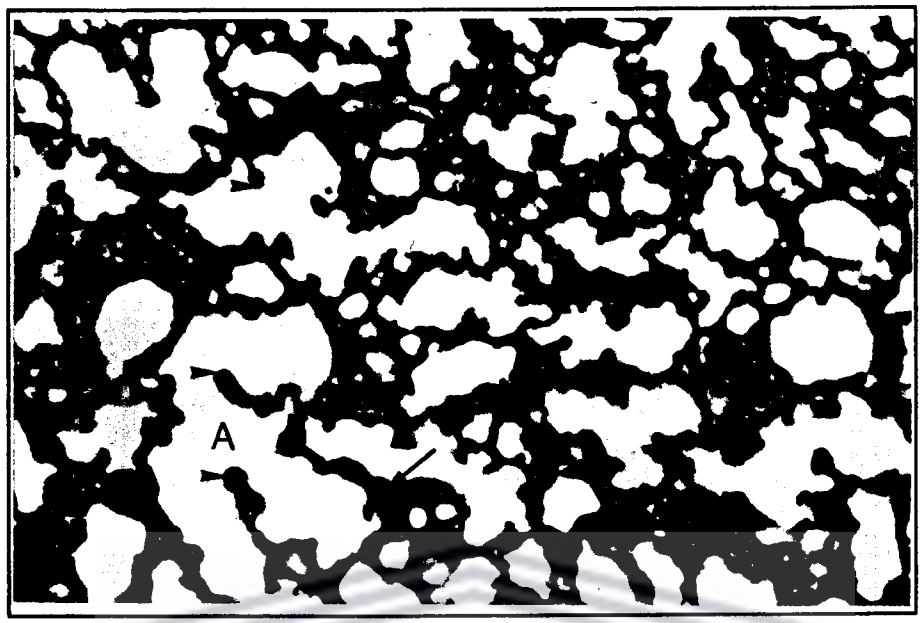

Figure 2.10 Abnormally enlarged alveoli (A) with stunted alveolar buds (arrowheads) are visible in day 14 nicotine exposed rat lung tissue. Type II cell (arrow); b=blood vessel. (140X magnification)

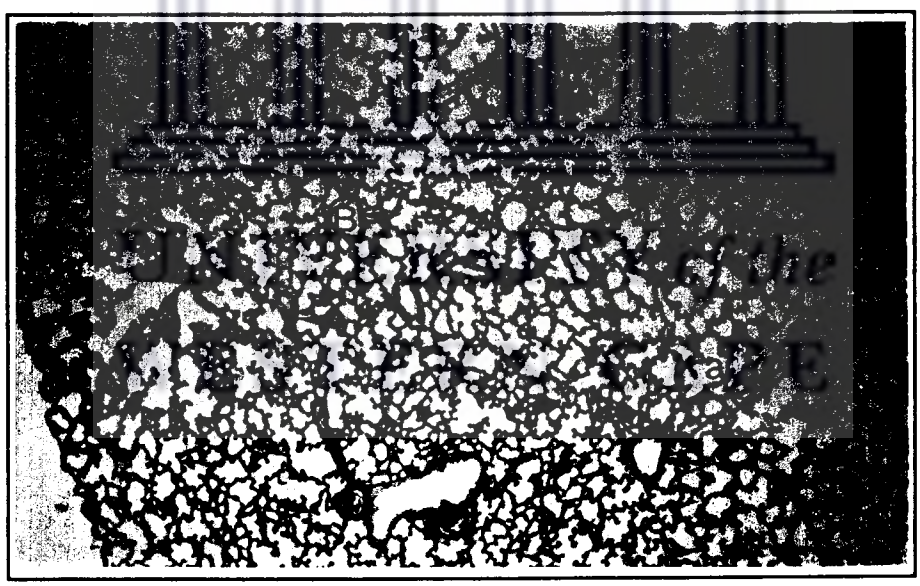

Figure 2.11 An illustration of day 14 nicotine exposed neonatal rat lung tissue showing connective tissue (small arrowhead), especially around the blood vessels (B). a=alveolus; Type I nucleus (large arrowhead) (40X magnification)

Figure 2.12 An illustration of day 14 nicotine exposed neonatal rat lung tissue showing stunted alveolar buds (small arrowheads) and expanded alveoli (a). Type I nucleus (large arrowhead). (110X magnification)

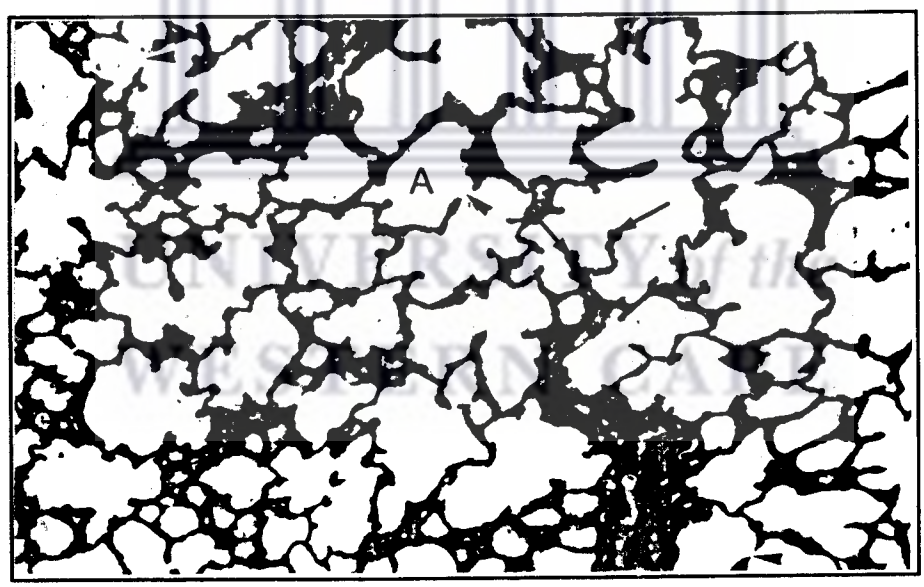

Figure 2.13 An illustration of day 21 nicotine exposed neonatal rat lung tissue showing broken septa (arrowheads), stunted septa (arrows) and enlarged alveoli (A). b=blood vessel. (140X magnification)

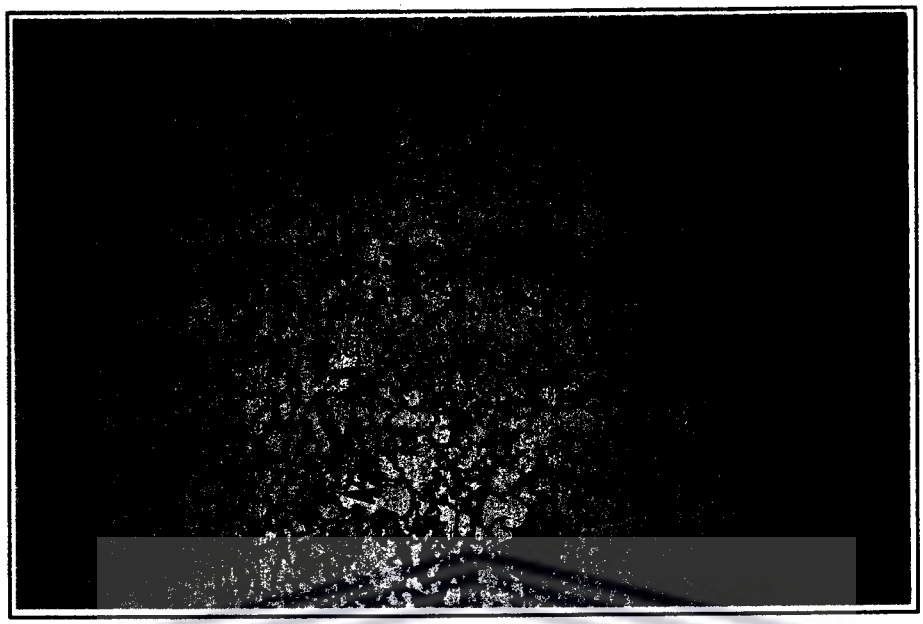

Figure 2.14 An illustration of day 21 nicotine exposed neonatal rat lung showing connective tissue distribution and collapsed alveoli in certain areas (arrowheads). b=blood vessel. (40X magnification)

2.4 DISCUSSION

In order for the lung to be an efficient gas exchanger, uninterrupted development of its alveoli is imperative for the maintenance of blood homeostasis. However, should disruption of the structural and spacial relationship of these functional units occur, it could result in the further damaging effects incurred due to respiratory inefficiency, which in turn affects the homeostatic equilibrium within the body.

The developmental process starts in the fetal stage and is continued postnatally. Any damage therefore incurred during the fetal stage of development will be carried through into the neonatal and adult stages. The damage will subsequently manifest itself by affecting structural aspects within the lung, thus also affecting the functionality the lung.

On comparing the control and nicotine exposed lungs of day 14 and day 21 neonates, (Figure 2.4 and 2.10, and Figure 2.6 and 2.14), it can be seen that the alveoli in the experimental group are enlarged and irregularly shaped. This is similar to the findings of Frasca et al (1974), who exposed dogs to cigarette smoke. They found thickened septa, with an increase in type 11 cell count. In another study done by Vidic et al (1989), a slower pace of secondary septal growth was observed, together with greater cellularity, collagen and LIC cell number. Due to these findings it was suggested that the differences in the developmental patterns of the control and experimental groups could be due to delayed differentiation of interstitial cells and a modified balance between the production and degeneration of stromal proteins within the neonates (Vidic et al,1989).

In a study done on sheep, the sequence of morphological changes were monitored after smoke inhalation (Linares et al, 1989). In the abovementioned study the pulmonary response, in many respects similar to those observed in smoke-inhaled humans, were found to be divided into 4 phases.

The exudative phase characterizes the first 48 hours after injury and is characterized by the exudation (flowing out) of fluids, resulting in interstitial edema and the infiltration of an abundance of inflammatory cells (primarily polymorphonuclear leucocytes). Intracellular edema in type I cells also occurs and the endothelial lining of capillaries and small arteries show loose junctions or interendothelial gaps. This allows for the development of alveolar interstitial edema which cause a decrease in alveolar compliance (Linares et al, 1989).

The degenerative phase (12 to 72 hours after injury) shows swollen or complete disruption of the epithelial lining. Type I cells show distinct necrosis, exposing the alveolar epithelial basement membrane and subsequently rendering the blood-air barrier ineffective. Type II cells also exhibit changes such as dilation of the endoplasmic reticulum, and changes in number and size of the lamellar bodies. Macrophages appear to accumulate at this stage for the resorption of necrotic material (Linares et al, 1989).

The proliferative phase (48 hours to 7 days after injury) is characterized by type II cell hyperplasia and macrophage stabilization. The type II cells form clusters which bulge into the alveolar space, some of which differentiate into type I cells (Linares

et al, 1989).

The reparative phase (more than 4 days after injury) may show a return to normal features of the pulmonary tissue or may develop other abnormalities such as septal scarring or interstitial and intra-alveolar fibrosis. The transformation of type II cells into type I cells is more common, and extensive fibroblast proliferation with collagen being laid down in the interstitium resulting in subsequent interstitial and/or intra-alveolar fibrosis (Linares et al, 1989).

In a recent study done by Lieberman et al (1992) it was demonstrated that cigarette smoking during pregnancy accelerates fetal lung maturation. This accelerated maturity is abnormal, since it alters or impair developmental aspects within the lung which is necessary to maintain pulmonary health after the postnatal period. This accelerated growth can also be compared to the thickened septa observed in dogs which had been exposed to cigarette smoke (Frasca et al,1974). The widespread thickening of septa could be as a result of the toxic effect that nicotine exerts on the alveolar epithelial cells, since thickened septa are also present in the nicotine-exposed rat lungs in the present study. This may therefore have significantly exaggerated the normal propensity of the alveolar epithelial cells in a bid to compensate for the damage and also maintain the epithelial barrier within the alveoli. Distortion of the septa in the nicotine exposed animals could be due to the aggressive proliferation of these cells as well as fibrosis which will in turn contract the pulmonary interstitium to simulate irregular alveolar collapse.

Smoking has also affected developmental aspects such as acute and prolonged impairments of lung functions in the neonates (Woolf, 1977), irreversible fetal cardiovascular damage, that is damage to the walls of the umbilical artery and vein as well as the placental villi (Asmussen, 1979) and fetal growth retardation (Hardy and Mellts, 1972). Smoking has also been associated with the development of emphysema (Auerbach et al, 1972), a disease characterized by destruction of the alveolar walls (Janoff, 1983). Since nicotine interferes with elastogenesis (Maritz and Woolward, 1992) as well as metabolic pathways (Maritz and Burger, 1992), it has consequently been implicated in the pathogenesis of pulmonary emphysema. The lung parenchyma changes observed in a study done on dogs after exposure to cigarette smoke (Auerbach and Hammond, 1967), shows the characteristics of emphysema found in persons who smoke heavily. These changes included rupturing of alveolar septa, fibrous thickening of alveolar septa, and pad-like attachments to alveolar septa.

Invasion of capillaries into the alveolar space is a critical change in the canalicular stage of the developing lung (Burri and Weibel, 1977). Fibroblasts play a role in organizing the relationship between the epithelial cells and the capillary endothelium (Maritz and Thomas, 1994). In the rat lung, capillary development precedes the formation of alveolar septa (Burri, 1974). Interference of nicotine with mesenchymal tissue (Maritz and Woolward, 1992) and its differentiation into fibroblasts (amongst others), may eventually result in the suppression of fibroblast activity in the regulation of capillary formation (Maritz and Thomas, 1994).

An overview of the findings can therefore be summarized as follows:

1. Nicotine exposure affects lung development by slowing down developmental processes, but also accelerating cellular multiplication. This can be observed in the increase in septal thickness in the various age groups.
2. Alveoli appear to be less in number in the nicotine exposed animals, with secondary septa showing stunted growth. Breaks in alveolar septa can also be seen in these lungs, thus giving the alveolar space an enlarged appearance.

The following chapter will deal with morphometric studies done to quantify the various morphological differences described in this chapter. These studies will deal with the following aspects:

3. Cellularity
4. Lipid-containing interstitial cells
5. Type I : Type II cell ratio
6. Capillary density

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CHAPTER 3

INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL LUNG SEPTAL CELLULARITY, TYPE I : TYPE II CELL RATIO, LIPID-CONTAINING INTERSTITIAL CELLS AND CAPILLARY DENSITY.

3.1 INTRODUCTION

The developmental events of early postnatal morphogenesis of the rat lung contribute in different ways to the transformation of an immature lung into a structurally and functionally efficient organ. However, should this developmental process in any way be interfered with, it will adversely influence the functional, metabolic and structural development of the lung, subsequently rendering it less resistant to disease. Due to the functionality of the lung, it is exposed to a continuous assault by a wide variety of air-borne chemicals present in the atmosphere. Some of these chemicals which gain access, by inhalation, to the alveolar zone as well as the capillaries within the alveolar septa of the lung, are toxic agents which will in effect cause acute and/or chronic lung injury.

The smoking of tobacco products has expanded enormously in the 350 years following its rediscovery by white men. What was first believed to be a cure for many diseases, turned out to contribute substantially to the occurrence of chronic pulmonary disorders. Cigarette smoke contains more than 1 000 chemicals, of which nicotine is the primary habit-forming substance (Mennies, 1983). Once inhaled, nicotine is rapidly absorbed into the bloodstream and triggers a number of physiological responses such as elevated

catecholamines, free fatty acids and cortisol levels; increased heart rate and blood pressure; and it also increases platelet stickiness and aggregation (Mennies, 1983). Other physical effects which the chemicals in cigarette smoke has been associated with are chronic obstructive pulmonary disease (COPD), peripheral vascular disease, coronary heart disease, cancers, peptic ulcers, allergic disorders, low birthweight babies, still-births, and sudden infant death syndrome (SIDS) (Mennies, 1983).

3.2 EFFECT OF SMOKING ON THE RESPIRATORY SYSTEM

Three types of diseases are usually classified under the generic term 'chronic obstructive pulmonnary disease' (COPD), viz. chronic bronchitis, pulmonary emphysema and bronchial asthma. A schematic representation of the pathogenesis of COPD is presented in Figure 3.1.

The majority of patients suffering from COPD are cigarette smokers (Crowdy and Sowden, 1975), with changes occurring in the bronchi and lung parenchyma being proportional to the total amount of smoke inhaled. Cigarette smoke inhibits ciliary activity of the bronchial epithelium and the phagocytic activity of the macrophages of the pulmonary alveoli (Dalham and Rylander, 1970). This results in defective clearance of inhaled foreign material, and results in increased incidence of respiratory infection. Pathological changes which have been observed and which are associated with smoking include goblet cell distention, alveolar septal rupture, thickened bronchial epithelium and mucous gland hypertrophy (Auerbach et al, 1967).

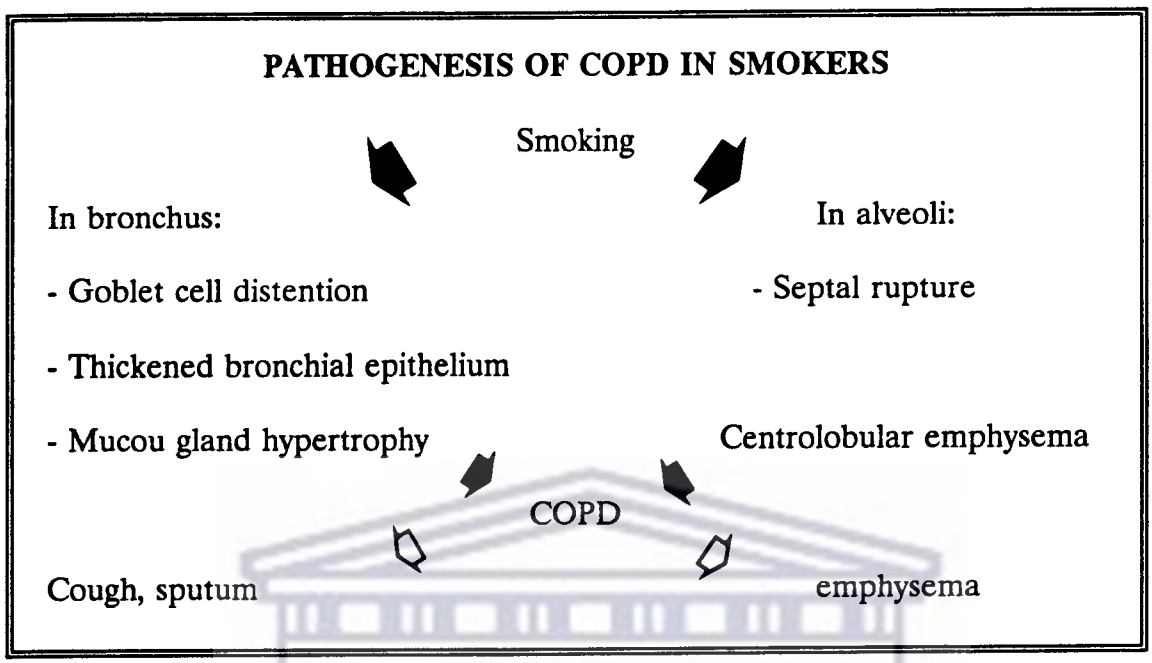

Figure 3.1 A schematic representation of the pathogenesis of COPD. Adapted from Van Lancker (1977).

3.3 SMOKING AND CHILD DEVELOPMENT

Maternal cigarette smoking has been associated with a host of pregnancy complications including increased incidence of spontaneous abortions, prematurity, fetal and neonatal death and fetal growth retardation (Pirani, 1978). Several studies, as depicted in a report by the Surgeon General of the United States (1980) have also suggested that maternal smoking during pregnancy unfavourably affects the child's subsequent growth, intellectual development and behaviour. The mortality in babies of smokers is significantly higher than in babies of nonsmokers for both stillbirths and neonatal deaths (Van Lancker, 1977). Cigarette smoking during pregnancy has been associated with reduced birth weight, and low weight is associated with a wide range of defective immune functions

which may predispose to respiratory infection (Chandra, 1975). It has been suggested that frequent respiratory illnesses in the first years of life in children of smokers may induce structural alterations in their lungs, thus subsequently affecting pulmonary function (Chandra, 1975).

Other findings also suggest that smoking during pregnancy may cause congenital damage to the developing respiratory system by affecting its bronchial tree or the developing lung vasculature (Asmussen, 1979). Altered and impaired fetal lung growth was found in studies done on mothers who smoked during pregnancy. These hypoplastic fetal lungs contained fewer, larger saccules, reduced volume proportion of parenchymal tissue and septal crests, and markedly reduced surface area for gaseous exchange (Collins et al, 1985). The parenchymal changes observed in the experimental lungs showed thinned saccular walls which may result in generalized defective mesenchymal growth or defective growth in capillaries or elastic tissue (Collins et al, 1985). It has been shown in a previous study by Maritz and Woolward (1992) that maternal nicotine exposure during pregnancy and lactation resulted in the virtual absence of elastic tissue from the lung parenchyma of 7 day old neonatal rats. This was confirmed in this study as observed in Figures 2.9; 2.11; 2.12 and 2.14 in Chapter 2. Recent studies also indicate that maternal nicotine exposure induce cell damage at alveolar level (Paul, 1987).

3.4 ASPECTS TO BE INVESTIGATED

This chapter will therefore address the effect of maternal nicotine exposure on neonatal lung alveolar status by concentrating on the following aspects:

3.4.1 Septal cellularity with respect to total cell number, including lipid-containing interstitial cells, type I and II cells.

3.4.2 Septal vasculature with respect to total number of capillaries in the alveolar septum.

3.4.3 Cell differentiation with respect to total number of type I and type II pneumocytes and type I:type II cell ratio.

By investigating the abovementioned morphological aspects of the alveolar septum, it may assist in elucidating the structural changes which have been observed in the preparations discussed in the previous chapter.

3.5 MATERIALS AND METHODS

3.5.1 ANIMALS

Treatment of animals is the same as stipulated in Chapter 2. After the removal of the lung tissue, the tissue samples are processed as follows:

3.5.2 TISSUE PREPARATION

3.5.2.1 Principle

In order to examine the tissue sections microscopically, it is essential to fix the tissue sample using an appropriate fixative, therefore losing as little detail as possible. Fixation involves a number of chemical events which prevents autolysis, bacterial attacks and changes in the shape or volume of the tissue. The tissue sample is treated with 2 fixatives, namely glutaraldehyde, which forms cross-links between protein molecules as well as the unsaturated lipids within the tissue. Osmium is also used as it stains cell

membranes, lipid inclusions and nucleoproteins, thus enhancing contrast. A phosphate buffer is used as the fixative vehicle, and it is thought that the buffer exerts the major influence during fixation, particularly when glutaraldehyde is used (Bone and Ryan, 1972). In order to prevent fixation artifacts, the following factors must be taken into account: pH, type of buffer, temperature, size of tissue for optimal penetration of fixative, changes in tissue volume, osmolality of fixative, concentration of fixatives, and duration of fixation.

3.5.2.2 Reagents

1. 2,5% Glutaraldehyde (pH=7,4). Add 90 ml of Working buffer to 10 ml of 25% Glutaraldehyde.

   Working Buffer (Phosphate Buffer). Add 210 ml of Solution A to 40 ml of Solution B. Make up to 500 ml with distilled water.

   Solution A: Make 1L of 0,2 M Na_2HPO_4 , pH=7,4. Dissolve 28,39 grams of $Na_2 HPO_4$ in 500 ml heated distilled water then make up to 1L.

   Solution B: Make 500 ml of 0,2 M $NaH_2PO_4.H_2O$ (hydrous), pH=7,4. Dissolve 13,78 grams of $NaH_2PO_4.H_2O$ in 500 ml distilled water.

2. Palade's Buffer (Veronal Acetate Buffer):

   Dissolve 2,89 grams of barbital sodium and 1,15 grams of anhydrous sodium acetate (or 1,90 grams of hydrous sodium acetate) in 70 ml distilled water. Make up to 100 ml.

3. 0,1 Normal HCl. Used as supplied by manufacturer.

4. 2% Osmium Tetroxide. Used ampules as supplied by manufacturer.

5. Palade's 1%Osmium Fixative Solution. Add 12,5 ml of 2% Osmium Tetroxide, 5 ml Palade's Buffer, 5 ml of 0,1 Normal HCl and 2,5 ml distilled water.

6. 2% Uranyl acetate. Dissolve 2 grams of uranyl acetate in 100 ml of 70% alcohol.

7. 2% Uranyl Nitrate. Add 2 grams of uranyl nitrate to 100 ml 96% alcohol. Keep solution in a dark bottle.

8. Absolute alcohol. Use distilled water to dilute to the various concentrations.

9. Spurr's Epoxy Resin. The standard embedding medium is formulated as follows: Add 10,0 grams ERL 4206 resin, 6,0 grams DER 736 (flexibiliser), 26,0 grams NSA hardener and 0,4 grams S1 accelerator together and mix with a magnetic stirrer until all air bubbles are released from the resin mixture.

10. 2 mM Sodium Methoxide. Dissolve 10 grams of sodium (solid metal) in 200 ml methanol. Evaporation will occur, therefore solution must be made up to 200 ml after fuzzing has stopped. Add 10 ml benzene.

11. Reynold's Lead Citrate . Dissolve 2,66 grams of lead nitrate in 20 ml distilled water. Dissolve 3,52 grams of sodium citrate in 20 ml distilled water. Add the two solutions together and make up to 60 ml using distilled water. Mix well and allow to stand for 2 to 3 hours. Make up a 4% NaOH solution by adding 4 grams NaOH to 100 ml distilled water. Add 16 ml of NaOH to the lead citrate solution and then make up to 100 ml.

12. 1% Toluidine Blue. Dissolve 1 gram Toluidine Blue in 100 ml distilled water.

3.5.2.3. Procedure for primary fixation

The terminal aspect of the middle lobe of the right lung is used for this morphological study. After removal, the tissue sample is immediately placed in a solution of 2,5% gluteraldehyde in 0,2M phosphate buffer (pH=7,4) for 1 minute in order to stabilize the

tissue protein through the process of cross-linkage. Thereafter, slices of 1mm thickness are cut off the tissue block and further diced into cubes of approximately $1mm^3$. These cubes are then placed overnight into glutaraldehyde fixative at 4 degrees Celcius.

3.5.2.4 Program for standard secondary tissue fixation and embedding

1. Post-fixation in 1% osmium tetroxide for 1-1,5 hours.
2. Rinse twice with distilled water.
3. Place in 2% uranyl acetate for 30 minutes (enhances general contrast and acids as tertiary fixatives).
4. Place in 70% alcohol for 5 minutes (dehydration of tissue).
5. Place in 96% alcohol for 5 minutes (dehydration).
6. Place in 2% uranyl nitrate for 10 minutes (dehydration).
7. Place in 100% dehydrated alcohol for 10 minutes (dehydration).
8. Place in 100% dehydrated alcohol for 15 minutes.
9. Place in 100% dehydrated alcohol for 20 minutes.
10. Place in a 1:1 mixture of Spurr's resin and alcohol for 1,5 hours (start of embedding process).
11. Place in clean Spurr's resin for 1 hour at room temperature.
12. Place in clean Spurr's resin for 1 hour at 60 degrees Celcius.
13. Embed in oven-dried embedding capsules and place overnight in incubator at 60 degrees Celcius.
14. Remove capsules from incubator and allow to set for ±2 hours.
15. Microtomy.

3.5.3 MICROTOMY

3.5.3.1 Principle

The thickness of a resin section is a major factor in determining the resolution obtained in an electron microscope. The production of ultra-thin sections of approximately 50 to 70 μm in thickness (which usually appear silver-grey to gold in colour), is critical in the preparation of material to be viewed using electron microscopy.

3.5.3.2 Steps to follow in Microtomy

The following steps are followed in cutting ultra-thin sections:

1. Trimming the resin blocks. The blocks are trimmed with a wax-free razor blade to form a pyramid with the face of the block being the shape of a trapezium, suitable for 1 μm thick sectioning.
2. Thin sectioning. The Sorvall MT-5000 Ultra-Microtome is used to cut 1 μm thick sections from the trimmed block using a glass knife. These sections are stained with 1% Toluidine Blue and examined using light microscopy. Before exposing the 1 μm sections to the dye, the slide with the sections is heated and then dipped into 2 mM Sodium Methoxide for 1 minute and then rinsed twice in absolute alcohol for 1 minute.
3. Trimming blocks for ultramicrotomy. The face of the sectioned block is retrimmed to a size suitable for ultramicrotomy after examination of the 1 μm sections.
4. Ultramicrotomy. Ultra-thin sections of approximately 50 to 70 μm are cut with the ultra-microtome and collected onto a 400 mesh copper grid. These sections

are then double stained using uranyl acetate and lead citrate (Bancroft and Stevens, 1982) to enhance contrast by increasing the electron density of the specimen. This is accomplished by depositing metals of high atomic number into or onto tissue structures in the sections. The salt of many metals (for example osmium, lead, mercury and uranium) are used to increase contrast of a general nature within the section.

Each of the above-mentioned steps will be dealt with in more detail and diagrams will also be included to illustrate the various steps.

3.5.3.3 Trimming the blocks

Blocks which are embedded in capsules having a flat base are trimmed into a pyramid with the tissue situated at the apex of the pyramid. The block is stabilized within the chuck of the ultra-microtome and rotated to trim away the excess resin around the sample, using a wax-free razor blade. Before thin sections can be cut, a glass knife has to be prepared to serve as the cutting edge. For the production of knives, a LKB Bromine 7800 knifemaker was used. The knifemaker stores the 25mm wide glass strip at a preset position and applies controlled pressure on the glass to form a break. The rhombus produced is then positioned diagonally in order to form a 45° cutting edge (Figure 3.2). The best cutting edges are straight, free from any edge blemishes for at least one third of their length, and have a small spike at one end (Figure 3.3). Adhesive aluminium tape is used to form a trough in which to float the sections. Molten dental wax is used as a sealant to close the small hole left at the bottom of the trough (Figure 3.4)

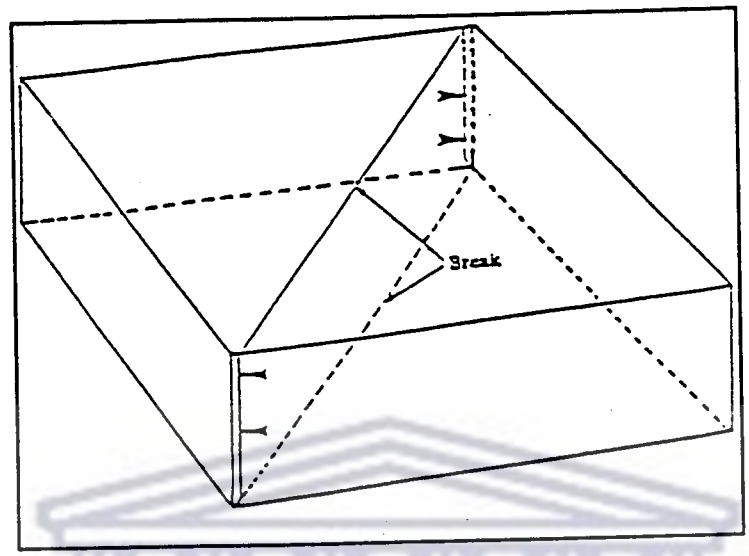

Figure 3.2 The arrows indicate the cutting edges formed when a glass square is broken diagonally.

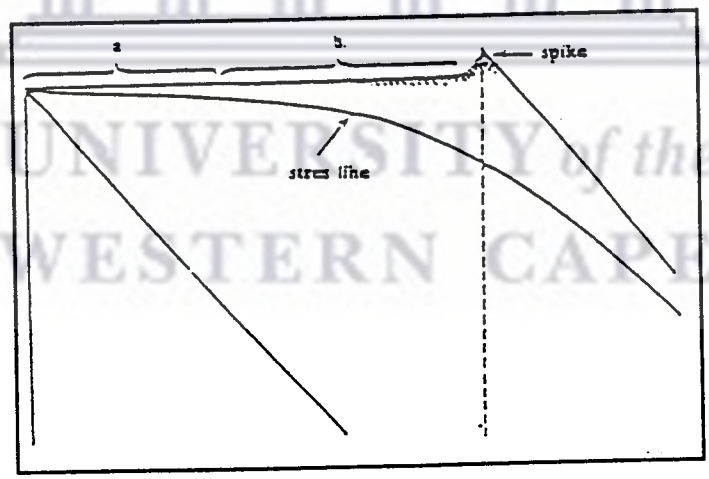

Figure 3.3 Diagrammatic representation of a good knife cutting edge. The area "a" is normally suitable for ultra-thin sectioning;"b" is only suitable for trimming and semi-thin sectioning.

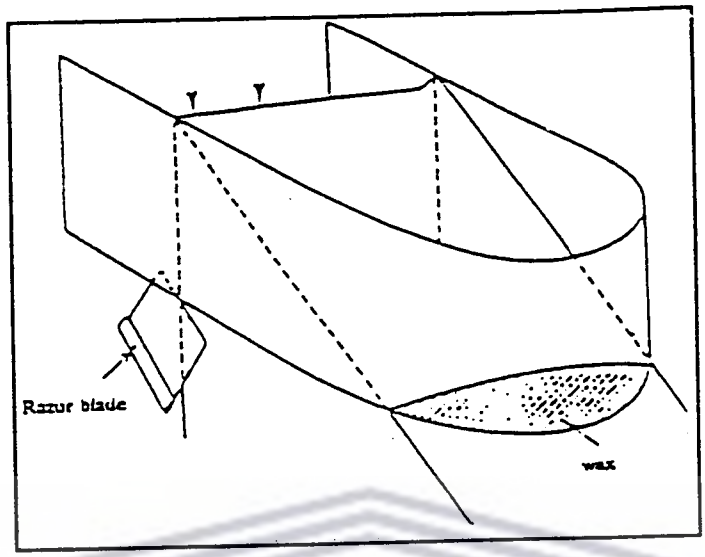

Figure 3.4 Adhesive tape applied to a glass knife to form a trough; the cutting edge is marked with arrows.

3.5.3.4 Thin sectioning

Sections of 1 μm thickness are cut from the trimmed block, picked up with a toothpick and placed onto a drop of water on a clean, labelled slide. The slide is placed onto a warm plate in order for excess water to evaporate. Thereafter slide is placed into a jar containing 2 mM Sodium Methoxide which facilitates penetration of the dye used (1% Toluidine Blue) (Giddings et al, 1982), after which it is rinsed twice in absolute alcohol thus ensuring the removal of the Sodium Methoxide and also facilitating penetration of the stain. In order to stain the slide, it is placed on to a staining rack and a few drops of 1% Toluidine Blue are poured over the sections. Using a Bunsen burner, a flame is held directly under the slide until vapour is given off from the dye. The slide is left for 1 to 2 minutes to allow penetration of the dye, after which it is rinsed and blotted dry before placing mounting medium, Canada Balsam, and a coverslip on the slide. Upon

viewing the section using light microscopy, a suitable area is selected which will be used for obtaining ultra-thin sections for electron microscopy.

3.5.3.5 Trimming blocks for ultra-microtomy

While viewing the sections through the light microscope, the corresponding block is orientated in the chuck of the ultra-microtome. The source of illumination in the ultra-microtome is fixed on the block face in such a manner that the detail on its face is apparent. The selected area is marked on the block face by slightly scoring with a razor blade and the block is trimmed into a smaller pyramid using the scores made as a guide. The size of the face depends on the consistency of the resin, the type of tissue and the quality of knife being used.

3.5.3.6 Ultra-microtomy

After lining the block face in both the vertical and horizontal planes, using the knife as a guide, the block is advanced until it is in close proximity of the cutting edge of the knife. The ultra-microtome is set to advance at 60 nm intervals and will subsequently advance automatically and cut sections of the specified thickness. The first few sections will be thick and uneven, but the variation will eventually lessen and a consistent thickness will be attained. Allow for enough sections to be cut to form a ribbon and stop sectioning so that these can be picked up using a 400 mesh copper grid. The grids are then placed on filter paper in closed petri dishes to dry. The contrast of these sections is further enhanced by using heavy metal salt stains.

3.5.4 STAINING ULTRA-THIN SECTIONS

Two dental wax strips of approximately 1x4 cm is used as staining blocks. These are placed in both halves of a clean, wet, piece of blotting paper with a petri dish covering the blotting paper. Place the uranyl acetate droplets on the first strip of wax, equivalent to the number of grids being stained. These drops must be filtered through a millipore filter to prevent sedimented crystals from being deposited onto the specimens. The grids are then placed onto the drops for 3 minutes and then rinsed twice in distilled water by repeated and rapid dipping of the grids into the beakers of distilled water. The grids are then dried with clean filter paper and each is placed on a filtered drop of Reynold's lead citrate for 3 minutes.

The grids are again rinsed by repeated and rapid dipping of it in two beakers of distilled water respectively. The grids are dried on filter paper and placed into a closed petri dish, ready for viewing.

3.5.5 SAMPLING OF BLOCKS AND TECHNIQUES USED IN DETERMINING THE DIFFERENT PARAMETERS INVESTIGATED IN THIS CHAPTER

Specimens were randomly chosen after the resin blocks had been placed individually in different envelopes. These blocks were used to determine all the parameters which were investigated, namely cellularity, type I : type II cell ratio, Lipid-Containing Interstitial Cell (LIC) and capillary density.

3.5.5.1 Cellularity

Cellularity is determined according to the method of Eidelman et al (1990). The 1μm sections are viewed at 400x magnification. Calibration of the eyepiece graticule and stage graticule at 400x magnification results in the following equation:

4 divisions = 0,01 mm

Septa are measured using lengths of 10 divisions, using the stained nuclei as markers of cells lying within the septum being counted.

$$\frac{10x0,01mm}{4}=0,025mm$$

0,025 mm x 40 = 1 mm

Five different septa from each of 10 rat samples were counted. The results are expressed as the number of cells per millimeter septum.

3.5.5.2 LIC count

The counting method of Eidelman et al (1990) was used in determining the LIC number per mm septum. Sections of 1 μm thickness were used for this purpose and viewed at 400x magnification. The lipid-containing interstitial cells (LIC) were identified by the presence of lipid droplets within the cells which distinguishes the LIC from the other septal cells. The lipid within these cells did not take up the Toluidine blue stain, thus remaining as white spherical droplets within the cells. The results are expressed as the number of LIC per millimeter septum. The ratio of LIC : total cell count was also calculated.

3.5.5.3 Capillary Density

Sections of 1 μm thickness were used to determined this parameter by viewing the slides at 400x magnification. The blood within the blood vessels stain dark blue to purple and this clearly differentiates the blood vessels from the rest of the septal interstitium. Lengths equivalent to 1 mm were counted and the results are expressed as capillaries per mm septum.

3.5.5.4 Type I and Type II cell count

Structural characteristics were used as a basis for identifying the epithelial type I and type II cells. Type I cells are characterized by attenuated cytoplasm with transporting vesicles and a large flattened nucleus. These cells are associated with the blood-air barrier and will occur in the vicinity of a capillary occurring on the periphery of a septum. The type I cells occur on the alveolar side of the septum.

The type II cells are characterized by lamellar bodies within the cell as well as being triangular to cuboidal in shape. They are very common at septal junctions. Counting is done using negatives of photographs which had been taken of septa of both the control and experimental rat lungs. These negatives are put in an enlarger and projected onto a large white surface, thus magnifying the septa and the cells within them. Ten random samples are used, and all the type I and type II cells within the projected picture are counted. The results are expressed as percentage type I cells and percentage type II cells as well as the ratio of the two cell types.

3.5.6 STATISTICAL ANALYSIS

Statistical analysis is executed using the Wilcoxon test for unpaired data. Using the Medcalc statistics programme, a probability level of $P<0,05$ is designated as significant in this study. Ten rat pups from ten different litters are used in each of the control and experimental groups, respectively. All results are expressed as MEAN ± SEM.

3.6 RESULTS

The cellularity of the alveolar septa in the control group gradually increases with 27,68% from 48,12 ± 1,62 cells/ mm septum on day 1 after birth to 61,44 ± 1,77 cell/mm septum on day 14 after birth ($P<0,005$). No further increase occur between days 14 and 21 (Table 3.1).

The cellularity of the nicotine exposed lungs increase with 17,06% from 60,96 ± 1,54 cells/mm septum on day 1 after birth to 71,36 ± 1,65 cells/mm septum on day 14 after birth ($P<0,005$). No further increases occur between days 14 and 21 after birth (Table 3.1). Comparisons are made within the individual group with respect to the different age groups. Significant differences have been found between control and nicotine groups when comparing the different age groups. Cellularity on day 1, 7, and 14 after birth of control rat pups are lower ($P<0,001$) than in the lungs of the nicotine exposed rat pups.

The lipid-containing interstitial cells (LIC) in the control group shows an increase in number from 3,40 ± 0,37 cells/mm septum on day 1 to 7,46 ± 0,96 cells/mm septum on day 21. There is no significant difference between day 1 and day 14, but a difference does occur between day 14 (4,28 ± 0,49) and day 21 where the number of LIC/mm

septum is 7,46 ± 0,96 (P<0,01). In the nicotine group, the number of LIC/mm septum on day 1 is found to be 4,50 ± 0,35, and this was not significantly higher than the 4,94 ± 0,55 cells/mm septum found on day 14. However, on day 21 (6,16 ± 0,42) the LIC/mm septum is higher than on day 14 (4,94 ± 0,55) (P<0,05). Increase in LIC count in the nicotine group is not as rapid as that in the control group, with an increase of 119,41% from day 1 to day 21 in the control pups, compared to only 36,89% in the nicotine group. During the last week of gestation, that is between day 14 and day 21, 73,4% of the increase in the control group occurs, while only 24,7% of the increase occur for the same period in the nicotine group. On comparing the LIC/mm septum count of the two groups, it is found that the nicotine group displays a significantly high number (P<0,05) than that of the control group for day 1. The nicotine group is 35,5% higher than that of the control group. No significant differences are found between the control and nicotine groups within the other age groups. A significant difference (P<0,05) is found between day 1 and day 21 in the nicotine exposed group.

For both the control and nicotine exposed animals, the cell/LIC ratio decrease as the lung matures. In the control lung the ratio decrease by 42,82% between days 1 and 21, after birth. Over the same period, the ratio in lung tissue of nicotine exposed rat pups decrease by 20,66%

Data in Table 3.2 indicates that the percentage of type I cells in the control group gradually increase from 58,96 ± 1,06 on day 1 to 64,82 ± 0,85 on day 21. This shows a significant increase of 9,94% (P<0,01). In the nicotine group the type I pneumocytes

decrease significantly (P<0,01) between day 1, from 43,12 ± 0,53 to 40,47 ± 0,62 on day 21. When comparing the control group to that of the nicotine exposed lungs, it is found that the control group have 15,8% (P<0,001) more type I pneumocytes than the nicotine group. Due to the gradual increase in the percentage of type I cells in the control lung and the percentage decrease in the nicotine exposed lung, this difference in percentage pneumocyte in the lungs of the two groups increased to 24,3% on day 21 after birth.

Type II pneumocytes are found to decrease in number in the control group from 40,93 ± 1,26 on day 1 to 35,92 ± 1,09 on day 21. This is a difference of 12,24% (P<0,01) between these two age groups, while day 7 and day 14 does not differ with the day 1 group. Comparing type I to type II pneumocytes for the control group, there is an increase of type I cells with age while type II cells decrease with age. Comparing this trend to that in the nicotine group, it is found to be the exact opposite. There is an increase of 4,82% from 56,88 ± 0,53 on day 1 to 59,62 ± 0,61 on day 21 (P<0,01). Between day 1 and day 7, the type II pneumocyte count increase by 3,99% (P<0,05). The number of type II cells then tend to plateau during the last two weeks of lactation. When comparing the type 11 cell count of the control group to that of nicotine group, significant differences occur in all the age groups (P<0,001), with the percentage difference increase from 38,97% on day 1 to 65,98% on day 21. This is due to decrease numbers in the control group while increasing in the nicotine group.

Due to the differences between type I and type II pneumocytes composition within the septum, the type I : type II cell ratio will also be affected. This resulted in a 24,48%

increase ($P<0,01$) in the type I : type II cell ratio from 1,43 ± 0,05 on day 1 to 1,78 ± 0,05 on day 21 in control rat lung. The decrease in the type I : type II cell ratio of the nicotine lungs from 0,76 to 0,68 ($P>0,01$) and the increase of the ratio in the control lungs is reflected by the percentage difference in the ratios increasing from 46,85% to 62,0% ($P<0,01$) for day 1 and day 21 respectively (Table 3.3). Figure 3.5 illustrates graphically the difference in trends between the control and nicotine exposed groups.

The data in Table 3.4 represents data showing the effect maternal nicotine exposure has on the number of capillaries present per unit length of neonatal lung septum. In lung tissue of control rat pups a significant increase ($P<0,01$) from 5,82 ± 0,22 capillaries/mm on day 1 to 8,44 ± 0,51 capillaries/mm on day 14 after birth is observed. This indicates a 45,0% growth in the capillary density per millimeter septum. No significant changes occur between day 14 and day 21.

In the nicotine exposed group, the capillary count per unit length of alveolar septum increase by 37,7% ($P<0,05$) from 4,72 ± 0,30 capillaries/mm on day 1 to 6,50 ± 0,56 capillaries/mm on day 21 after birth. The capillary density of control rat lung is higher ($P<0,001$) than in the lungs of nicotine exposed rat pups. While the capillary count of the lung septa of control rat pups stabilize on day 14 after birth, it continue to increase in the nicotine exposed lungs beyond day 14 after birth.

TABLE 3.1: THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL RAT LUNG CELLULARITY.								
AGE (Days)	CELLULARITY (Cells/mm± SEM)			LIC (LIC/mm ± SEM)			•CELL/LIC	
	CON	NIC	%Δ	CON	NIC	%Δ	CON	NIC
1	48,12±1,62	60,96±1,54	26,68	3,40±0,37	4,50±0,35	32,35	14,15	13,55
7	51,52±1,70	59,12±1,72	14,75	3,42±0,53	3,36±0,33	-1,75	15,06	17,34
	P>0,005	P>0,05		P>0,05	P>0,05			
	7,07%	-80,31%		0,6%	-25,33%			
14	61,44±1.77	71,36±1,65	16,15	4,28±0,49	4,94±0,55	15,42	14,35	14,45
	P<0,005	P<0,005		P>0,05	P>0,05			
	27,68%	17,06%		0,05%	9,78%			
21	59,76±2,09	66,24±2,62	10,84	7,46±0,96	6,16±0,42	-17,43	8,05	10,75
	P>0,05	P>0,05		P<0,01	P<0,05			
	24,19%	8,66%		119,4%	36,89%			

•CELL/LIC :Ratio of cells/mm septum to LIC/mm septum. CON=Control; NIC=Nicotine; %Δ=percentage difference relative to day 1 pups; LIC=Lipid-containing interstitial cell.

TABLE 3.2: INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL RAT LUNG TYPE I AND TYPE II CELL COUNT.								
AGE (Days)	CELLS COUNTED		% TYPE I PNEUMOCYTES			% TYPE II PNEUMOCYTES		
	CON	NIC	CON	NIC	%Δ	CON	NIC	%Δ
1	471	804	58,96±1,06	43,12±0,53	-26,87	40,93±1,26	56,88±0,53	38,97
7	472	761	60,85±1,21	40,85±0,87	-32,87	39,05±1,25	59,15±0,87	51,47
			P>0,05	P>0,05		P>0,05	P<0,05	
			3,21%	-5,26%		-4,59%	3,99%	
14	663	732	62,25±1,10	41,92±1,06	-32,59	37,75±1,10	58,08±1,06	53,85
			P>0,05	P>0,05		P>0,05	P>0,05	
			5,58%	-2,78%		7,77%	2,11%	
21	691	736	64,82±0,85	40,47±0,62	-37,57	35,92±1,09	59,62±0,61	65,98
			P>0,05	P>0,05		P>0,05	P>0,05	
			9,94%	-6,15%		-12,24%	4,82%	

CON=Control; NIC=Nicotine; %Δ= % difference relative to day 1 pups.

TABLE 3.3: INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON THE TYPE I:TYPE II CELL RATIO OF NEONATAL RAT LUNG TISSUE.			
AGE (Days)	TYPE I : TYPE II CELL RATIO		
	CONTROL n = 10	NICOTINE n = 10	%Δ
1	1,43 ± 0,05	0,76 ± 0,02	-46,85
	P<0,01	P<0,01	
7	1,56 ± 0,06	0,72 ± 0,01	-53,85
	9,09%	-5,26%	
14	1,66 ± 0,09	0,70 ± 0,02	-57,83
	16,08%	-7,89%	
21	1,78 ± 0,05	0,68 ± 0,02	-62,0
	24,48%	-10,53%	
	P<0,01	P<0,01	

%Δ=percentage difference relative to day 1 pups; n=number of animals used.

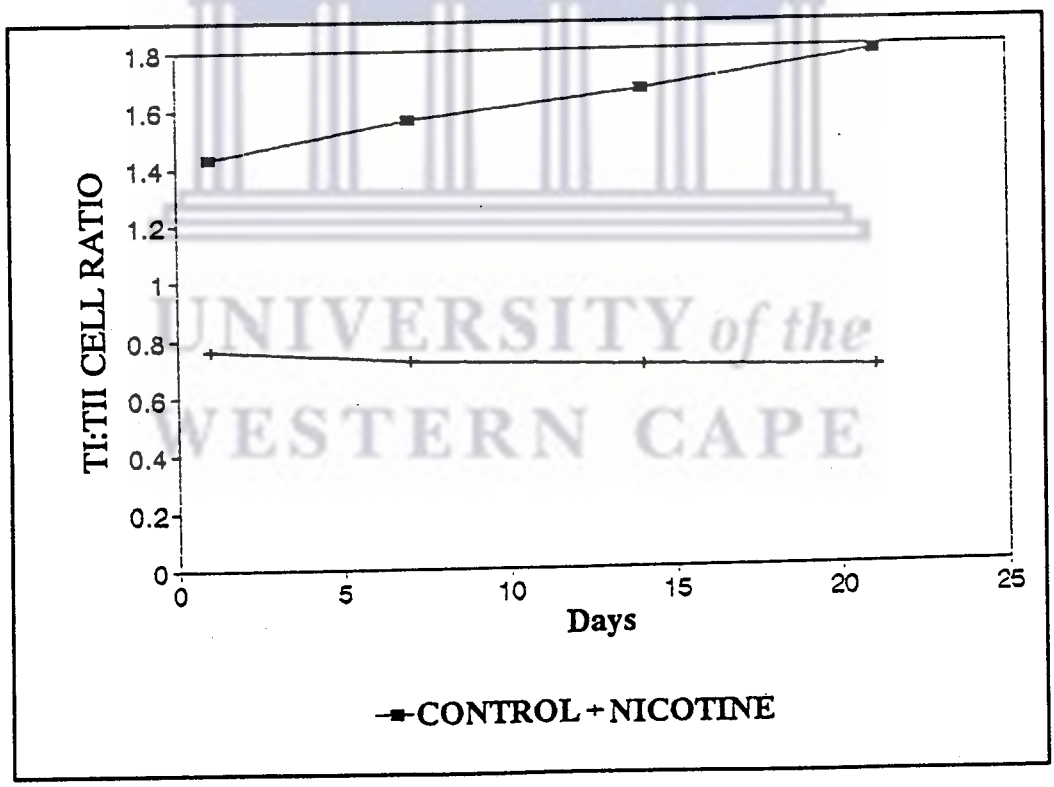

Figure 3.5 Graphic representation of type I:type II cell ratio. Note that the control group increase linearly, while the nicotine exposed group decreases linearly.

TABLE 3.4: THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL LUNG SEPTAL CAPILLARY DENSITY (CAP/mm ± SEM)

AGE (Days)	CONTROL n = 10	NICOTINE n = 10	%Δ
1	5,82 ± 0,22	4,7 ± 0,3	-18,9
	P<0,001	P<0,05	
7	6,74 ± 0,51	4,9 ± 0,4	-26,4
	15,8%	5,1%	
14	8,44 ± 0,51	5,6 ± 0,3	-33,9
	P<0,001	18,2%	
	45,0%		
21	8,96 ± 1,31	6,5 ± 0,6	-27,5
	53,9%	37,7%	

Comparisons made within control and nicotine groups; %Δ=percentage difference relative to day 1 pups; n = number of animals used.

3.7 DISCUSSION

The basis of disease is injury to the smallest living unit of the body, namely the cell. Components of the alveolar septa include the type I and type II pneumocytes, fibroblasts myofibroblasts, smooth muscle cells, connective tissue and capillaries (Kauffman et al, 1974). Each of these components of the alveolar septum has an important function in maintaining alveolar integrity and overall lung efficiency. The formation of alveolar septa is seen to be one of the most important processes in the formation of alveoli. Therefore, when the development processes of any of these components is adversely affected, the functional integrity of these components will be reflected by the eventual integrity and functionality of the alveolar septa and thus the alveoli of the lung.

When the mother smokes during pregnancy and lactation, the neonate is exposed to those components within the cigarette smoke which cross the placenta and is also taken up during the production of mother's milk. Nicotine is one of the components of tobacco smoke which is considered to be, pharmacologically, the most active constituent of cigarette smoke (Daldstrom et al, 1990). During pregnancy, nicotine crosses the placenta and exposes the fetus to concentrations similar to that in the mother's blood (Luck and Nau, 1984; Greenberg et al, 1984). Nicotine accumulates in the respiratory tract of the developing fetal lung. Alveolar sacs originate from these respiratory tracts (Kuhn, 1982). It is therefore conceivable that fetal lung alveolar development will be adversely affected by nicotine.

Most of the alveoli are formed between day 4 and day 13 after birth in the rat lung (Burri, 1974). Studies done by Maritz and Burger (1992) illustrated that maternal nicotine

exposure during pregnancy and lactation interfered with carbohydrate metabolism and the structural development of neonatal rat lung by affecting elastic and collagen fibre content in the alveolar septum. Smoking also attracts polymorphonuclear (PMN) leucocytes and macrophages to the airspaces. PMN leucocytes have a high elastase content which have been associated with increased risk of developing pulmonary emphysema due to its ability to attack collagen and other connective tissue macromolecules (Janoff, 1983). Nicotine also activate the release of superoxide from PMN and PAM, thereby inducing membrane damage (Hannan et al, 1989).

Changes on placental vascularization as well as more fibrous and compact placenta's, due to an increased collagen content, was found in studies done on smoking mothers (Asmussen, 1979).

3.7.1 SEPTAL CELLULARITY

Pulmonary septal parenchyma is basically composed of 5 different cell types, namely type I cells, type II cells, endothelial cells (found lining the blood capillaries), alveolar macrophages and interstitial cells which include the lipid-containing interstitial cells and the fibroblasts (Burri et al, 1973).

The type I and type II cells forms the respiratory epithelium (Evans et al, 1975). The type I cells cover most of the alveolar surface, forming part of the blood-air barrier, while the type II cells are dispersed throughout the alveoli, usually in alveolar corners (Adamson and Bowden, 1974).

The endothelial cells forms the lining of the blood capillaries found in the septa. These cells are in close proximity to the type I cells since they form part of the blood-air barrier across which gas exchange occur (Burri, 1974). These cells are extremely sensitive to membrane and cytoplasmic changes that can lead to cell destruction (Kinnula et al, 1991).

The alveolar macrophages serves as the cleaner of the alveolus by removing all cellular debris as well as the remains of damaged lung tissue (Hannan et al, 1989). Macrophages have also been implicated in emphysema due to their ability to produce large quantities of oxygen radicals which causes blebbing and may eventually lead to cell rupture (Frank, 1991).

The majority of interstitial cells (92,7%) are fibroblasts (Vidic et al, 1989). These include 2 types, namely the myofibroblasts, found at the septal apex, which are essential for the synthesis and secretion of stromal elastin, and the lipid-containing interstitial cell (LIC) (lipofibroblast), which is found at the septal base. The function of the LIC is uncertain (Vidic et al, 1989).

Data obtained in this study shows significant differences in septal cellularity between the control and nicotine exposed groups, with cell numbers being higher in the latter group. The increase in cell number with time corresponds with the time period of alveolar formation, that is day 4 to day 13 (Burri, 1974) in both groups. The higher cell count in the nicotine group corresponds with an increase in type II pneumocyte number which also occurred between days 1 and day 21 after birth (Table 3.2). Cellularity of the septa tended

to plateau on day 14 in both groups, since the process of alveolar formation would be approximately complete and the lung would therefore take on a more mature structure. The percentage difference between the two groups with respect to cellularity, decreases with age. This could partly be ascribed to a greater increase in type II cells in the nicotine exposed lungs compared to the control lungs. Together with the increase in type II cells there is also a decrease in the number of type I cells in the nicotine exposed lungs. This indicates that the rate of type I cell destruction is faster than the rate of proliferation and differentiation by the type II cells to replace damaged type I cells in the nicotine exposed group.

Various gases such as nitrogen dioxide, ozone and high concentrations of oxygen cause a similar pattern of lung damage (Palmer et al, 1975) as observed in the experimental rat lungs in this study. In all these models there appears to be stimulation of type II cell proliferation, thus increasing septal cellularity. Smith et al (1932) showed that after exposure to high O_2 concentration the alveolar walls of the young rat lungs resemble in degree of cellularity, those of fetal lungs, and following prolonged exposure to hyperoxia, the cellularity in the alveoli of old rats increased, to resemble that of young rats. A study done on the exposure of dogs to cigarette smoke showed alveolar cell hyperplasia (Frasca et al, 1974).

A study done by Eidelman and co-workers (1990) has also shown an increase in cellularity of the alveolar walls in lungs which had been exposed to cigarette smoke. The pathophysiologic significance of the increased cellularity and destruction of alveolar walls

was demonstrated by their role in airflow limitation and losses of elastic recoil pressure (Eidelman et al, 1990). This was therefore verification that injury caused by cigarette smoke adversely influenced the functionality of the lungs. Therefore, also based on the findings of the present study, it is plausible that nicotine in tobacco smoke contribute to the higher cellularity in lungs which were exposed to tobacco smoke. Lannan et al (1993) found a decrease in epithelial cell size over time with cigarette smoke condensate compared with control. This change in cell size may result from changes in the cell cytoskeleton and in cell attachment. If these changes do occur in vivo then it may result in consequent loss in alveolar integrity which contributes to subsequent lung injury.

3.7.2 ALVEOLAR EPITHELIAL CELLS

The cell population of the lung can be described with respect to the three principal tissue compartments of the septum : epithelium, endothelium and interstitium. The epithelium is composed of two cell types, namely type I cells and type II cells. Type I pneumocytes play a major integumentary role in maintaining the blood-air barrier (BAB) at the alveolar level. This cell also has a high degree of structural differentiation and great susceptibility to alveolar injury (Kauffman et al, 1974).

Type II cells are round or cuboidal cells characterized by the osmiophilic lamellar bodies and by a tuft of microvilli at their free surface. Focal proliferation of type II pneumocytes is a common response to alveolar injury. Proliferation is followed by differentiation of one or both of the sister cells to replace the damaged type I pneumocyte, since type I pneumocytes are incapable of undergoing mitosis in order to regenerate the damaged

epithelium (Crapo et al, 1980).

Table 3.2 shows an increase in type II cell numbers in the experimental group whilst there is a decrease in the control group ($P<0,001$). The trend within the control group is consistent with literature (Burri, 1974; Mason et al, 1977) and therefore indicates normal lung development. In the control lungs there is an overall increase, with age, in type I cell number, and this increase corresponds with the period of alveolarization since these cells make up 90% of the alveolar surface cover (Naimark, 1977) and forms a part of the BAB needed for gaseous exchange (Burri, 1974). However, the nicotine exposed lungs show a significant decrease in the number of type I cells when compared to the control lungs. Since damage of type I cells are followed by type II cell proliferation (Evans et al, 1975), and no undue proliferation of type II cells are observed in the control lungs, it is conceivable to believe that damage had occurred in type I cells. Similar results were obtained in studies done by Evans et al (1975) who exposed rats to nitrogen dioxide. They found proliferation of type II cells after exposure and then increases in type I cells as the number of type II cells decrease in experimental group. Frasca et al (1974) also found Type II cells almost completely lining some alveoli, after the experimental group had been exposed to cigarette smoke. In a study done on mice by Adamson and Bowden (1974), they found that following oxygen poisoning, the epithelial injury involved type I cells while the recovery phase was characterized by proliferation of type II cells. Due to the variation in type I and type II cell number, it will consequently affect the type I : type II cell ratio. Data in Table 3.3 indicates a decrease in the experimental group of the type I : type II cell ratio. This can be expected since the rate of type II cell proliferation exceeds that of type I

formation. However, in the control group there is an increase in ratio since the type I cells increase in number with age, while the type II cell decreased as a function of lung maturation.

Lactation lasts until approximately day 12 after birth, after which the pups start feeding on the Epol cubes. Their nicotine intake will therefore be reduced from this period onwards, and it is expected that this will also have an influence on the type I : type II cell ratio. However, in this study it is found that there is a decrease in the ratio despite the fact that the pups had weaned.

The continued decrease in the type I : type II cell ratio could be due to a number of factors. In a study done on pulmonary alveolar macrophages (Hannan et al, 1989), it was established that the chemical and physical changes induced by the toxic effect of tobacco smoke, were perpetuated in new macrophages which enter the alveolar spaces. This may therefore also be possible for the cells which had been exposed to the deleterious effect of nicotine. Massaro et al (1975) has found that type I cells are dependent on glycolysis for the maintenance of their structural integrity. Maritz (1987) has shown that irreversible inhibition of the glycolytic pathway occurs in lungs of neonates which was exposed to nicotine during gestation and lactation. This therefore results in type I cell damage with subsequent type II cell proliferation (Evans et al, 1975). Approximately 30% of the type II cells transform into type I cells following the period of cell proliferation after exposure to a toxic agent (Evans et al, 1975). Maritz and Burger (1992) has also shown that nicotine interferes with ATP hydrolysis which is necessary for the maintenance of Na^+-K^+-ATP-ase

(Paul, 1987) within the cell membrane. However, nicotine also has an inhibiting effect on Na^+-K^+-ATP-ase (Meyer et al, 1971). This could therefore affect the cell volume since this pump plays a vital role in maintaining cell volume. It is therefore conceivable that due to the inhibition of glycolysis, swelling of type I cells occur as well as the formation of membrane-blebs. This may eventually result in the rupturing of blood-air barrier (BAB) (Meyer et al, 1971). Since the type II cells are unable to proliferate at a fast enough rate to replace the damaged type I cells, it will therefore also affect the integrity of the BAB, thus affecting the diffusion capacity of the lung. If it is indeed so that type I cells are damaged, it may eventually not only result in the rupturing of the BAB, but also the rupture of the alveolar walls and thereby create emphysema-like damage.

3.7.3 LIPID-CONTAINING INTERSTITIAL CELLS (LIC)

During postnatal alveolar formation in the rat, cell proliferation and cell differentiation occur as mentioned earlier. Type II cells divide and differentiate into alveolar type I cells, where the latter cover 90% of the alveolar surfaces (Naimark, 1977). Like the alveolar epithelial cells, the interstitial cells also divide and differentiate into 2 cell types, namely myofibroblasts and lipofibroblasts (Kauffman et al, 1974). The lipofibroblast or LIC occur in greatest abundance during the period of alveolar formation, that is between day 4 and day 13 (Maksvytis et al, 1981), and then seemingly vanishes when this phase of rapid lung growth ends (Maksvytis et al, 1981). There are two possible theories about the role of LIC in alveolarization. Firstly, the LIC may serve to regulate the conformation of elastin and collagen within the septum via the activity of lysyl oxidase which is produced by LIC or other interstitial cells during lung development (Mennies, 1983). Lysyl oxidase is an

extracellular enzyme which is essential for the cross-linking of newly formed collagen and elastin. Secondly, the contractile activity of LIC filaments may be important in the control of alveolar shape and lung morphogenesis during early postnatal lung development (Kacew and Narbaitz, 1978). Cytoplasmic filaments similar to those in the contractile cells of the adult lung have been observed in neonatal lung alveolar LIC (Maksvytis et al, 1981). Any changes therefore affecting their morphology or metabolism, would influence the development of the alveoli.

Although the role and fate of the LIC and its lipid content is uncertain, the time of its appearance suggests that it is involved in alveolar formation and lung growth during early postnatal lung development. Whether LIC exist in adult lung is also unclear. It may be that LIC continue their functions in the adult lung despite the absence of large amounts of cytoplasmic lipid. It is also hypothesized that LIC are the progenitor of the contractile interstitial cells of the adult lung, which have a central position in the septum similar to LIC and which contain actin myofilaments that traverse the alveolar wall (Brody and Kaplan, 1983).

The results obtained in this study indicate a significant increase in LIC number with age in the nicotine exposed group as well as in the control group. The LIC count differs significantly on day 1 between control and experimental groups, and this indicates that nicotine has affected LIC during fetal development, rather than during neonatal lung development. In a study done on lipid metabolism after exposure to cigarette smoke, significant alterations were observed in various categories of lipids present in the body

(Latha et al, 1988). Significant increases in cholesterol, triglycerides and phospholipids occurred within the heart, aorta and the lungs (Latha et al, 1988). The activity of both glucose-6-phosphate dehydrogenase and malic enzyme showed a significant increase in the heart and lungs. Both these enzyme provide NADPH for lipogenesis and an increase in their activity may indicate increased lipogenesis in the heart and lungs. The number of LIC may not differ from that of the control, but if cognisance is taken of the above findings, then the lipid content of the LIC cells are expected to increase. LIC predominantly accumulate linoleic component in unsaturated triglycerides (Maksvytis et al, 1981). This together with other fatty acids accounts somehow for the production-to-degradation balance of elastin in the parenchymal interstitium. The conformational and mechanical properties of stromal elastin are linked to the elastin-binding capacity of various fatty acids (Mukherjee et al, 1976). Degradation of insoluble elastin by elastase is also enhanced by exposure to sodium linoleate, which inhibits lysyl oxidase activity (Vidic et al, 1989). Maritz and Woolward (1992) found that maternal nicotine exposure interfered with elastic tissue quantity, by decreasing the amount found in the nicotine exposed group compared to that in the control group. Inhibition of lysyl-oxidaze which is formed by LIC (Vidic et al, 1989), would adversely affect the elastic tissue network in the lung parenchyma, since cross-linking would be inhibited.

3.7.4 CAPILLARY DENSITY

Capillary growth may be the controlling force which initiates the postnatal restructuring of the newborn lung and directs the alveolar septal growth that occurs during this period. The capillarization and the full deployment of capillaries in the newly formed septa, is the latest

process involved in the morphological transformations during early postnatal lung development and thus signifies the critical change in the canalicular stage of development (Fukuda et al, 1983). Fukuda et al (1983) also observed that fibroblasts play a role in organizing the relationship which exists between epithelial cells and capillary endothelium. Burri (1974) found that in the rat lung, capillary development precedes alveolar septa formation. Therefore, any interference in capillary development would be reflected in alveolar formation within the developing lung. Burri et al (1973) found that during day 4 and day 7, the alveolar and capillary surface area were enlarged by 66% and 75% respectively. Alveolar and capillary surface areas increased three-fold between days 7 and 21, therefore indicating that the number of capillaries within the lung multiplies as the lung matures in structure.

Data from the present study supports the findings of previous researchers (Fukuda et al, 1983; Burri et al, 1973), that is capillary density increased as a function of age in the normal development of the rat lung (Table 3.4). Maternal nicotine exposure had an adverse effect on capillary density by decreasing the number of capillaries within the septum, compared to that found in the control group. It also slowed down the rate of capillary growth since the difference in growth between the control and nicotine group increased from 18,9% on day 1, to 27,5% on day 21. During day 14 and day 21, 45% of the capillary growth occurs in the control group, while 37,2% capillary growth occurs in the nicotine exposed group. Since capillary formation and septal formation is integrated, one can expect a decrease in alveoli within the lungs of the nicotine exposed group. Studies have indeed illustrated that there is a decrease in radial alveolar count in nicotine exposed rat lungs (Maritz and

Woolward, 1992). The mechanism by which nicotine exerts its influence is not known as yet.

In a study done by Collins et al (1985) it was determined that maternal nicotine exposure results in a decrease in the mesenchyme within the fetal rat lung. It was also found that mesenchymal cells differentiate into fibroblasts and produce a "fibroblast pneumocyte" factor which serves as a stimulus for fibroblast activity (Motoyama et al, 1988). Since fibroblasts determine the relationship between epithelial cells and endothelial cells of the capillary, exposure of mesenchyme to nicotine in the fetal lung would therefore manifest itself by suppressing fibroblast activity during lung maturation. Also, studies by Asmussen (1979) has shown that maternal smoking causes severe damage to the fetal cardiovascular system, namely umbilical artery, umbilical vein and vessels of the placental villi. The data also indicated that placentas of the smoking mothers were small and poorly vascularized. This should therefore affect the fetal blood supply and organ development in utero. Nicotine has also been shown to cause injury to endothelial cells (Hladovac, 1978). In a study done by Auerbach and co-workers (1972), it was found that cigarette smoke is highly associated with rupturing of the alveolar septa, fibrosis or thickening of alveolar septa, thickening of walls of arteries and arterioles, and pad-like attachments to the alveolar walls. These changes were also found to increase with age (Auerbach et al, 1972). Ruptured BAB were also observed by Maritz et al (1993) in a study done on the effects of maternal nicotine exposure on alveolar epithelial status in the developing rat lung. Since type I cells are reliant on glycolysis for the maintenance of their cell membranes, inhibition of this biochemical pathway by nicotine (Maritz and Burger, 1992) could be one of the major

causative factors of BAB destruction, thus affecting lung diffusion capacity by reducing alveolar and capillary surface area. Despite the destructive effect nicotine has on blood vessels, it also slows down capillary growth (Table 3.4), and it would therefore seem as if this is another mechanism by which nicotine affects lung development.

In conclusion, maternal nicotine exposure during pregnancy and lactation has a definite effect on lung development. This has resulted in changes in the structural and morphometric parameters of the alveolar septa within the developing neonatal rat lung. These changes include significantly higher cell counts in the nicotine exposed rat lungs. Also due to type II cell proliferation, the type I : type II cell ratio in the nicotine exposed animals show a decrease. An increase in capillary density is observed in both groups, however, the increase is more gradual within the nicotine group. This could result in insufficient oxygen being made available to the rest of the body since less capillaries are available for gaseous exchange within the lungs. This could then lead to hypoxic conditions within the cells which results in the malfunction of metabolic processes within these cells. Apart from the abovementioned changes in cell counts, it is clear that the septal cells in the neonatal lungs exposed to nicotine during pregnancy and lactation, is smaller than in the control lungs (Lannan et al, 1993) . Due to these changes, the efficiency of the lung as an organ of gaseous exchange will be affected and it may also render the lung more susceptible to lung disease.

In this chapter it was established that maternal nicotine exposure induced changes in neonatal rat lung which resembles damage to type I cells in particular. To determine

whether maternal nicotine exposure indeed induce damage to septal cells and in particular the alveolar epithelial cells, electron microscopy techniques must be employed. It is therefore the aim of chapter 4 to describe and discuss the effect of maternal nicotine exposure on neonatal lung cells, with particular reference to the alveolar epithelial cells.

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CHAPTER 4

CELL INJURY INCURRED DUE TO MATERNAL NICOTINE EXPOSURE ON NEONATAL RAT LUNGS AND ITS MANIFESTATIONS.

4.1 INTRODUCTION

A knowledge of the normal is a necessary prelude to the study of the abnormal, which deals with the alterations in structure and function of the body and its organs, tissues, and cells caused by toxic agents. In the previous chapter, significant differences with respect to cellularity, blood capillary density and type I : type II cell ratio, were observed between the control and nicotine exposed groups. Cell division is related to the demand for growth and replacement in tissues, and the increase in cellularity after nicotine exposure corroberate this principle. The reduction in type I : type II cell ratio suggests the replacement of type I cells which have been selectively damaged due to nicotine exposure, and proliferation of type II cells. This identifies the type II cells as a comparatively injury-resistant cell population which provides a reservior of dividing cells for the replacement of the injury-susceptible type I cells.

Type II cells are also the source of the alveolar surfactant (Crim and Simon, 1988). A deficiency in surfactant leads to alveolar collapse with its attendant physiologic consequences of hypoxemia and poorly compliant lungs (Crim and Simon, 1988). Studies using animals models have also demonstrated alterations in surfactant metabolism after acute lung injury. Le Mesurier et al (1981) found that certain

chemical injury to the lower respiratory tract of rats is characterized by a reduction in the yield of pulmonary surfactant. Additional ultrastructural abnormalities were also observed in type II cells which exhibited hypertrophied cytoplasm, particularly the lamellar organelles (Le Mesurier et al ,1981).

The biosynthesis of organelles involves the transition from one level of organization, the molecular, to a higher level, the supramolecular. The central concept in organelle biosynthesis, is that of self-assembly. This means that the macromolecules will more or less spontaneously associate with one another in specific patterns to build up more complicated structures. The cell lives by and through its organelles - distinctive structures which continuously maintain or replace themselves and assemble new structures as required for the alteration of existing cells, or the formation of new ones through cell division. Intracellular membranes include virtually all the organelles which occur in the cytoplasm of the basic eukaryotic cell. The organelles, which include mitochondria, endoplasmic reticulum, lysosomes, peroxisomes, and Golgi apparatus are defined by membranes of various types. These membranes, in addition to serving specific functions, also serve to separate them from the undifferentiated cytoplasm in which they reside. By means of the structural and functional differentiation of the internal membrane into various types of organelles, intracellular activities of great variety and complexity has been made possible within each cell. Interference with the development of organelles or with the associated enzymes, will influence the function of the cell. This again may result in cell death and interfere with the maintenance of organ function and structure.

One such organelle of great complexity is the mitochondrion. The inner membrane is the seat for oxidative phosphorylation, a process of primary importance to the energy metabolism of cells. Oxidative phosphorylation involves two processes. The first is a proton translocation via the respiratory chain (a multi-enzyme system), that goes from the inside of the membrane to the outside, thereby establishing a proton concentration gradient as well as a membrane potential. The second process involves a proton pump which reverses the proton flux, pumping protons from the outside to the inside of the cell, and in so doing allows the generation of ATP (adenosine triphosphate) from ADP (adenosine diphosphate) and inorganic phosphate (P_i). Any movement of the protons across the mitochondrial membrane is dependent on these two processes since the membrane is impermeable to protons (Siekevitz, 1975).

The endoplasmic reticulum is a clear example of both specificity and dynamic change in intracellular membranes. As stated by Siekevitz (1975), the structure of the endoplasmic reticulum consist of an interconnecting system of membrane-enclosed cisternae which performs a variety of chemical functions, namely protein synthesis (structural and enzymatic proteins), steroid hormone synthesis, phospholipids and detoxification of harmful exogenous substances (as found in liver cells).

From the above it is clear that the intracellular membranes play a central role in both health and disease. Many chemical substances gain access into the cell via the plasma membrane and thereafter modify the intracellular processes in the cytoplasm

or some organelle. Cigarette smoke has been associated with numerous functional and structural changes which occur in the pulmonary alveolar macrophage (PAM). Among the functional changes associated with cigarette smoking, is decreased adherence and increased superoxide release, all involve the plasma membrane (Hannan et al, 1989). The mobility of the membrane components (membrane fluidity) was shown to decrease in smoke-exposed animals (Hannan et al, 1989). Since fluidity of the membrane is associated with changes in cell function, this could therefore be one of the mechanisms via which smoking leads to disease. Patel and Block (1988) have shown that the oxidant gas, nitrogen dioxide, can cause changes in the endothelial cell membrane phospholipid content, consequently decreasing its membrane fluidity.

Type II cells only account for a small percentage (12%) of the total lung cells, but it performs numerous important functions (see Chapter 1) necessary for the efficient functioning of the lung. Type II cells, most importantly responds to lung injury, particularly to type I cells by proliferation to reestablish and maintain the respiratory epithelium. I have therefore deemed it important to specifically investigate these cells to determine the structural changes incurred due to maternal nicotine exposure. The effects of nicotine on various intracellular components of the type II cells was investigated. These components are the mitochondria, endoplasmic reticulum, lamellar bodies and general features pertaining to the cell structure. Mention will also be made of other observations made about other features of the alveolar septa which sets trends within the different age groups. Changes to type I and endothelial cells will also be investigated.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

Animals undergo the same treatment as discussed in Chapter 2.

4.2.2 TISSUE PREPARATION AND ULTRAMICROTOMY

The same procedure is followed as stipulated in Chapter 3.

4.2.3 TRANSMISSION ELECTRON MICROSCOPY (TEM)

4.2.3.1 Principle

The ultra-thin sections are viewed using a Hitachi H-800 Transmission Electron Microscope. TEM involves the passage of a high-voltage (75kV) homogeneous electron beam through a specimen which is thin enough to transmit at least 50% of the incident electrons. The transmitted electrons are then refracted by a series of electromagnetic lenses to form a magnified, two-dimensional image of the specimen. The image can be viewed on the fluorescent viewing screen and it may be recorded photographically. The electron microscope consist of 3 major systems, namely:

1. an illuminating system
2. an imaging system
3. an image translating system

1. The illuminating system comprises a source of radiation (produced by the electron gun) and the condensor lens assembly which focuses the electron beam onto the plane of the specimen.

2. The imaging system consist of a number of lenses which will eventually produce the magnified image of the specimen. The lenses are electromagnetic coils which creates a magnetic field caused by current running through them and enabling them to deflect electrons. In order to focus the electron beam onto a given plane, the current passing through the coil is changed. An increase in current will bring the electron beam to focus closer to the objective lens, while decreasing the current will increase the focal length.

3. The image is formed partly by electrons being scattered by the specimen and partly from electrons which have passed through the specimen. The scattered electrons forms a pattern in the emergent beam which is translated into an image on the fluorescent viewing screen. The amount of scatter is a direct function of the product of specimen thickness and atomic number of the material through which it is passing, and an inverse function of the velocity of the electron beam.

4.2.4 PHOTOGRAPHIC PROCESSING

Agfa E.M. photographic sheet film are utilized for the recording of ultrastructural detail as viewed on T.E.M. Since the photographic emulsions absorb atmospheric moisture, the film sheets must be vacuum desiccated before being introduced into the microscopic vacuum.

4.2.4.1 Negative Development

The developing procedure is standardized at 20 degrees Celcius for 4 minutes in

developer (1 + 9 dilution). The film is agitated every 30 seconds after which it is washed in running water for 2 minutes. The film sheets are then transferred into the fixer (1 + 3 dilution) for another 2 minutes and then again placed in running water for 5 to 10 minutes. The film sheets are then given a quick rinse in water containing wetting agent and then hung out to dry at room temperature.

4.2.4.2 Printing

The micrographs may now be used to make photographic prints. Ilford Multigrade photographic paper is used for all prints. A Berkey Omega Universal D5V 4x5 Enlarger, together with an Intensity Exposure Timer is used to improve the contrast of the pictures. Since the Timer assists in filter selection and exposure time, it helped to reduce the number of trials that needed to be run before a favourable contrast was obtained. After determining the desirable contrast, a sheet of photographic paper is exposed at the set time, using the corresponding filter. After exposure, the paper is placed into the paper developer for 1 minute. Thereafter it is placed into stopbath for 30 seconds and then into fixer for 2 minutes. It is then placed into a waterbath for 5 minutes and left to dry on a drying rack at room temperature.

4.2.5 DETERMINATION OF LAMELLAR BODY QUANTITIES

No less than 5 type II cells per specimen are counted and the cells are randomly selected. The lamellae bodies within the cells are distinguished by their concentric lamellae and their position at the apical end of the cell. Counting was conducted in a clockwise direction, using the first lamellar body as a marker to indicate a

complete rotation.

4.2.6 STATISTICAL ANALYSIS

Statistical analysis is done on the quantitative data obtained for the lamellar body count. The Wilcoxon test for unpaired data is utilized, using the MedCalc package. A probability level of $P<0,05$ is designated as significant in this study. All results are expressed as MEAN ± SEM.

4.3 RESULTS

Swelling, blebbing, distention, fragmentation, obvious rupturing of a cell membrane and discontinuous septal parenchyma are all perceived as lung tissue damage in this present study.

Table 4.1 illustrates the highly significant differences ($P<0,001$) in lamellar body count found between the control and experimental rat pups in all age groups. Within the control group there are significant differences ($P<0,001$) between day 1 and days 7, 14 and 21. The number of lamellar bodies increase from 2,83 ± 0,25 on day 1 to 4,84 ± 0,25 on day 21. This means that there is an increase of 71.02% from day 1 to day 21 after birth.

In the nicotine exposed lungs, there is a similar pattern of increase. Highly significant differences are found between day 1 pups and that of days 7, 14 and 21. The number of lamellar bodies increases from 4,37 ± 0,26 on day 1, to 6,33 ± 0,31 on day 21. This shows an increase of 44,85% between the ages of day 1 and day

21 after birth. It is also important to note that lamellar body count in type II cells of nicotine exposed neonatal rat lung tissue decreases by 24,91% ($P<0,001$) between day 14 and day 21 after birth. This implies that the 44,85% increase in lamellar body count between days 1 and 21 after birth is not a true reflection of the changes in lamellar body status of these cells in the nicotine exposed neonatal rat lungs. Further analysis show that the lamellar body count increases by 92,91% between day 1 and day 14 after birth, compared to the 57,96% within the control group during the same period. In the control lung the lamellar body count plateau on day 7 (Figure 4.1), while it linearly increases in nicotine exposed lung up to day 14 whereafter it decreases. On comparing day 7 and day 14 control neonatal rat lungs, no significant difference is found. This also held true for days 14 and 21 in the control neonatal rat lung. In the nicotine exposed neonatal rat lung, a highly significant difference ($P<0,001$) is found between days 7 and 14 and days 14 and 21, respectively.

On comparing the control and experimental groups, day 1 nicotine exposed lungs contain significantly more lamellar bodies ($P<0,001$) than the same age group in controls. Day 7 nicotine exposed lungs have a lamellar body count of 6,46 ± 0,34 while day 7 control have a lamellar body count of 4,69 ± 0,23, which is significantly less ($P<0,001$) than the nicotine group. Day 14 nicotine exposed pups, having a lamellar body count of 8,43 ± 0,65, show a significantly higher ($P<0,001$) number than the day 14 control lungs, 4,47 ± 0,23. The percentage difference between the two groups reaches its highest level of 88,59% at this stage. It is also important to note that the quantities for day 14 control and day 1 nicotine exposed rat pups are

very similar. This has also been found in data obtained for cellularity and LIC (in the previous chapter).

Within the type II cells, the lamellar bodies are found near the apical surface of the cell and the mitochondria more towards the basal part of the cell, as well as along the longitudinal axis of the cell. Typical lamellar bodies are illustrated in Figure 4.2. Mitochondria in the day 1 to day 21 control lungs are generally characteristic in structure. The mitochondria are spherical or oval shaped, having both an external and internal membrane. The internal membrane projects folds, the cristae, into the interior of the mitochondrion. The cristae enclose 2 spaces, the intercristal space (that is the space enclosed by the internal membrane) and the intracristal space (that is the outer space between the outer and inner membrane). The cristae are flat and shelf-like, with electron dense granules occurring in the matrix between the cristae (Figure 4.3)

Mitochondria in the type II cells of the nicotine exposed pups differ from those found in the control group. These mitochondria show swelling and distention, and the cristae are disrupted and fragmented (Figure 4.4). The cristae also appear to be less in number than found in the control group. The matrix between the cristae stain lighter than that of the control group, with the matrix being more granular in the nicotine exposed lungs. The internal membrane appeared to be completely broken down with membrane fragments occurring in the matrix. The mitochondria are closely packed within the type II cells of the day 7 control group (Figure 4.5) and resembles that of type II cells of 1 day old control rat pups (Figure 4.2).

Microvilli also occur on the luminal surface of the type II cell (Figure 4.5) and this characteristic feature of the type II cells of lung tissue of 21 day old control rats is also illustrated in Figure 4.3. Some type II cells in the nicotine exposed lungs showed microvilli, while others which occurred along the surface of the alveoli appear to have lost their microvilli (Figure 4.6). These cells become more elongated, typical of an intermediate between type I and type II cells. Macrophages could also be seen in the alveolar space and close to cellular debris present in the alveolar space (Figure 4.6). A characteristic of the septa of the lung tissue of nicotine exposed rat pups is the presence of groups of type II cells (Figure 4.7). This is not found in septa of lung tissue of control rat pups. Swelling of the endothelial cells occur as well as swelling of the mitochondria within these cells (Figure 4.8). Blebbing of type I cell membranes also occur (Figure 4.8). Swelling and formation of membrane blebs of endothelial and type I cells are often a feature of these cells in nicotine exposed lung tissue of rat pups of all age groups under investigation. Ruptured blood-air barriers are also found in the nicotine exposed lung tissue of the rat pups (Figure 4.9).

TABLE 4.1: LAMELLAR BODIES PER TYPE II CELL (MEAN ± SEM)			
AGE (Days)	**CONTROL**	**NICOTINE**	**%▲**
1	2,83 ± 0,25 (n = 23)	4,37 ± 0,26 (n = 35)	54,42 P<0,001
7	4,69 ± 0,23 65,72% P<0,001 (n = 36)	6,46 ± 0,34 47,83% P<0,001 (n = 39)	37,74 P<0,001
14	4,47 ± 0,23 57,95% P<0,001 (n = 38)	8,43 ± 0,65 92,91% P<0,001 (n = 35)	88,59 P<0,001
21	4,84 ± 0,25 71,02% P<0,001 (n = 37)	6,33 ± 0,31 44,85% P<0,001 (n = 42)	30,79 P<0,001

Day 1 was used as a base value and days 7, 14, and 21 were compared with day 1 for statistical purposes. %▲= Percentage difference; n=number of type II cells counted.

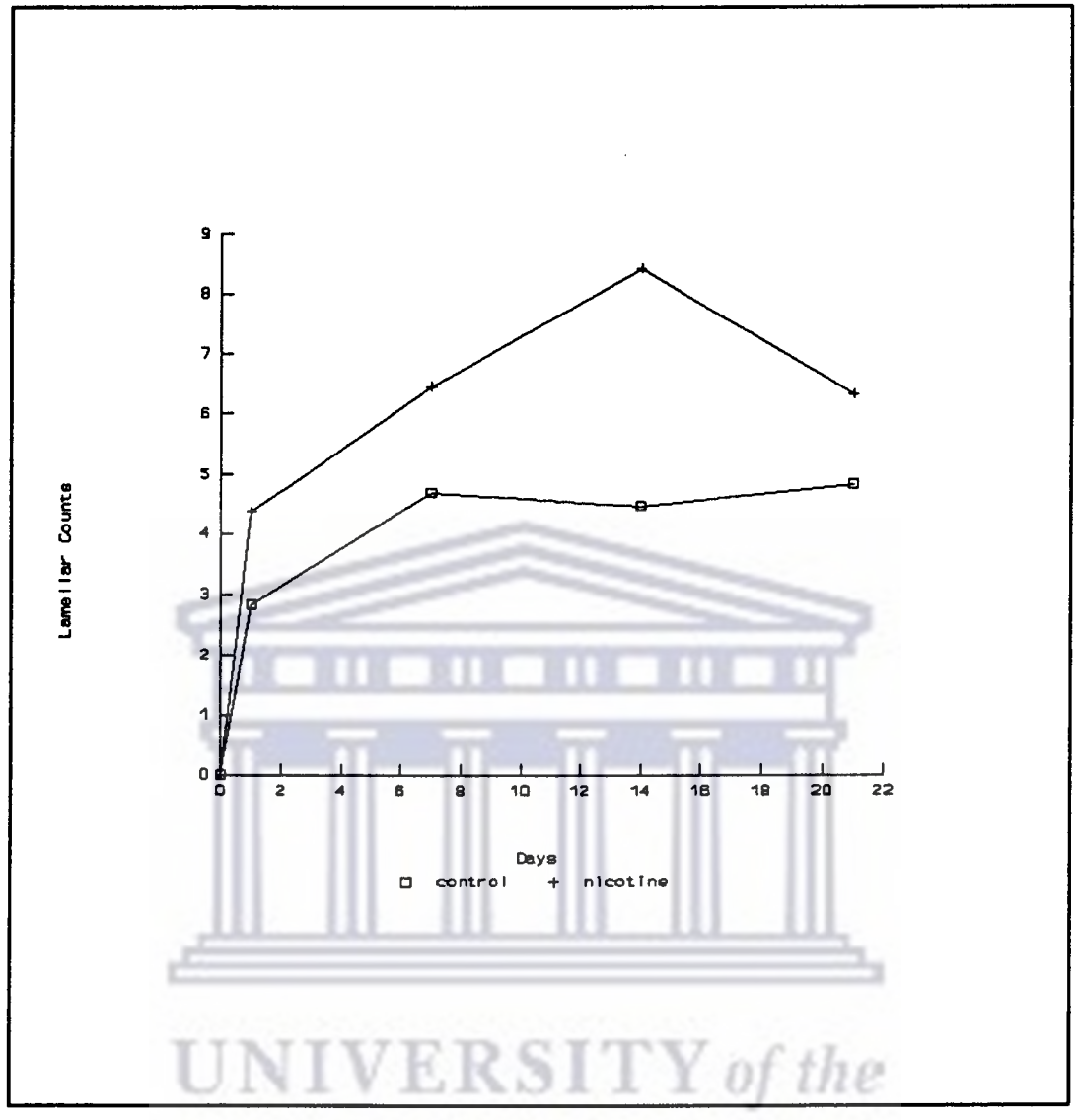

Figure 4.1 Graphic representation of the lamellar body count per Type II cell. Note that in control lungs, the lamellar body content plateau, while it increases linearly in nicotine exposed lungs.

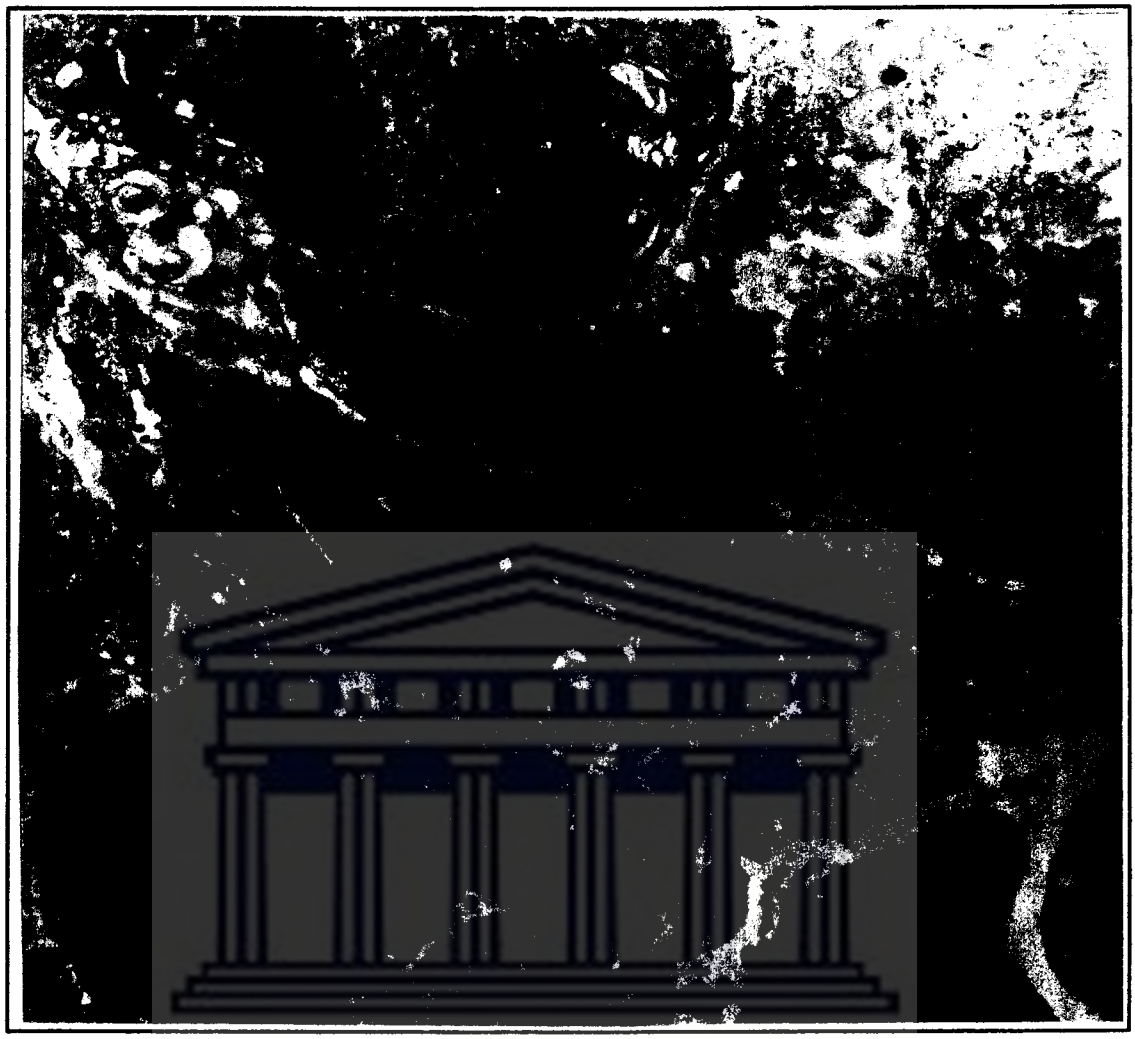

Figure 4.2 Type II (T2) pneumocytes of 1 day old control rat lung tissue showing lamellar bodies (L) and mitochondria (arrowheads). n=nucleus. (10 000X magnification; 1 cm=1μm).

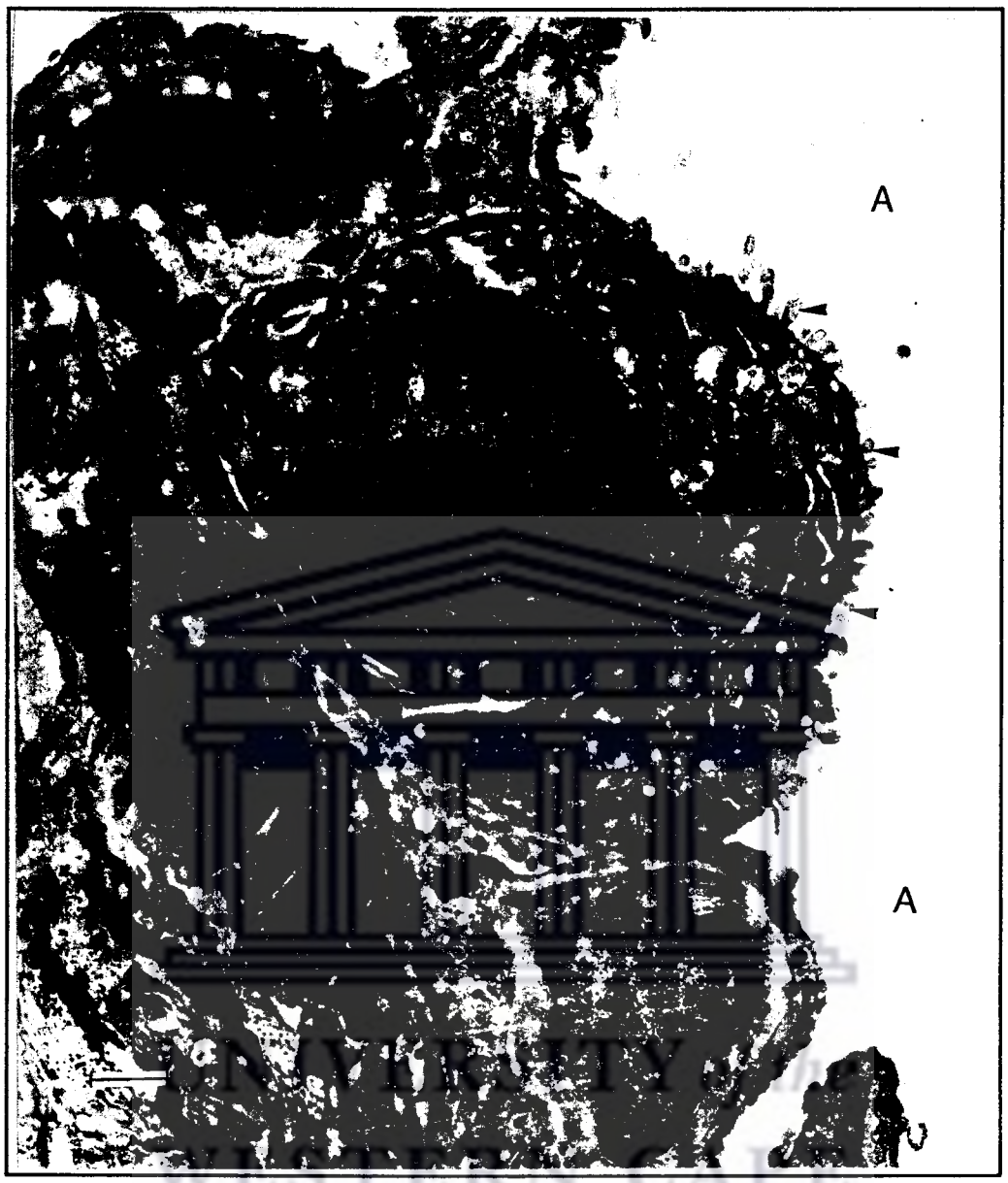

Figure 4.3 Type II (T2) cell of lung tissue of 21 day old control lung tissue showing the characteristic microvilli (arrowheads) on the alveolar (A) surface of the cell. Also note the peripherally arranged chromatin (long arrow) in the nucleus (n). Mitochondria (m) are also visible. (10 000X magnification; 1cm=1,0μm)

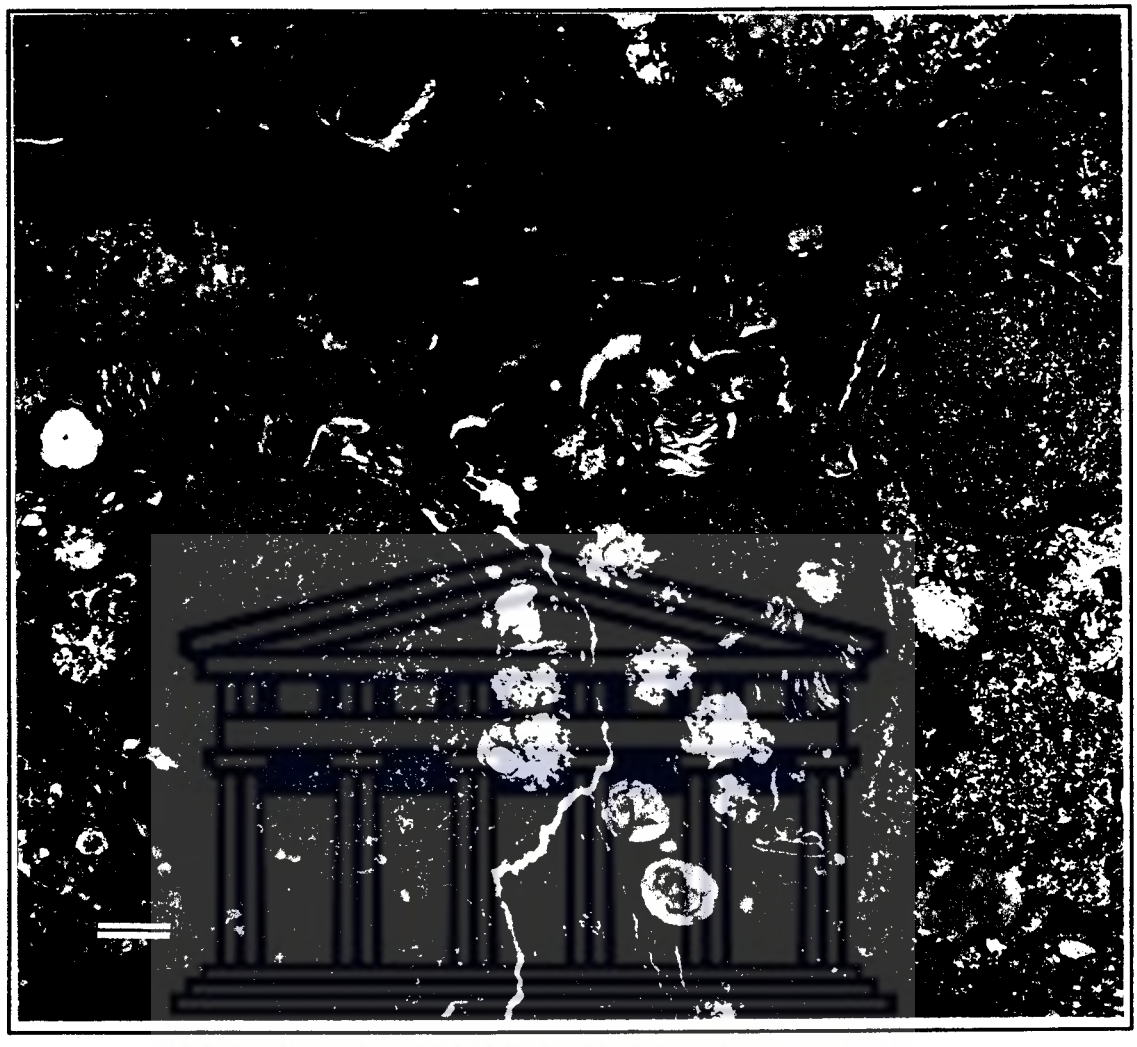

Figure 4.4 Type II (T2) pneumocytes of 1 day old nicotine exposed rat lung tissue showing swollen mitochondria (arrowheads), and prominent lamellar bodies (L). No microvilli can be seen. n=nucleus. (15 000X magnification; 1 cm=0,67μm).

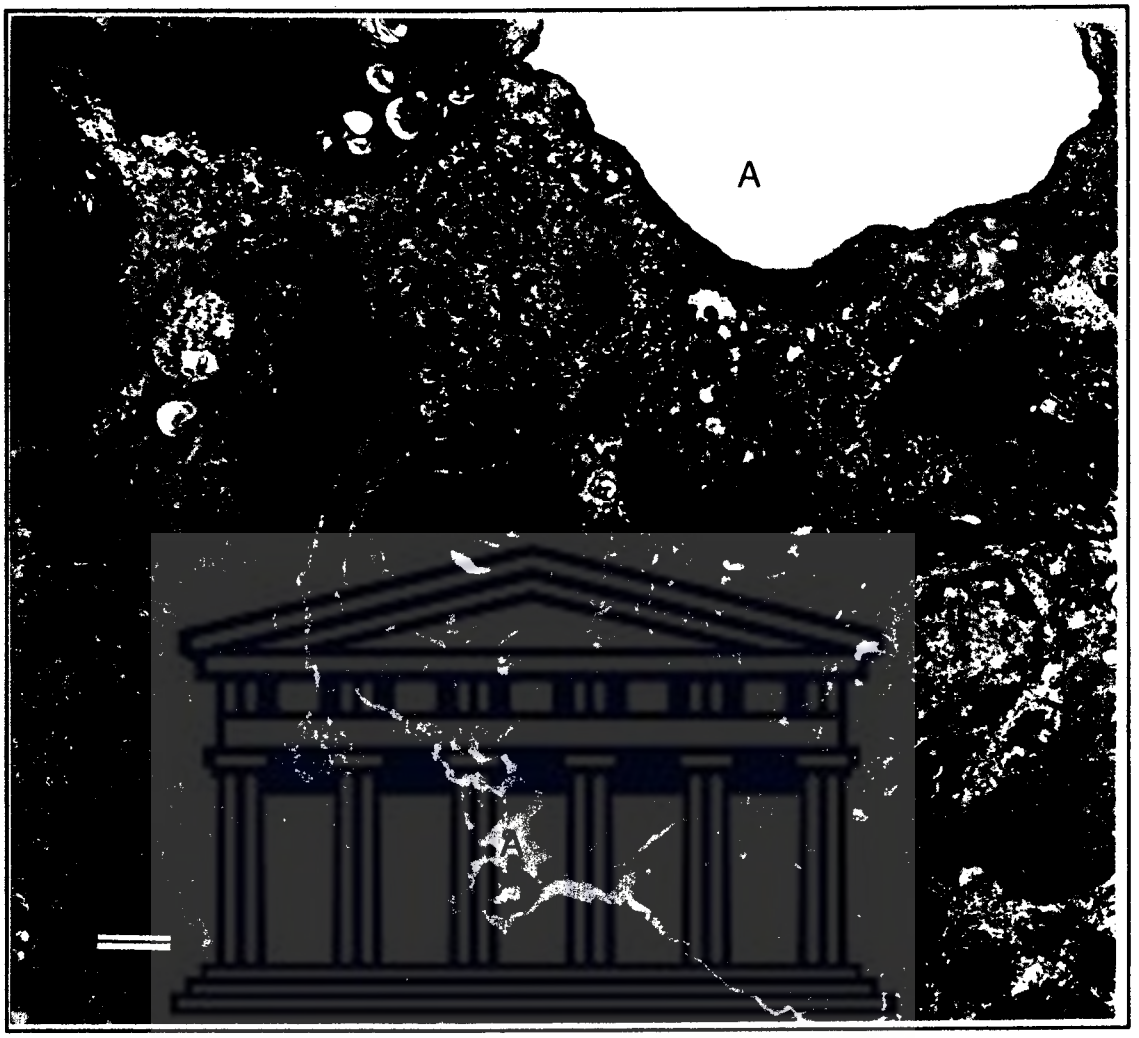

Figure 4.5 The location of mitochondria (m) and lamellar bodies (L) in type II (T2) cells of lung tissue of 7 day old control rat pups are illustrated above. Note the intense staining of the mitochondria compared to that of the nicotine exposed rat lung in Figure 4.6. Note the presence of microvilli (arrowheads). A=alveolar space. (7 000X magnification; 1cm=1,43μm)

Figure 4.6 Type II (T2) cell of day 7 nicotine exposed lung showing the absence of microvilli and swollen mitochondria (arrowheads). Macrophages (M) and cell debris (long arrow) often occur in the alveolar (A) space. (9 000x magnification; 1cm=1,11μm)

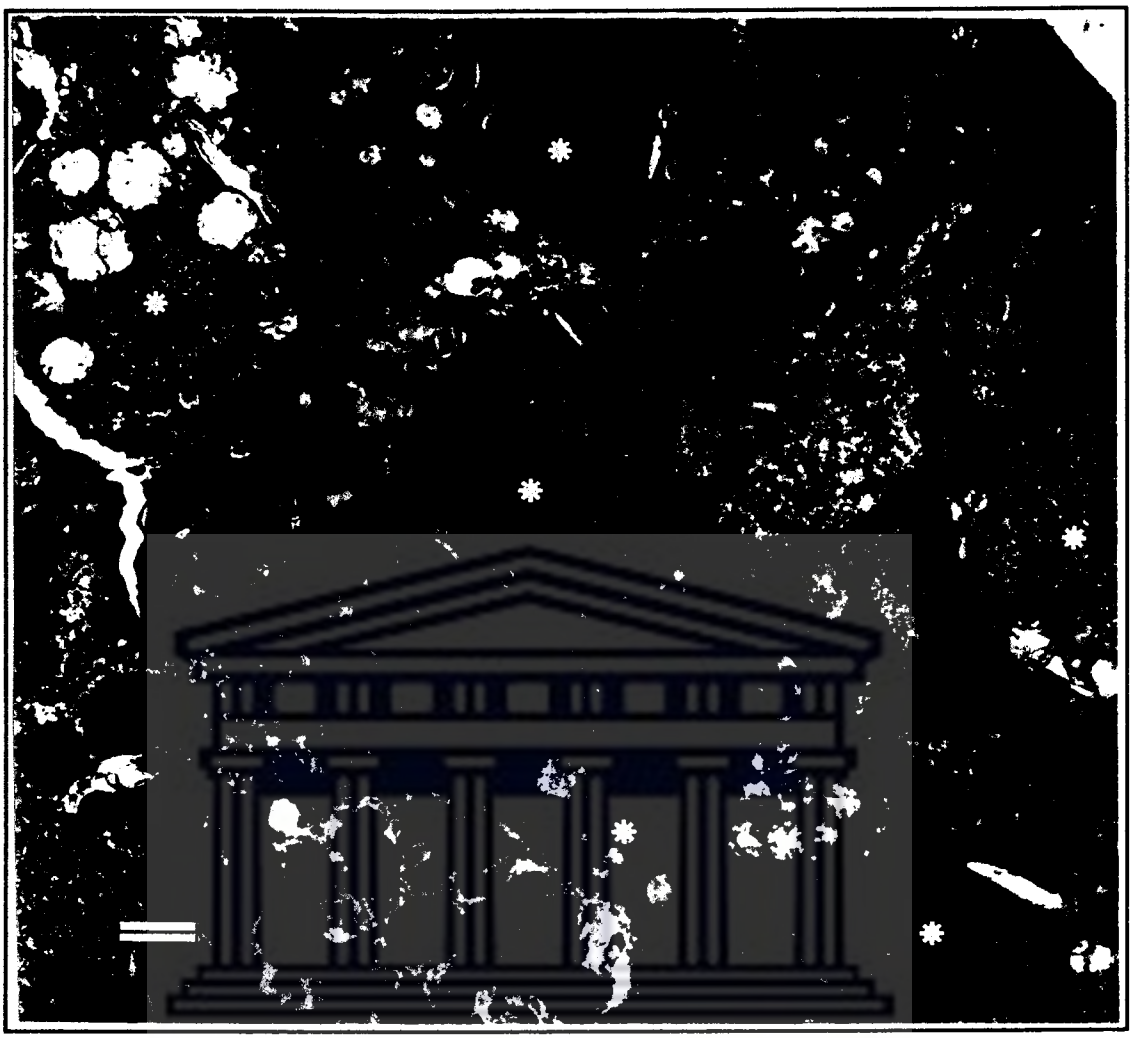

Figure 4.7 Section through the septum of lung tissue of a 21 day old nicotine exposed rat pup illustrating several type II cells (asterisks) grouping together in the interstitium. Note the swollen mitochondria in all the type II cells (arrowheads) and the absence of microvilli. (9 000X magnification; 1 cm=1.11μm).

Figure 4.8 Illustrating the swelling and blebbing (arrows) of the type I cell of the blood-air barrier in the lung of 1 day old nicotine exposed rat pup. Swollen mitochondria (arrowheads) occur in the distended capillary endothelial cell (asterisks). Note the reduction in the diameter of the capillary (Cap). n=nucleus. (18 000X magnification; 1 cm=0,5μm).

Figure 4.9 An illustration of the total destruction of the blood-air barrier (arrowheads) in a day 7 nicotine exposed lung. (24 000x magnification; 1 cm=0,42μm).

4.4 DISCUSSION:

Under most circumstances cells try to maintain a steady state. However, if their environment is altered in any way, they will adapt to the change without their function being significantly impaired. Alteration in functional demands could lead to an increase or decrease in undivided cell size and/or an increase or decrease in cell number. If the degree of change is so great that the cell become incapable of adapting to the changes, then some loss in its normal range of functions will most likely occur. The degree of loss may be so great as to involve vital cellular functions and consequently lead to cell death. The maintenance of the cell's steady state involves a number of basic metabolic functions. These metabolic functions and structural elements are so intricately interwoven in the cell, that whatever the precise point of attack may be, will also affect other functions and elements of the cell (Woolf, 1977).

Damage to cells within the alveolar septa are observed in the lung tissue of the nicotine exposed rat pups. It is evident from the state of the cells that the osmotic and fluid homeostasis had not been maintained. This is illustrated by:

1. swelling of mitochondria within the type II cells. This was also found in the mitochondria of endothelial cells.
2. Swelling and blebbing of type I cells as well as endothelial cells.

Furthermore:

1. The type II cells showed smooth laminar surfaces thus lacking the characteristic microvilli.
2. The nicotine exposed lungs show grouping of type II cells within the septal

interstitium.

3. The lamellar bodies within the type II cells show an increase in number (Table 4.1).

Another important observation is that all the changes induced in type II cells in nicotine exposed neonatal rat lungs, occur during the fetal and early postnatal stage of development. From day 7 after birth, onward, the abovementioned changes are only maintained and do not worsen. Signs of early damage include swelling and blebbing which can be reversed after the removal of the causative factor, namely nicotine. Should the cause remain, it will eventually result in cell death (Figure 4.9). However, despite the fact that type II cells are more resistant to damage, irreversible damage also occur in some of these cells because the mitochondria showed disrupted cristae as well as membranous figures. Since type II cells serve as progenitors of type I cells, the death of type I cells resulted in type II cell proliferation. However, if these type II cells are irreversibly damaged, it is plausible that the rate of proliferation and differentiation is not effective enough to replace the damaged type I cells.

On comparing the quantitative results (Table 3.2) with the structural changes observed, it is evident that the increase in type II cell numbers and the decrease in type I cell numbers in the lungs of rat pups exposed to nicotine, is a clear indication that type II cells are proliferating in an effort to replace damaged type I cells (grouping observed of type II cells, Figure 4.7). From the ruptured blood-air barriers (Figure 4.9) it can therefore be suggested that the rate of proliferation and

differentiation is not sufficient to curtail the damage to type I cells. The rate of proliferation may also be slowed due to insufficient energy being available for mitosis. Glucose and glycogen are important energy sources used during mitosis (Bullough, 1952). Since maternal nicotine exposure suppresses glycogenolysis in neonatal lung (Maritz, 1987), the release of glucose-6-phosphate which is necessary for energy release via the glycolytic pathway is also suppressed. This will therefore adversely affect the developmental process of the lung and can result in retardation and even abnormal alveolar formation.

The proliferation and differentiation of type II cells and general lung development is influenced by epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) in fetal lung. Type II cells are a source of TGF-α in fetal lung, and TGF-α and EGF act parellel (Strandjord et al, 1994). Therefore, it is plausible that the damage to the type II cell can affect fetal lung morphogenesis and maturation.

Many functions are mediated via the plasma membrane, whether it serves as a cell membrane or as a membrane binding an organelle for example mitochondria. The transport of metabolites via energy-dependent transport systems require intact membranes in order to preserve osmotic and fluid homeostasis (Siekevitz, 1975). In this study is has been shown that the laminal surface of the type II cells lack their characteristic microvilli, thus suggesting a change in the functional characteristics of the membrane. Furthermore, the severe mitochondrial swelling observed show irreversible damage, which theoretically interferes with the energy metabolism

within these cells. This could lead to gradual deterioration of the cell and all its functions. Further investigation into this specific aspect will be undertaken.

Cells may also acquire an imbalance in its intracellular volume, thus causing swelling and blebbing, due to partial failure of the Na^+-K^+ pump. It was indeed illustrated by Meyer et al (1971) that nicotine suppresses the Na^+-K^+-ATP-ase in cell membranes. Maritz and Burger (1992) has found that nicotine interferes with the hydrolysis of ATP which is necessary for the maintenance of the Na^+-K^+-ATP-ase in the cell membrane. Since ATP hydrolysis is adversely affected, it will lead to partial failure of the sodium-potassium pump. The differential with respect to sodium and potassium ions within the cell is maintained by this ATP energy-dependent membrane transport system. The control of volume of the cell and its membrane-bound organelles is largely exerted by this pump. Due to the partial failure of the pump, the potassium ions diffuse out of the cell into the extracellular fluid and the reverse applies to the sodium ions. The influx of large amounts of sodium ions will be accompanied by the influx of water into the cell (and mitochondria), thus leading to increased volume and swelling. Whether the swelling observed is a direct effect of nicotine, or whether it is related to the inhibition of glycolysis with resulting membrane impairment, is not known. However, since maternal nicotine exposure induce swelling of mitochondria and since exposure to cigarette smoke also cause mitochondrial swelling, it is conceivable that the swelling could be due to the presence of nicotine in the cigarette smoke (Kyle and Riesen, 1970).

Apart from its influence on ATP metabolism and Na^+-K^+-ATPase, and thus most likely on the membranes, nicotine can also indirectly affect membrane integrity. Nicotine has two major metabolites, namely cotinine and nicotine-N-oxide (Rotenberg and Adir, 1983), where nicotine-N-oxide is a free radical. Apart from being the source of nicotine-N-oxide, nicotine also enhances superoxide anion O_2^- generation and its release from neutrophils (Jay et al, 1986; Gillespie et al, 1987). The mechanism whereby nicotine augments neutrophil superoxide anion generation is not clear. It is suggested that it may induce release of this oxidant by causing general membrane fluidization (Jay et al, 1986). The formation of free radicals is a common effector pathway associated with membrane injury (Jennings and Reimer, 1981). Since nicotine-N-oxide is a free radical, nicotine metabolism may increase the concentration of free radicals within the cells. The damage observed in mitochondria may therefore be caused by the high concentration of free radicals present within the cells of lung tissue of rats exposed to nicotine. Uncontrolled activity of free radicals leads to blebbing of the membranes and a failure to maintain the normal fluid and ionic relationships between the intracellular and extracellular compartments (Frank, 1991) These features have been found in this study, especially at the level of the blood-air barrier pertaining to both the capillary endothelial cell and the type I cell (Figure 4.9).

Nicotine may also effect its damage on membranes via nicotine-N-oxide which could affect various components of the membrane. The plasma membrane is composed of proteins, enzyme systems, and receptors associated with a lipid bilayer. The mobility of the membrane components is termed membrane fluidity and this

represents an inherent biophysical property of the membrane. Changes in fluidity is associated with changes in cell function. Constituents of cigarette smoke (for example free radicals) are potentially capable of altering fluidity, as was found by Stephens et al (1989) and Jay et al (1986). Fluidity of the membrane can be affected via the fatty acids which undergoes reaction with the free radicals (nicotine-N-oxide) thus causing tighter packing within the membrane, thereby changing the structural and functional characteristics of the membrane.

For secretagogues such as ß-adrenergic agonists and catecholamines to activate adenyl cyclase with the sebsequent formation of cAMP from ATP, the presence of specific receptor sites are required on the membrane. In addition to the intracellular ATP, ATP present in the bronchiolar fluid is also involved in the regulation of surfactant secretion, by binding to the P_2-purinoceptors on the surface of type II cells (Rice and Singleton, 1986; Gilfillan and Rooney, 1987).

Type II cells contain lamellar bodies in which the pulmonary surface-active material, surfactant (Mason et al, 1977) is found. In this study a build up of lamellar bodies within type II cells was found in nicotine exposed rat lung tissue. Surfactant is responsible for maintaining the low surface tension at the air-liquid interface in the alveoli, thus preventing it from collapse at low lung pressures. It is known that a balance exists between release of lamellar bodies, and thus of surfactant, into the alveolar space and the removal thereof in order to prevent an accumulation within the alveoli which could lead to alveolar collapse (Fisher and Chander, 1985). The secretory activity of the surface-active extracellular lining, has been shown to be an

energy-dependent process (Massaro et al, 1975) involving the microtubular and microfilament systems within the cell (Marino and Rooney, 1980). In a study done by Rice et al (1985) it was demonstrated that cytochalasins, which disrupt microfilaments, leads to enhanced phosphatidylcholine release in association with alteration of filamentous actin around the lamellar bodies. Actin also form part of c-AMP-dependent protein kinase present in type II cells, and the actin phosphorylation during the perinatal period of lung development occurs with enhanced surfactant synthesis and release (Whitsett and Lessard, 1984).

It is generally accepted that oxidative metabolism provides the energy for secretory processes (Massaro et al, 1975), and studies have shown that there is suppression of glycolysis due to nicotine exposure (Maritz, 1987). This could subsequently affect surfactant secretion due to depletion of ATP concentration within the cell. In the present study it is shown that maternal nicotine exposure increases the number of lamellar bodies per type II cell. This could be by either suppressing excretion or stimulating the rate of synthesis of the lamellar bodies. Other studies have shown that exposure to cigarette smoke results in seemingly excessive numbers of lamellar bodies within hypertrophied type II cells, as well as reduced surfactant yield in endobronchial lavage, of up to 50% of that of the control group (Le Mesurier et al, 1981). These findings were also associated with areas of alveolar collapse. Studies have shown that oxygen metabolites does impair surfactant metabolism, since it progressively decreases the incorporation of the amount of precursors into phosphatidylcholine by type II cells (Crim and Simon, 1988). It is therefore feasible that a decrease in surfactant yield by type II cells would cause an increase in

alveolar fluid surface tension, which will consequently promote the collapse of small alveoli, a feature which was also observed in the present study (Figure 2.14). The pathogenic changes which are caused by surfactant deficiency are demonstrated most clearly in children suffering from infant respiratory distress syndrome (Crim and Simon, 1988). Maternal smoking also adversely modifies fetal lung growth (Collins et al, 1985), increase incidence of pneumonia and bronchitis, impaired lung function (Colley et al, 1974), and general respiratory disorders in children of smoking parents (Maritz, 1987).

Catalase and superoxide dismutase form part of the antioxidant enzyme system (AOE) and serve as oxygen metabolite scavengers in the extracellular environment as well as within cells. Additional evidence also shows that the protective effect of these scavengers on type II cells are lost with prior inactivation of these enzymes after oxygen metabolite exposure. It is interesting to note that damage very similar to that found in this present study has also been observed in studies done on ischemic injury. It was found that ATP concentration was low, thus resulting in membrane damage and swollen mitochondria with distorted cristae (Jennings and Reimer, 1981). Also, the chromatin of the nuclei become peripherally arranged and membrane blebs are visible. It is therefore tempting to speculate that the adverse effects of maternal nicotine exposure on neonatal lung, is due to the effect of nicotine-N-oxide and oxygen radicals released by macrophages in response to nicotine exposure. Further studies to investigate this is underway.

4.5 CONCLUSION:

A considerable body of evidence has been and continues to be accumulated, which supports involvement of nicotine in the pathogenesis of smoking-induced lung injury. Despite all the morphological changes brought about after chronic nicotine exposure during gestation and lactation, the exact mechanism of action has yet to be elucidated. However, the following structural pathogenesis incurred by neonatal rat lungs due to maternal nicotine exposure has been established and confirmed in this study:

1. There are significantly higher numbers of cells in the septa of the nicotine exposed animals than in the control lungs.
2. Type I cells are significantly less in the nicotine exposed lungs.
3. Type II cells are significantly higher in the nicotine exposed lungs.
4. Lamellar body count in nicotine exposed lungs are significantly higher than in the control lungs.
5. The number of capillaries per unit length of septum is significantly lower than that of the control lungs.
6. Mitochondrial changes within the nicotine exposed lungs includes swelling and distorted cristae.
7. Plasma membranes in the nicotine exposed lungs show swelling and blebbing.

It is important to note that the lesions induced in type II cells due to maternal nicotine exposure resembles that caused by cigarette smoke (Latha et al, 1988; Stephens et al, 1989). It is therefore plausible that maternal smoking during

pregnancy and lactation will interfere with type II cell integrity, thus affecting lung development and may thus result in the pathogenesis of progressive alveolar collapse and eventually emphysema. Due to the interference by nicotine during the fetal and early postnatal period of rapid lung modelling and growth, the lungs may be rendered more sensitive to the harmful effects of foreign substances to which the lung is exposed. This may render the lung more susceptible to respiratory disease.

Although current information derived from biochemical (Gillespie et al, 1987) and morphological studies provide a reasonable explanation for the link between smoking and chronic obstructive pulmonary disease, the effectors of lung injury present in cigarette smoke, specifically nicotine, and the pathways via which it mediates its effect, remains a challenge for future investigation.

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4.7 SUMMARY

Formation of alveolar septa is one of the most essential processes in the morphogenesis of alveoli. This study deals with the structural aspect of lung development, specifically investigating the morphology of the alveolar septum. The aim of this study is therefore to investigate the effect of maternal nicotine exposure on neonatal rat lung septal status by investigating cellular characteristics of the alveolar septa as well as capillary density. Emphasis is put on the type II cell since it plays a very important role in maintaining alveolar epithelial integrity and lung modelling. Female Wistar rats are mated and then randomly assigned to control and experimental groups. Those assigned to the experimaental group are treated with nicotine on a dosage of 1 mg nicotine/kg body wt/day. The nicotine is administered via subcutaneous injection until the birth of the pups, whereafter the dams are injected intraperitoneally. The present study illustrates that the cellularity of the alveolar septa of the lungs of the nicotine exposed rat pups were higher than that of the control animals. The change in septal cellularity between days 1 and 21 after birth in the nicotine exposed lungs were lower than that of control lungs, thus indicating that the increased cellularity observed could be due to the interference of maternal nicotine exposure with fetal lung development. The present study also showed damage being induced in type I cells, with subsequent type II cell proliferation. These changes would therefore also affect the type I : type II cell ratio. The present study illustrated that the type I : type II cell ratio for nicotine exposed lungs decreased and the difference between the control and experimental groups increased as a function of age. The inability of the type II cell to effectively replace the damaged type I cells, resulted in the rupturing of some of the blood-air

barriers. Swollen mitochondria were observed in both the capillary endothelial cells and the type II cells. This is an indication of irreversible damage within the cells, thus also supporting the argument that irreversibly damaged capillary endothelial cells could also contribute to the rupturing of the blood-air barriers and that the type II cells are ineffectively replacing damaged type I cells. The build up of lamellar bodies observed within the type II cells could be due to an imbalance in the rate of synthesis of lamellar bodies or suppression of surfactant excretion. Surfactant is responsible for maintaining the low surface tension in the alveoli, thus preventing alveolar collapse at low pressures. Areas of collapsed alveoli has been observed in the regions where type II cells containing increased lamellar bodies were found.

Maternal nicotine exposure during pregnancy and lactation suppresses capillary formation in the fetal and neonatal rat lung. Since capillary formation is essential for septal formation, which in turn plays an important role in alveolarization, it is therefore expected that less alveoli will subsequently be formed.

The interference of nicotine with lung development therefore resulted in changes in septal morphology as well as septal morphometry, thus adversely affecting the structural characteristics of the lung. This could lead to inefficient functionality and increased susceptibility to respiratory disease due to abnormal lung development.

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APPENDIX A

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THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL LUNG ALVEOLAR EPITHELIAL STATUS: AN ELECTRON MICROSCOPE STUDY.

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ABSTRACT

The aim of the present study was to investigate the effect of maternal nicotine exposure (1 mg nicotine/kg body mass/day) on neonatal lung alveolar epithelial cells. Rats (Wistar descendants) were used. The data illustrate that maternal nicotine exposure during pregnancy and lactation resulted in alveolar fenestrations, blebbing and rupturing of the blood-air barrier. The type I pneumocyte appears to be more sensitive to the effect of nicotine than the type II pneumocytes.

INTRODUCTION

Nicotine, a major component of tobacco smoke, is implicated in the occurrence of abnormal lung conditions (Maritz, 1993). Research indeed illustrated that maternal nicotine exposure during pregnancy and lactation interfered with carbohydrate metabolism (Maritz, 1989; Maritz and Burger, 1992) and structural development of neonatal rat lung (Maritz and Woolward, 1992; Maritz et al 1993). The radial alveolar count, which serves as an estimate of the number of alveoli, is also markedly reduced in the lungs of those neonatal rats exposed to nicotine via the mother's blood and milk (Maritz and Woolward, 1992).

The epithelium lining the alveoli is primarily made up of large squamous type I cells and smaller type II cells (Evans et al, 1975). The type I cells cover more than 90% of the alveolar surface (Naimark, 1977). The type II cells are dispersed throughout the alveoli between the type I cells (Evans et al, 1975). The lung alveolus is also the site for gas exchange and damage to the alveoli will thus render gas exchange less effective.

Studies by Kauffmann (1980) show that after birth the lung enters a phase of rapid cell proliferation during formation of alveoli. In rats this phase of rapid cell proliferation and alveolarisation occurs between days 4 and 13 after birth (Brody and Vaccaro, 1979). In the human, exposure of the fetal and rapidly developing neonatal lung to various chemicals via the mother's blood or milk, for example due to maternal smoking, may interfere with normal lung development and maturation and render the lung more susceptible to disease. An increased incidence of respiratory disorders has indeed been observed in children of smoking parents (Yarnell and St leger, 1979, Schilling et al, 1978). In some epidemiological studies a relationship is drawn between diseases of the distal airways of children of smoking parents and chronic bronchitis and emphysema of the same individuals in adulthood (Wall et al, 1985).

The basis of all disease is injury to the smallest living unit of the body, namely the cell. Since nicotine interferes with cell energy metabolism and thus with the ability of the cell to maintain its integrity, the aim of this study was to investigate the effect of maternal nicotine exposure on neonatal lung alveolar epithelial cells and thus alveolar status, using the electron microscope. This may give some insight into the etiology of smoke related lung disease.

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MATERIALS AND METHODS

Animals: White virgin female rats (Wistar descendants) of 200-250g were used in the investigation and were fed a stock diet (Epol rat cubes) throughout the experiment. Room temperature was kept at 22°C and a day night cycle of 12 hours was maintained.

We maintained our own breeding program. Animals were mated overnight and were afterwards randomly assigned to control and experimental groups. The length of gestation averaged 22.5 days for both control and experimental groups. The treatment procedure and dosage (1 mg nicotine/kg/mass day) was the same as described by Maritz (1987). Control animals received normal saline instead of nicotine. Rat pups were randomly selected from each of 4 control litters and 4 nicotine exposed litters to give a total of 12 control and 12 nicotine exposed rat pups. The animals were killed by decapitation 24 hours after the last exposure. The lung tissue was then quickly removed and samples collected for morphologic studies. Two to three week old rat pups were used.

Sample Preparation:

a) Transmission electron microscopy. The terminal aspect of the middle lobe of the right lung was removed, fixed in 2.5% glutaraldehyde in 0.25 M potassium phosphate buffer (pH 7.2), for 1 hour and cut into 1 mm portions. These were fixed for a further 30 minutes, rinsed in buffer and postfixed in 1.5% osmium tetroxide for 14 hours at 4°C. After rinsing in distilled water, the samples were placed in 1.0% uranyl acetate for 15 minutes before dehydrating in graded acetone and embedding in Spurr's resin.

For both control and nicotine exposed lungs 70mm thick section were prepared by using standard techniques. Staining was done by the method of Sato (1967).

b) Scanning electron microscopy. The dissected lung tissue was postfixed in 2.5% Sorenson phosphate buffered glutaraldehyde (pH 7.4), dehydrated with graded ethyl alcohol solutions, and dried by the critical point method (Van der Horst et al, 1989) using a Hitachi HPC-2 critical point dryer. The dried specimen was coated with a layer of gold (20 nm), using an Edwards S150B sputter coater. The prepared samples were examined with a Hitachi model x650 scanning electron microscope.

RESULTS

a) Scanning electron microscopy. The results show clearly that exposure of female rats to nicotine during pregnancy and lactation, interfered with neonatal alveolar development. This is illustrated by numerous alveolar fenestrae (fig. 1) in the lungs of the nicotine exposed rat pups. No fenestrae occured in the alveoli of lung tissue of control rat pups of the same age (fig. 2). Apart from fenestrations, membrane blebs (fig. 3) as well as areas of more severe cell damage also occur. These include rupturing of the blood-air barriers (fig. 4) which implies that type I epithelial cells and capillary endothelial cells were destroyed. In some instances this cell destruction resulted in damage of almost the entire alveolar surface (fig. 5).

b) Transmission electron microscopy. Transmission electron micrographs also illustrate ruptures in blood-air barriers (fig. 6) Some septae in lungs of nicotine exposed rat pups were completely destroyed.

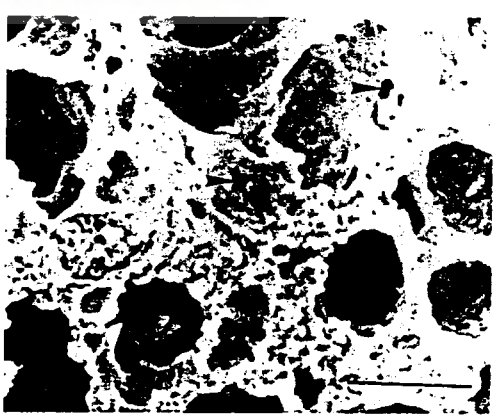

Figure 1. Fenestrations in alveoli (arrowheads) of nicotine exposed lung. Bar = 40μ.

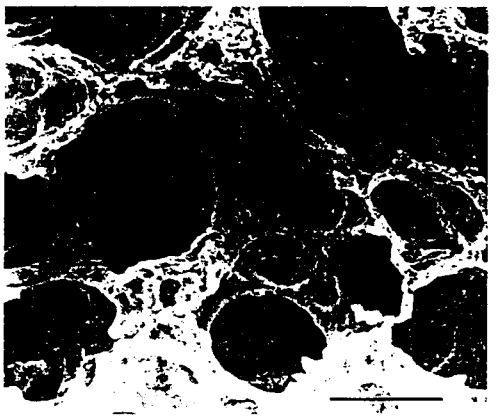

Figure 2. Alveolar surface of control lung. Bar = 60 μm.

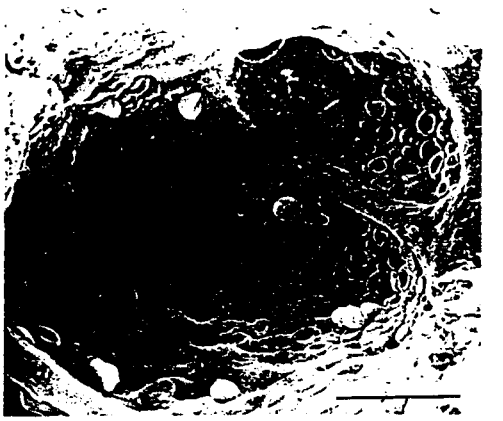

Figure 5. Grossly damaged alveolar surface Bar = 20 μm. Arrowhead = alveolar ridge.

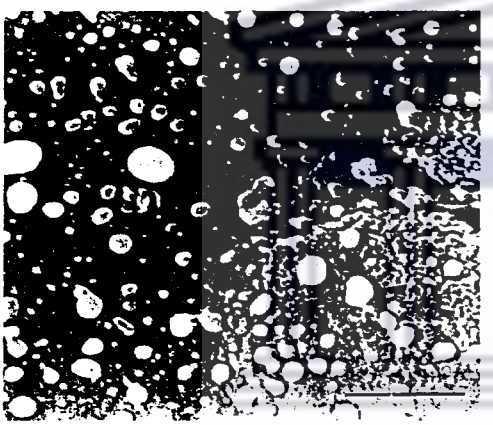

Figure 3. Alveolar surface of nicotine exposed lung. Membrane blebs occur (arrowheads). Bar = 3.0 μm.

Figure 6. Transmission electron micrograph illustrating ruptured blood-air barrier. a = alveolar space, c = capillary. Small arrowhead = blood-air barrier. Large arrowhead = ruptured blood-air barrier. (x1500).

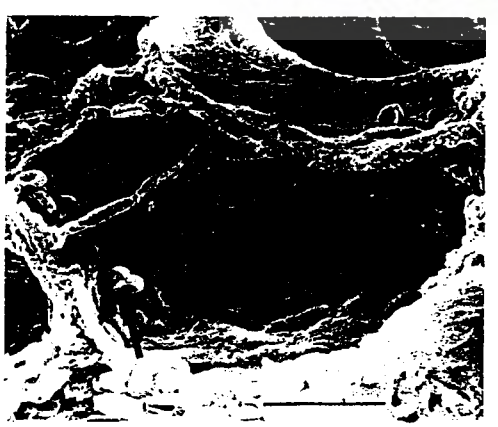

Figure 4. Ruptured blood-air berrier. Erythrocytes (arrow) leaking out of the capillary. Bar = 20 μm.

Figure 7. Destroyed septum of a nicotine exposed neonatal lung. Arrowheads indicate breaks in capillary walls. (x5900)

DISCUSSION

When the mother smokes during pregnancy and lactation, the fetus and neonate is exposed to those components of cigarette smoke which cross the placenta and which occur in the milk of the nursing mother. One of these components is nicotine which is considered to be the pharmacologically most active constituent of tobacco smoke (Dahlstrom et al, 1990). During pregnancy, nicotine passes the placenta and exposes the fetus to concentrations similar to those in the blood of the smoking mother (Luck and Nau, 1984; Greenberg et al, 1984). After birth the infant may still be exposed to these substances through passive smoking and breast feeding (Luck and Nau, 1984 and 1985). Nicotine also accumulates in the respiratory tract of the developing lung (Szutz et al, 1978). During the fetal stage of lung development, alveolar sacs originate from these respiratory tracts (Kuhn, 1982). In the neonatal rat lung these sacs rapidly develop into alveoli between day 4 and 13 after birth. These alveoli are lined by type I and type II epithelial cells (Brody and Vaccaro, 1979). More than 90% of this surface area consists of type I cells (Naimark, 1977). It is therefore clear that any interference with the normal alveolar epithelial cell differentiation and maturation or damage thereof will have an influence on the alveolar status of the lung.

The present study indeed demonstrates alveolar damage which range from membrane blebs to total rupturing of blood air barriers. Of particular interest are the fenestrations that occur in the alveolar walls of the 3 week old rat pups exposed to nicotine via the placenta and mother's milk. According to various reports these fenestrations can be related to the onset and presence of emphysema (Boren, 1962; Kuhn and Tavasoli, 1976; Cosio et al, 1986). It is conceivable that fusion of these fenestrations may result in more severe alveolar damage as observed in this study. Whether the results of this study are indeed an indication of the presence of emphysema is not clear. However, recent research indicates that maternal nicotine exposure induced emphysemalike lesions in the lungs of the offspring (Maritz et al, 1992). It is clearly plausible that damage to the lungs at this early stage of lung development as a result of nicotine exposure is a more important determinant of emphysema than the subsequent exposure to tobacco smoke in adult life.

From the findings of this study it appears that the type I cell is the principle site of injury in nicotine exposed neonatal rat lung. The mechanism whereby nicotine induces type I cell injury is not known. However, survival of these and other cells is dependent upon the conversion of nutrients into energy. The type I cell depends on glycolysis for the maintenance of its integrity (Massaro et al, 1975). Apart from being the most important energy generating pathway in type I cells, glycolysis also supplies sufficient ATP to maintain membrane-linked Na^+-K^+-ATPase (Paul, 1983).

This latter pump system plays a vital role in maintaining cell volume and inhibition thereof results in hydropic cell swelling and membrane blebs (Contran et al, 1989). If the source of this inhibition is not eliminated, irreversible cell damage will occur. It is therefore clear that the viability of the alveolar cells and thus the maintenance of the alveolar integrity is linked to the ability of the type I and type II cells to utilize glucose to yield the required energy. In recent studies it was illustrated that maternal nicotine exposure during pregnancy and lactation irreversibly suppressed glycolysis and ATP hydrolysis in the lungs of the offspring (Maritz, 1987; Maritz and Burger, 1992). Furthermore, studies by Meyer et al (1971) showed that nicotine inhibits Na^+K^+-ATPase. It is therefore reasonable to believe that this interference with energy metabolism largely contributed to the observed damage to the type I epithelial cells.

The exact consequences of the change in the status of the alveolar epithelial cells of the developing lung is not clearly established. However, minimal lesions in the infant lead to gross lesions in adults (Emery, 1970). It is therefore clear that whatever the mechanism of action of nicotine, the outcome of maternal smoking will most likely present itself in more severe pathology of the lung of the adult offspring.

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APPENDIX B

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THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON THE INTERALVEOLAR SEPTAL STATUS OF NEONATAL RAT LUNG.

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ABSTRACT

The aim of this study was to determine the effect of maternal nicotine exposure (1 mg nicotine/kg body mass/day, subcutaneously) on the status of the alveolar septa of the 1 to 21 day old offspring. The data obtained showed swelling of type II and interstitial cell mitochondria. The type I:type II cell ratio decreased as a result of type II cell proliferation. The number of capillaries per unit length of septum was also significantly lower than that of control lung. Ruptured blood-air barriers also occur in the nicotine exposed lungs of rats of all age groups. The results show that maternal nicotine exposure interfered with the morphometric and morphologic characteristics of the septa of lung tissue of the offspring.

INTRODUCTION

Morphological studies in humans and animals illustrated three stages of prenatal lung development namely, the glandular stage, the canalicular stage and the alveolar or saccular stage (Emery, 1969; Meyrick and Reid, 1977). One of the most important processes in pulmonary development is the formation of alveolar septa. These septa subdivide the terminal saccules thereby providing a large surface area for gas exchange (Fukuda et al, 1983). The majority of the lung's functional gas exchange units develop over a defined period of time during early postnatal life (Thurlbeck, 1975). After this period of alveolarisation which encompasses the first 8 to 10 years of life in the human (Thurlbeck, 1975) and the fourth through 13th days in the rat (Burri et al, 1973), lung growth involves enlargement rather than multiplication of alveolar gas exchange units.

During the period of postnatal alveolar formation in the rat, cell proliferation and cell differentiation occur. Surfactant producing type II epithelial cells proliferate and differentiate into alveolar type I cells which cover the newly formed alveolar surfaces (Kaufman et al, 1974). The interstitial cells also proliferate and apparently differentiate into two types of cells (Kaufman et al, 1974; Vaccaro and Brody, 1978). One type which appears at the tip of developing secondary septa, is actively involved in the synthesis and secretion of connective tissue. This fibroblast has been associated with septal formation during postnatal lung development. The second type, a lipid-containing interstitial cell, appears throughout the walls of alveoli during early postnatal growth. The lipid-containing interstitial cell (LIC) is present in the lung at birth, but it appears in greater numbers during the period of alveolar proliferation and disappears when lung growth ends.

The invasion of capillaries into the alveolar zones of the developing lung is the critical change in the canalicular stage (Burri and Weibel, 1977). Fibroblasts play a role in organizing the relationship that exists between epithelial cells and capillary endothelium. This relationship is established after the primordia of alveolar septa are formed in the late glandular stage (Fukuda et al, 1983). In the rat lung, capillary development precedes the formation of alveolar septa (Burri 1974).

Intricate cell to cell communication and control mechanisms are therefore involved in normal lung development. Growth and maturation is also energy dependent. Interference with any one of these cells and/or control mechanisms may therefore also interfere with normal lung growth and maturation. Recent studies indeed illustrated that maternal nicotine exposure during pregnancy and lactation inhibits energy metabolism in neonatal lung (Maritz, 1988; Maritz and Burger, 1992). As a consequence of this finding it was speculated that type I cell

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damage will occur, which again will result in a decrease in the type I:type II cell ratio. This implies that the cellular status of the alveolar septum and possibly its intactness will change. The aim of this study was therefore to determine whether maternal nicotine exposure during pregnancy and lactation will indeed influence the septal status of neonatal rat lung. Changes in the status of the septa or interference with the development thereof will be reflected in the lung alveolar status.

MATERIALS AND METHODS

Animals: White virgin female rats (Wistar descendants) of 200-250 g were used in this study. The animals were fed a stock diet (Epol rat cubes) throughout the experiment. Room temperature was kept at 22°C and a day-night cycle of 12 hours was maintained.

We maintained our own breeding program. Animals were mated overnight and were afterwards randomly assigned to control and experimental groups. The length of gestation averaged 22,5 days for both control and experimental groups. The treatment procedure and dosage (1 mg nicotine/kg body mass/day) was the same as described by Maritz (1987). Control animals received normal saline instead of nicotine. Lung tissue from 1,7,14 and 21 day old rat pups were used in this investigation. Rat pups from each of the 4 age groups were randomly selected from each of 11 control litters and 11 nicotine exposed litters. From each of the litters, 3 rat pups were sacrificed and lung tissue collected for this investigation. The rat pups were killed by decapitation 24 hours after the last exposure of the mother. The lung tissue was then quickly removed and samples collected for morphologic studies. Two samples were taken form each of the lung lobes. For each of the animals, three blocks were randomly selected and from each block one good section was considered.

Sample Preparation:

a) Transmission Electron Microscopy

The lung tissue was fixed in 2,5% glutaraldehyde in 0,25 M potassium phosphate buffer (pH 7.2), for 1 hour and cut into 1 mm portions. These were fixed for a further 30 minutes, rinsed in buffer and postfixed in 1,5 % osmium tetroxide for 14 hours at 4°C. After rinsing in distilled water, the samples were placed in 1,0% uranyl acetate for 15 minutes before dehydrating in graded acetone and embedding in Spurr's resin.

For both control and nicotine exposed lungs 70 mm sections were prepared by using standard techniques. Staining was done by the method of Sato (1967).

b) Cellularity of Alveolar Walls

For determination of total cellularity, type I cells, type II cells, and LIC, as well as capillary density, 1μ sections were made and stained with tolluidine blue (Woolward, 1991). Cellularity was determined according to the method of Eidelman et al (1990). Briefly, at a magnification of x400 the length of the septum visualized in the center of the microscopic field was measured with a micrometer. The total number of nuclei, taken as representative of cells lying within this septum was counted (for each of the control and nicotine exposed lungs). The results were expressed as the number of cells per mm of septum. The same method was employed to determine LIC and capillary counts per unit length of septum. Lipid containing interstitial cells (LIC) was identified by the presence of lipid droplets. LIC were counted and expressed as number of LIC per mm septum and as a ratio of the total cell count. Epithelial type I and type II cells were differentiated on the basis of structural characteristics. Type I epithelial cells were characterized by attenuated profiles and transporting vesicles. Type II epithelial cells were characterized by cuboid profiles and the presence of lamellar bodies. Counting was done blind by 2 independent observers. To prevent double counting, only one section per tissue block was used for counting. Statistical analysis of differences between means was carried out by the use of the Wilcoxon test for unpaired data. A probability level of $P<0,05$ was designated as significant in this study. Results were recorded as mean $\pm$ SEM.

RESULTS

The data summarized in table I illustrates that the cellularity of the septa of the neonatal lung increased as a function of age up to day 14 after birth. In the control lung the total cell count per mm septum

Table 1: The influence of maternal nicotine exposure on neonatal lung cellularity.

Age Days	Cellularity (Cells/mm ± SEM)		LIC (LIC/mm ± SEM)		Cell/LIC*	
	Control	Nicotine	Control	Nicotine	Control	Nicotine
1	48,12 ± 1,62	60,96 ± 1,54	3,40 ± 0,37	4,50 ± 0,35	14,15	13,55
7	51,52 ± 1,70 P > 0,05	59,12 ± 1,72 P > 0,05	3,42 ± 0,53 P > 0,05	3,36 ± 0,33 P > 0,05	15,06	17,34
14	61,44 ± 1,77 P < 0,005	71,36 ± 1,65 P < 0,005	4,28 ± 0,49 P > 0,05	4,94 ± 0,55 P > 0,05	14,35	14,45
21	59,76 ± 2,09 P >0,05	66,24 ± 2,62 P > 0,05	7,46 ± 0,96 P < 0,01	6,16 ± 0,42 P < 0,05	8,05	10,75

*Cell/LIC = Ratio of total cell number/mm septum to total LIC/mm septum.

Cellularity (cells/mm septum):

Day 1,7 and 14 : Control vs nicotine - $P < 0,001$

Day 21 : Control vs nicotine - $P < 0,01$

LIC:

Day 1 : Control vs nicotine - $P < 0,05$

Day 21 : Control vs nicotine - $P > 0,05$

Table 2: Influence of maternal nicotine exposure on neonatal lung type I and type II cell count.

Age Days	Total number of cells counted		% Type I cells		% Type II Cells	
	Control	Nicotine	Control	Nicotine	Control	Nicotine
1	471	804	58,96 ± 1,06	43,12 ± 0,53	40,93 ± 1,26	56,88 ± 0,53
7	472	761	60,85 ± 1,21 P > 0,05	40,85 ± 0,87 P > 0,05	39,05 ± 1,25 P > 0,05	59,15 ± 0,87 P < 0,05
14	663	732	62,25 ± 1,10 P > 0,05	41,92 ± 1,06 P > 0,05	37,75 ± 1,10 P > 0,05	58,08 ± 1,06 P > 0,05
21	691	736	64,82 ± 0,85 P > 0,05	40,47 ± 0,62 P > 0,05	35,92 ± 1,09 P > 0,05	59,62 ± 0,61 P > 0,05

Type 1 cells:

Day 1 to 21: Control vs nicotine - $P < 0,001$

Control day 1 vs control day 21 - $P < 0,01$

Nicotine day 1 vs nicotine day 21 - $P < 0,01$

Type II cells:

Day 1 to 21: Control vs nicotine - $P < 0,001$

Control day 1 vs control day 21 - $P < 0,01$

Nicotine day 1 vs nicotine day 21 - $P < 0,01$

increased from 48,12 ± 1,62 on day 1 after birth to 61,44 ± 1,77 (P>0,005) on day 14 after birth. No difference in cell count was observed between days 14 and 21 after birth. One day after birth the septal cell count of 60,96 ± 1,54 cells/mm of lung tissue of nicotine exposed rat pups were 26,68% (P<0,001) higher than that of the control rat pups of the same age. The septal cell numbers of the nicotine exposed rat pups increased from 60,96 ± 1,54 on day 1 to 71,36 ± 1,65 on day 14 (P<0,005). No difference in septal cell numbers occurred between days 14 and 21 (P> 0,05) after birth. It is also interesting to note that the percentage difference in total cell count between control and nicotine exposed rat pups decreased as function of age from 26,68% on day 1 to 10,84% on day 21 after birth. This was due to a 24,2% increase in the cell number of control lung between days 1 and 21. In nicotine exposed lung it increased by 8,7% only.

The number of lipid-containing interstitial cells (LIC) in control lung (Table 1) increased from 3,40 ± 0,37 LIC/mm septum on day 1 after birth to 7,46 ± 0,96 LIC/mm septum on day 21 after birth (table 1). Analysis of the data show that there was no significant increase in LIC per unit length of septum between days 1 and 14 of lung tissue of control rat pups. However, between days 14 and 21 the LIC count increased by 73,4% (P<0,01).

The number of LIC per unit length of septum in lungs of nicotine exposed rat pups increased from 4,50 ± 0,35 on day 1 after birth to 6,16 ± 0,42 LIC/mm on day 21 after birth (P<0,05). This increase is more gradual than in control lung because no difference in LIC count per unit length of septum occurred between days 1 and 14 after birth (table 1). Further analysis of the data in table 1 show that the LIC count of control lung increased by 118,2% between days 1 and 21 of lactation, of which 73,4% occurred between days 14 and 21. The LIC count of the nicotine exposed lung on day 1 after birth was at 4,50 ± 0,53, 32,4% higher (P<0,05) than the 3,40 ± 0,36 of the control lung. Between days 1 and 21 after birth the LIC count increased by 36,9% only of which 24,7% was during the last week of gestation.

A comparison of the Cell/LIC ratio show that the ratios for both control and nicotine exposed lungs follow the same trend. Virtually no changes occurred between days 1 and 14 after birth. The ratio decreased between days 14 and 21 after birth due to an increase in LIC count and stabilisation of cellularity of the septa.

Table 3: The influence of maternal nicotine exposure on the type I:type II cell ratio of neonatal lung.

Age: (Days)	Type 1:Type II cell ratio	
	Control	Nicotine
1	*1,43 ± 0,05	**0,76 ± 0,02
7	1,56 ± 0,06	0,72 ± 0,01
14	1,66 ± 0,09	0,70 ± 0,02
21	*1,78 ± 0,05	**0,68 ± 0,02

* P<0,01 - Control day 1 vs control day 21
**P<0,01 - Nicotine day 1 vs nicotine day 21

The data in table 2 illustrates that the alveolar epithelial cell composition changes as a function of age. On day 1 after birth the type I cells constitutes 58,96 ± 1,06 of the total number of epithelial cells counted. On day 21 after birth, the percentage type I cells increased by 5,86% (P<0,01) to 64,82 ± 0,85%. Concomitantly with the percentage increase in type I cell count, the percentage type II cells decreased by 5,01% (P<0,05) from 40,93 ± 1,26% on day 1 after birth to 35,92 ± 1,09% on day 21 after birth. As a consequence of the change in type I

Fig. 1. Interstitial cells of a lung septum of a 14 day old rat pup exposed to nicotine during pregnancy and lactation: n = nucleus; arrowhead = mitochondria: double arrowheads = endoplasmic reticulum. (x11800).

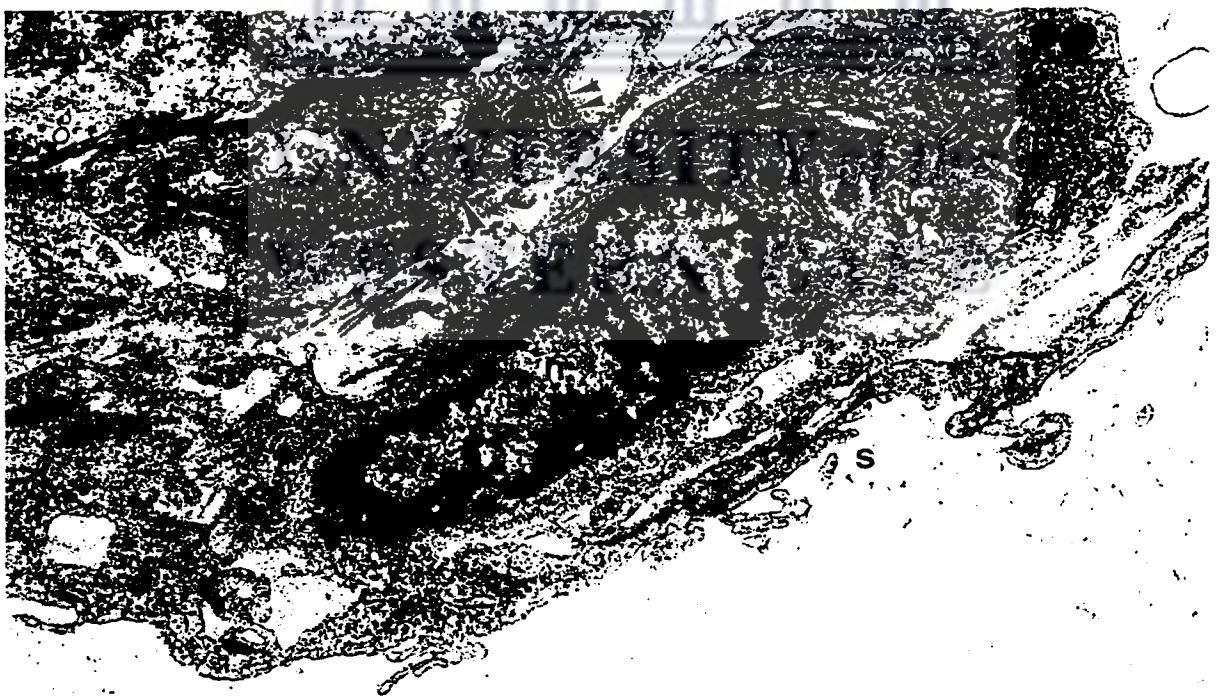

Fig. 2. Interstitial cells of a lung septum of a 14 day old control rat pup: n = nucleus: single arrowhead = mitochondria: double arrowhead = endoplasmic reticulum. (x11800).

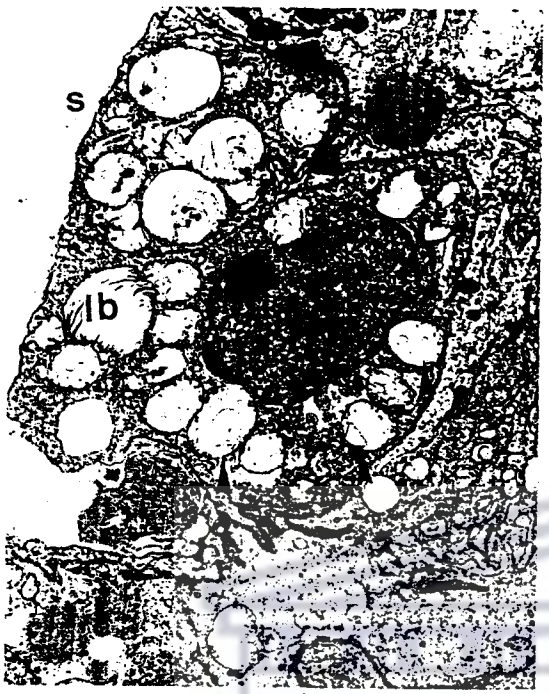

Fig. 3. Type II epithelial cell of lung tissue of nicotine exposed rat pups. Lb = lamellar body : arrow-heads = mitochondria : S = alveolar space. (x9000)

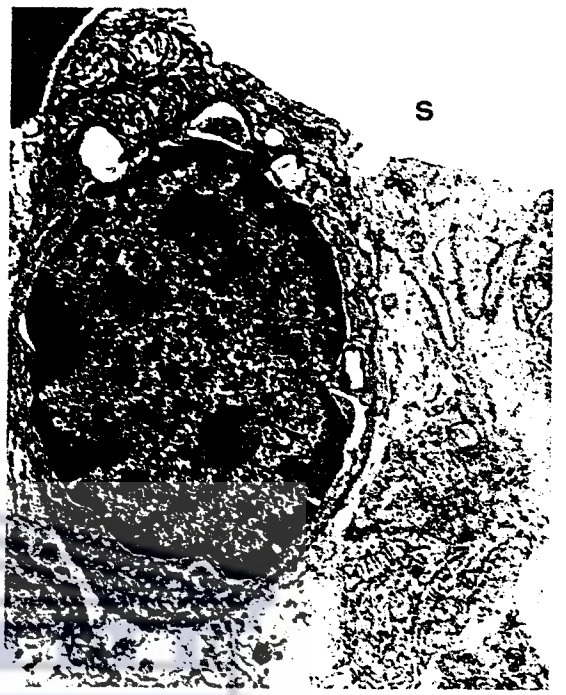

Fig. 4. Type II epithelial cell of lung tissue of control rat pups. Arrowheads = mitochondria. (x9000)

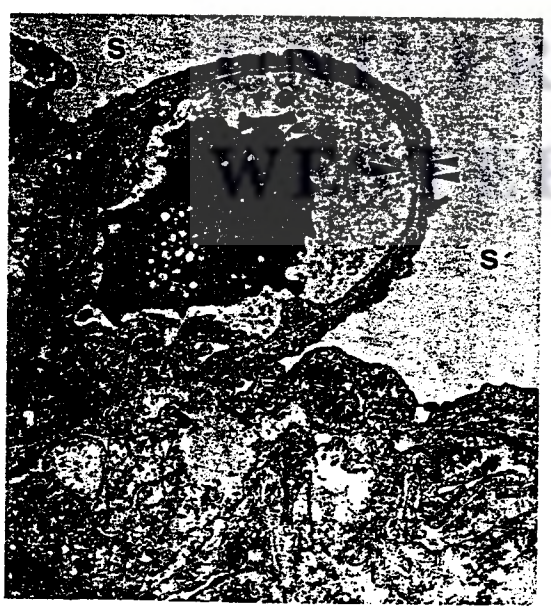

Fig. 5. Blood-air barrier of control lung. Double arrowhead = Type I epithelial cell : Single arrowhead = capillary endothelial cell. S = Alveolar space : P = platelet. (x10100)

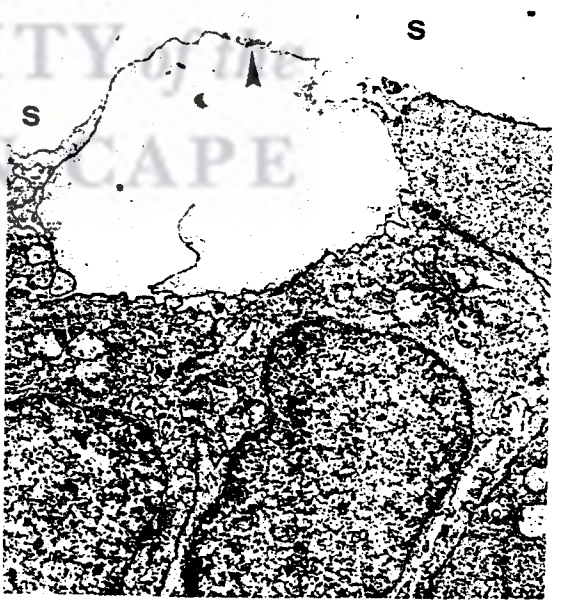

Fig. 6. Ruptured blood-air barrier (arrowhead) of lung tissue of nicotine exposed rat pups. S = alveolar space. (x10500)

and type II cell composition of the septum, the type I:type II cell ratio of control lung increased by 24,48% form 1,43 ± 0,05 to 1,78 ± 0,05 ($P<0,01$) between days 1 and 21 after birth (table 3).

Maternal nicotine exposure however interfered with the normal development of the alveolar epithelial cells since the percentage of type I decreased by 2,74% from 43,12 ± 0,53 on day 1 after birth to 40,47 ± 0,62 ($P<0,01$) on day 21 after birth. At the same time the percentage type II cells increased by 2,65% from 56,88 ± 0,53 to 59,62 ± 0,61 ($P<0,01$). This resulted in a 10,53% decrease in the type I:type II cell ratio from 0,76 ± 0,02 on day 1 to 0,68 ± 0,02 on day 21 after birth. Due to the gradual decrease in the type I:type II cell ratio in lungs of nicotine exposed neonatal rat pups versus the gradual increase of the ratio in control lung, the percentage difference in the ratios increased from 46,9% ($P<0,001$) on day 1 to 61,8% ($P<0,001$) on day 21 after birth (table 3).

Maternal nicotine exposure also resulted in the swelling of the mitochondria of the interstitial cells of the alveolar septa (fig. 1). No mitochondrial swelling occurred in the interstitial cells of the alveolar septa of the control animals. Note the close association between the mitochondria and endoplasmic reticulum of the interstitial cells of the septa of the control lung (fig. 2). Although no swelling of the endoplasmic reticulum was observed in the interstitial cells of the nicotine exposed lungs (fig. 1), it appeared less prominent than in the interstitial cells of the control lung (fig. 2).

Apart from the interstitial cells, mitochondrial swelling is also evident in the type II epithelial cells of the nicotine exposed rat lung (fig. 3). No mitochondrial swelling occurred in type II epithelial cells of the control lung (fig. 4).

The data in table 4 shows a gradual increase in the number of capillaries in control lung from 5,52 ± 0,22 capillaries/mm of septum on day 1 after birth to 8,44 ± 0,51 on day 14 after birth. This represent an increase of 45,0% ($P<0,005$). No further increases in septal capillary count occurred between days 14 and 21 after birth. In the lungs of the nicotine exposed rat pups the number of capillaries per unit of septum increased by 37,2% between days 1 and 21 after birth. Because of this slower increase in capillary count, in the nicotine exposed lungs, the difference between the nicotine exposed and control lung become more significant as the rat pups becomes older. On day 1 after birth the difference was 18,9% ($P<0,05$) and on day 21 it was 27,5% ($P<0,001$).

The blood-air barrier of control lung typically consists of a type I epithelium separated from the capillary endothelium by a basement membrane (fig. 5). Maternal nicotine exposure often resulted in ruptured blood-air barriers (fig. 6) in all age groups investigated. No rupturing of this barrier occurred in the lung tissue of the control rat pups.

DISCUSSION

The formation of alveolar septa is one of the most essential processes in the morphogenesis of alveoli.

Table 4: The influence of maternal nicotine exposure on neonatal lung septal capillary count (capillaries/mm ± SEM)

Age (days)	Control*	Nicotine
1	*5,82 ± 0,22	*4,72 ± 0,30
7	6,74 ± 0,51	4,96 ± 0,43
14	*8,44 ± 0,51	5,58 ± 0,25
21	8,96 ± 1,31	**6,50 ± 0,56

* $P<0,001$ - control day 1 vs control day 14
** $P<0,05$ - nicotine day 1 vs nicotine day 21

Alveolar septa of the adult lung can be classified into primary alveolar septa which are the walls of the saccules, and the secondary alveolar septa which protrude from the saccular walls and form the tissue crests that subdivide the terminal saccules (Burri and Weibel, 1977). It has been suggested that the time of formation of secondary septa differs from species to species. In the rat lung the bulk of the alveoli are formed between days 4 and 13 after birth (Burri, 1974).

The components of these alveolar septa include the type I and II alveolar epithelial cells, fibroblasts, myofibroblasts, smooth muscle cells, connective tissue, and capillaries (Kaufmann, 1974). Each of these components of the alveolar septum plays an important role in maintaining alveolar integrity and lung function. Interference with the development, integrity and function of any of these components will be reflected in the integrity of the septum and thus of the alveoli of the lung.

The present study illustrated that the cellularity of the alveolar septa of the lungs of the nicotine exposed rat pups were higher than that of the control animals. The fact that the change in septal cellularity between days 1 and 21 after birth was lower for the lungs of the nicotine exposed rat pups than for the control animals suggest that maternal nicotine exposure after birth was not responsible for the higher cellularity of the septa of the nicotine exposed rat pups. This argument is further supported by the fact that the increase in cellularity between days 7 and 14, which closely resembles the phase of rapid alveolarisation (Burri et al, 1973), was the same for the lung tissue of both the control and nicotine exposed rat pups. This implies that the observed effect of nicotine exposure was due to its interference with fetal lung development.

An analysis of the data shows the response of certain components of the alveolar septum to maternal nicotine exposure and its contribution to changes in septal characteristics.

Lipid-containing interstitial cell (LIC): Although the functions of these cells are not clearly defined, the mere presence thereof is an indication of there importance in septal and thus alveolar development. Two possibilities pertaining to the role of these cells in the restructuring process, which take place during this period of alveolar formation (Burri, 1974), could be considered. Firstly, it is possible that the LIC lipid serves to regulate the conformation of elastin or the activity of lysyl oxidase, produced by the LIC or other interstitial cells, during lung development. Secondly the LIC may also be involved in the control of alveolar shape through interaction of filament bundles with the alveolar basement membrane (Kapanci et al, 1974). It is therefore reasonable to believe that changes in their numbers and/or metabolism will affect lung development. From the results it is clear that maternal nicotine exposure during pregnancy fascilitate LIC proliferation in fetal lung but not in neonatal lung. The significance of this on early lung development is not clear. However, since maternal nicotine exposure had no influence in the LIC count in neonatal lung, it is more likely that maternal nicotine exposure will influence the role of the LIC on alveolar development via a metabolic route. Previous studies indeed illustrated that maternal nicotine exposure interfered with energy metabolism of lung tissue of neonatal rat pups (Maritz, 1986 and 1987; Maritz and Burger, 1992). Furthermore, the present study it was shown that maternal nicotine exposure resulted in mitochondrial swelling in the interstitial cells. Although no direct evidence exists to proof it, it is possible that the mitochondrial swelling contribute to the slower rate of carbohydrate utilisation of these cells and of the lung. It is also an early sign of cell damage which can culminate in cell death if the source of the problem,in this instance nicotine, is not eliminated (Contran et al, 1989). It is uncertain to what extent swelling of mitochondria and inhibition of energy metabolism is influencing the proposed functions of the LIC. Neither is it clear to what extent the previously reported interference with elastic tissue formation and deposition (Maritz et al, 1992) is related to the inhibition of energy metabolism of LIC and other fibroblasts.

Alveolar epithelial cells: Gradual differentiation of type II epithelial cells occur during lung development with the formation of the type I epithelial cells. Damage of the type I cell is also followed by type II cell proliferation and differentiation of one of the sister cells to replace the damaged type I cell (Crapo et al, 1980). In a recent study it was clearly demonstrated that maternal nicotine exposure induced type I cell damage (Maritz et al, 1993). It is therefore reasonable to believe that the decrease in the type

I:type II cell ratio obtained in this study, is due to type II cell proliferation in response to type I cell damage.

The type I:type II cell ratio on day 1 after birth can be seen as a reflection of the ratio in fetal lung. The low type I:type II cell ratio of the lung tissue of the one day old nicotine exposed rat pups therefore illustrate the response of fetal lung tissue to nicotine exposure during pregnancy. Since the milk intake of rat pups decreased considerably from day 12 after birth because of a switch to solid food (unpublished data), the nicotine intake will also be reduced to the same extent. It is therefore reasonable to expect that the type I:type II cell ratio will improve. However, contrary to expectations, the ratio for nicotine exposed lungs decreased and the difference between the control and experimental groups therefore increased as a function of age. This can be explained by the fact that the type I epithelial cells are dependent on glycolysis for energy (Massaro et al, 1975). Inhibition of this pathway will therefore result in type I cell death and consequently type II cell proliferation to replace the damaged type I cells. Previous research clearly illustrated that maternal nicotine exposure irreversibly inhibit glycolysis (Maritz, 1987) and ATP hydrolysis (Maritz and Burger, 1992) and that the degree of inhibition remain constant even 4 weeks after nicotine withdrawal (Maritz, 1987). Glycolysis also supply the ATP required to maintain the membrane-linked Na^+-K^+-ATP-ase (Paul, 1983). Furthermore, nicotine inhibits the membrane-linked Na^+K^+-ATP-ase (Meyer et al, 1971). This Na^+-K^+-ATP-ase pump plays a vital role in maintaining cell volume. Thus, by reducing its activity via inhibition of glycolysis will result in swelling of these cells and formation of membrane blebs (Contran et al, 1989). Blebbing of type I cells were indeed already shown (Maritz et al, 1993) suggesting impairment of this membrane pump. The irreversible inhibition of glycolysis (Maritz, 1987) and of ATP-hydrolysis (Maritz and Burger, 1992) therefore explains why no improvement of the type I:type II cell ratio occurred despite the lower nicotine intake via the mother's milk. This observation furthermore implies that the damage that was induced in the fetal stage, will become progressively worse over time.

As for the LIC, it is uncertain to what extent inhibition of glycolysis and swelling of type II cell mitochondria are influencing the functions of the type II cell. From the results in table 3 it appears that the number of these cells indeed increased in contrast to that of control lung where the type II cell count decrease between days 14 and 21 after birth. This imply that the type II cells of the nicotine exposed lungs still can proliferate. There is evidence however, that the rate of proliferation is not sufficient to compensate for the damage of the type II cells because of a slight decrease in the type I cell count as appose to the increase in control lung (table 3). The inability of the type II cells to replace all the damaged type I cells effectively prevent the continued maintenance of the blood-air barriers (fig. 4). The gradual decrease of the type I:type II cell ratio can also be attributed to the increase in type II cell count and decrease in type I cell count.

Capillaries of the septum: Studies by various researchers indicate that fibroblasts and capillary formation during the fetal stage, is crucial for septal formation in the developing lung (Burri, 1974; Burri and Weibel, 1977; Fukuda et al, 1983). Interference with the process of capillary formation will therefore be reflected in the capillary density of the septa and in alveolarisation of the developing lung. Interconnections between capillaries in septa increase during lung growth and maturation indicating that the capillaries multiplied as development goes on (Burri, 1974). The increase in the capillary count per unit length of septum as a function of age found in the present study (table 4) support the findings of previous researchers (Fukuda et al, 1983).

Maternal nicotine exposure during pregnancy suppressed capillary formation in the fetal and neonatal stage (table 4). Therefore, since capillary formation is essential for septal formation in developing lung (Fukuda et al, 1983), and since septal formation plays an important role in alveolarisation (Burri and Weibel, 1977), less alveoli can be expected in the lungs of the nicotine exposed neonatal lung. Earlier studies indeed illustrated a lower radial alveolar count and thus the number of alveoli in the lungs of rat pups exposed to nicotine via the placenta and mother's milk (Maritz et al, 1993). The mechanism whereby maternal nicotine exposure suppress capillary formation is not known. Some explanations can be offered. Firstly, in a previous study it was illustrated that maternal nicotine exposure resulted in a less densely developed

mesenchymal tissue in fetal lung (Woolward, 1991). The mesenchymal cells differentiate into fibroblasts and produce a "fibroblast pneumocyte" factor which is required for fibroblast activity (Motoyama et al, 1988). The fibroblasts play a role in the fetus in determining the relationship that exists between epithelial cells and the capillary endothelium (Fukuda et al, 1983). Thus, the interference by nicotine with mesenchymal tissue together with fetal and neonatal lung carbohydrate metabolism (Maritz, 1986 and 1987) and the mitochondrial integrity of the interstitial cells before and after birth, may suppress the role of the fibroblast in contributing to the regulation of capillary formation after the first day of gestation. Secondly, destruction of capillaries in the septa of lung tissue of nicotine exposed rat pups (fig. 6) may also contribute to the slower increase in the septal capillary count (table 4)

In conclusion, maternal nicotine exposure during pregnancy and lactation interfered with normal lung development resulting in changes in the morphologic and morphometric characteristics of the alveolar septa and therefore of the neonatal rat lung. Research to determine the mechanisms whereby nicotine induce these changes is required and the relationship thereof on the etiology of lung disease.

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APPENDIX C

MATERNAL NICOTINE EXPOSURE : REPONSE OF TYPE II PNEUMOCYTES OF NEONATAL RAT PUPS

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ABSTRACT

The influence of maternal nicotine exposure during pregnancy and lactation on the Type II cells of lung tissue of one day old neonatal rat pups was investigated. The results clearly show that maternal nicotine exposure resulted in an increase in the type II cell count in the lungs of the offspring. In addition the lamellar body content of the type II cells of the nicotine exposed rat pups were significantly ($P<0,01$) higher than that of the control animals. The type II cell mitochondria of lung tissue of nicotine exposed rat pups were swollen and no microvilli occurred on the alveolar surface. This clearly illustrates that nicotine interfered with type II cell integrity of the neonatal lung and may subsequently interfere with the normal development of the alveolar region of the lung.

INTRODUCTION

The alveolar type II pneumocyte plays a vital role in maintaining the integrity of the alveoli where the latter is the major site of gas exchange. In this respect the type II pneumocytes are involved in surfactant secretion which lowers the surface tension of the alveoli and thereby promotes alveolar stability (Mason, et al, 1977). The type II pneumocytes also help to maintain the alveoli in their dry state by actively transporting sodium from the alveolar hypophase into the pulmonary interstitium (Goodman et al, 1984). In addition, the alveolar type II pneumocyte protects the alveolar epithelium by reducing the extracellular hydrogen peroxide by a catalase dependent pathway (Engstrom et al, 1990).

Damage to type I pneumocytes is followed by type II pneumocyte mitosis and differentiation of one of the sister cells into type I pneumocytes to replace the damaged type I pneumocytes (Crapo et al, 1980). Since both these cell types are exposed to inhaled and blood borne pollutants, ty pe II pneumocytes may be damaged which could retard the process of repair via replacement of type I cells as explained earlier. This may render the lung more susceptible to damage by inhaled pollutants and may further have a negative effect on normal neonatal lung development. A study by Le Mesurier (1981) illustrates that exposure of rats to cigarette smoke, resulted in damage of the type II pneumocytes. More recently it was demonstrated that maternal nicotine exposure interferes with neonatal rat lung metabolism and development. These effects include suppression of glycogenolysis and glycolysis (Maritz, 1988), changes in the connective tissue framework (Maritz and Woolward, 1992) as well as to the cells of the blood-air barrier (Maritz and Woolward, 1990) and of the bronchi and bronchioles (Wang et al, 1984). It is therefore possible that the damage to type II pneumocytes of rats exposed to cigarette smoke (Le Mesurier, 1981) was due to the nicotine therein.

The aim of this study was therefore to determine whether maternal nicotine exposure will induce changes to type II pneumocyte characteristics and to compare any changes with the findings observed for animals exposed to

cigarette smoke.

MATERIALS AND METHODS

Animals: White virgin female rats (Wistar descendants) of 200-250 g were used in the present investigation and were fed a stock diet (Epol rat cubes) throughout the experiment. All animals received food and tap water as required. Room temperature was kept at 22°C and a day-night cycle of 12 hours was maintained.

We maintained our own breeding program for the control and experimental animals. Animals were mated overnight and were afterwards randomly assigned to control and experimental groups.

The dams received doses of 1 mg nicotine/kg body weight/day subcutaneously. This dose lies within the range of intake 0,16 to 1,8 mg/kg body weight/day of habitual smokers (Maritz and Woolward, 1992). Control animals received saline instead of nicotine. Nicotine exposure commenced on day 7 of gestation to avoid interference of nicotine with blastocyte implantation and initial embryonic growth and was continued during lactation.

Rat pups were randomly selected from 4 litters of both the control and nicotine exposed groups. The animals were killed by decapitation 24 hours after the last exposure. The lung tissue was then quickly removed and samples collected for studying type II pneumocyte morphology and proliferation.

Sample preparation for transmission electron microscopy (TEM).
The terminal aspect of the middle lobe of the right lung was removed, fixed in 2,5% glutaraldehyde in 0,2 M potassium phosphate buffer (pH 7,2) for 1 hour and cut into 1 mm portions. These were fixed for a further 30 minutes, rinsed in buffer and postfixed in 1,5% osmium tetroxide overnight at 4°C. After staining in 1,0% uranyl acetate and dehydration, the samples were embedded in Spurr's resin.

For both control and nicotine exposed lungs, ultrathin 70 nm sections were prepared using standard techniques and subsequently examined using a Hitachi model L-800 TEM. Cells from each of 10 control and 10 nicotine exposed lungs were randomly photographed at a X10 000 magnification and the average number of lamellar bodies per type II cell determined. Each of the 10 controls and 10 nicotine exposed lungs were sampled from a different litter.

The volume fraction of lamellar bodies of the type II cells were determined using point counting (Weibel, 1973) by placing a square lattice system containing test points, 1 cm apart, over micrographs printed at a magnification of X10 000. Results are expressed as ratios, number of test points falling over lamellar bodies to number of test points falling over cell cytoplasm including lamellar bodies; that is, volume of lamellar bodies to volume of cell cytoplasm ($V_{lamellar\ bodies}/V_{cytoplasm}$).

For the determination of type I/type II cell ratios, 1 μm sections were made and stained with tolluidine blue (Woolward, 1991). Epithelial type I and type II cells were differentiated on the basis of structural characteristics. Type I epithelial cells were characterized by attenuated profiles and transporting vesicles. Type II epithelial cells were characterized by their cuboidal profiles and the presence of lamellar bodies. Counting was done blind by 2 independent observers. Only those type II cells displaying both a nucleus and lamellar bodies were counted. To prevent double counting, only one section per tissue block was used for counting. At least 4 fields per sample were used to count alveolar type I and type II epithelial cells.

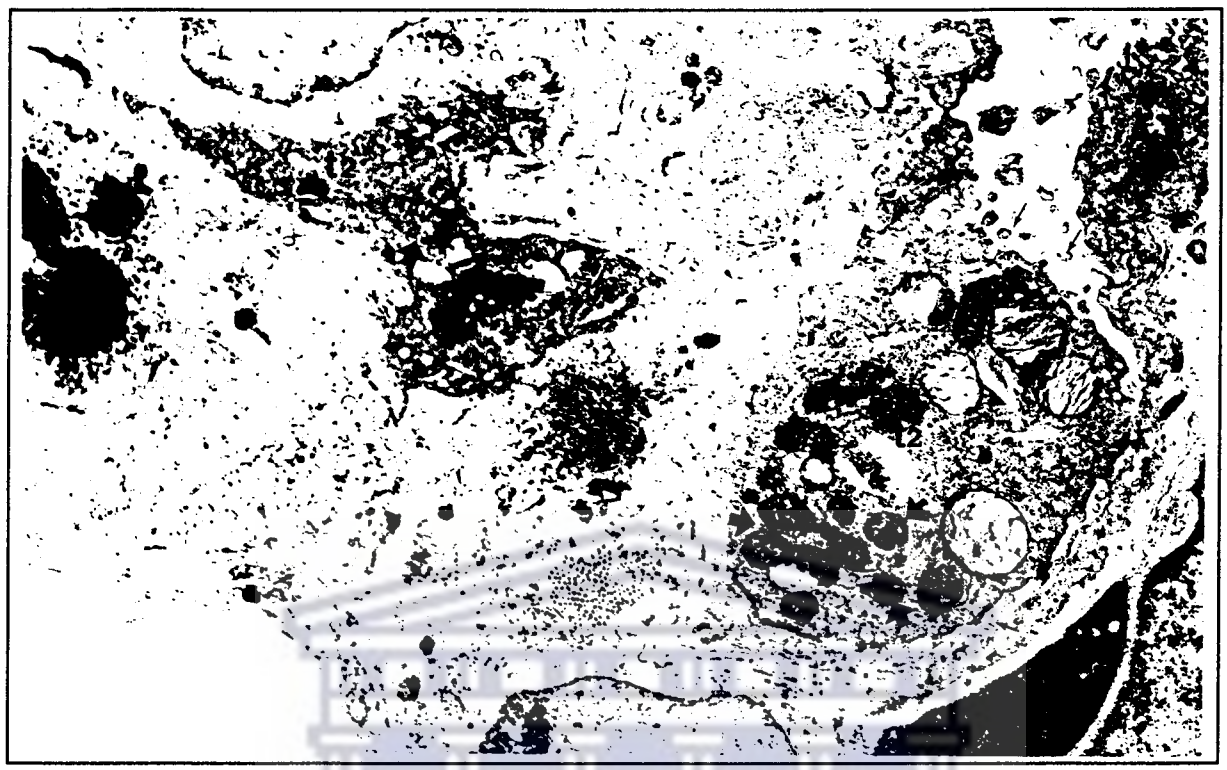

Figure 1: A type II pneumocyte (t2) of control rat pups. Note the small dense mitochondria (thick arrows) and microvilli (small arrows) on the alveolar surface (x10 000).

Statistical analyses

The unpaired t-test was used to statically evaluate the control and test groups. A probability level of $P<0,05$ was designated as significant in this study. Results were recorded as mean ± SEM.

RESULTS

The data in table I illustrates the type I cell count of control rat pups gradually increased from 58,96 ± 1,06 on day 1 after birth to 64,82 ± 0,85 ($P<0,01$) on day 21 after birth. In contrast to the control rat pups the type I cell count decreased in nicotine exposed rat pups from 43,12 ± 0,53 on day 1 after birth to 40,47 ± 0,62 ($P<0,01$) on day 21 after birth. In all instances the type I cell count of the nicotine exposed lungs were lower ($P<0,001$) than in the control lungs. The type II cell count of control rat lung on the other hand was always lower ($P<0,001$) than in lung tissue of nicotine exposed rat pups. In control lung the type II cell count gradually decrease by 12,21% from 40,93 ± 1,26 on day 1 to 35,92 ± 1,09 on day 21 after birth ($P<0,01$). In lung tissue of nicotine exposed rat pups, the type II cells increased by 4,82% ($P<0,05$) from 56,88 ± 0,53 to 59,62 ± 0,61 between days 1 and 21 after birth. The response of the alveolar epithelial cells to maternal nicotine exposure described above is reflected in a type I/type II cell ratio which is always significantly lower ($P<0,001$) in nicotine exposed lung than in control lung.

The data summarized in table II show that the lamellar body content of type II epithelial cells of lung tissue of both the

Table I: **The influence of maternal nicotine exposure on neonatal lung type I and type II cell count.**

Age (Days)	Total of number of cells counted		% Type I cells		%Type II cells	
	Control	Nicotine	Control	Nicotine	Control	Nicotine
1	471	804	58,96±1,06	43,12±0,53	40,93±1,26	56,88±0,53
7	472	761	60,85±1,21 P>0,05	40,85±0,87 P>0,05	39,05±1,25 P>0,05	59,15±0,87 P<0,05
14	663	732	62,25±1,10 P>0,05	41,92±1,06 P>0,05	37,75±1,10 P>0,05	58,08±1,06 P>0,05
21	691	736	64,82±0,85 P>0,05	40,47±0,62 P>0,05	35,92±1,09 P>0,05	59,62±0,61 P>0,05

Type I cells	:	Day 1 to 21 : control vs nicotine	: P<0,001
		Control day 1 vs control day 21	: P<0,01
		Nicotine day 1 vs control day 21	: P<0,01
Type II cells	:	Day 1 to 21 : control vs nicotine	: P<0,001
		Control day 1 vs control day 21	: P<0,01
		Nicotine day 1 vs nicotine day 21	: P<0,01

Table II: **The influence of maternal nicotine exposure on the lamellar body content of type II cells of lung tissue of the offspring ($V_{lamellar\ body}/V_{cytoplasm}$)**

Age (Days)	$V_{lamellarbody}/V_{cytoplasm}$ (X ± SEM)		P control vs Nicotine
	Control	Nicotine	
1	0,08±0,008 (n=23)	0,15±0,008 (n=35)	<0,001
7	0,12±0,006 (n=36) (P<0,001)*	0,22±0,01 (n=39) (P<0,001)*	<0,001
14	0,13±0,007 (n=38) (P>0,05)*	0,25±0,02 (n-37) (P>0,05)*	<0,001
21	0,14±0,007 (n=37) (P>0,05)*	0,22±0,01 (n=42) (P>0,05)*	<0,001

*P values in parenthesis compare means of each group with the previous age group
n = number of type II cells from tissue samples from 10 lungs from 10 litters
Control : Day 4 vs day 21 : P<0,001
Nicotine: Day 1 vs day 21 : P<0,001

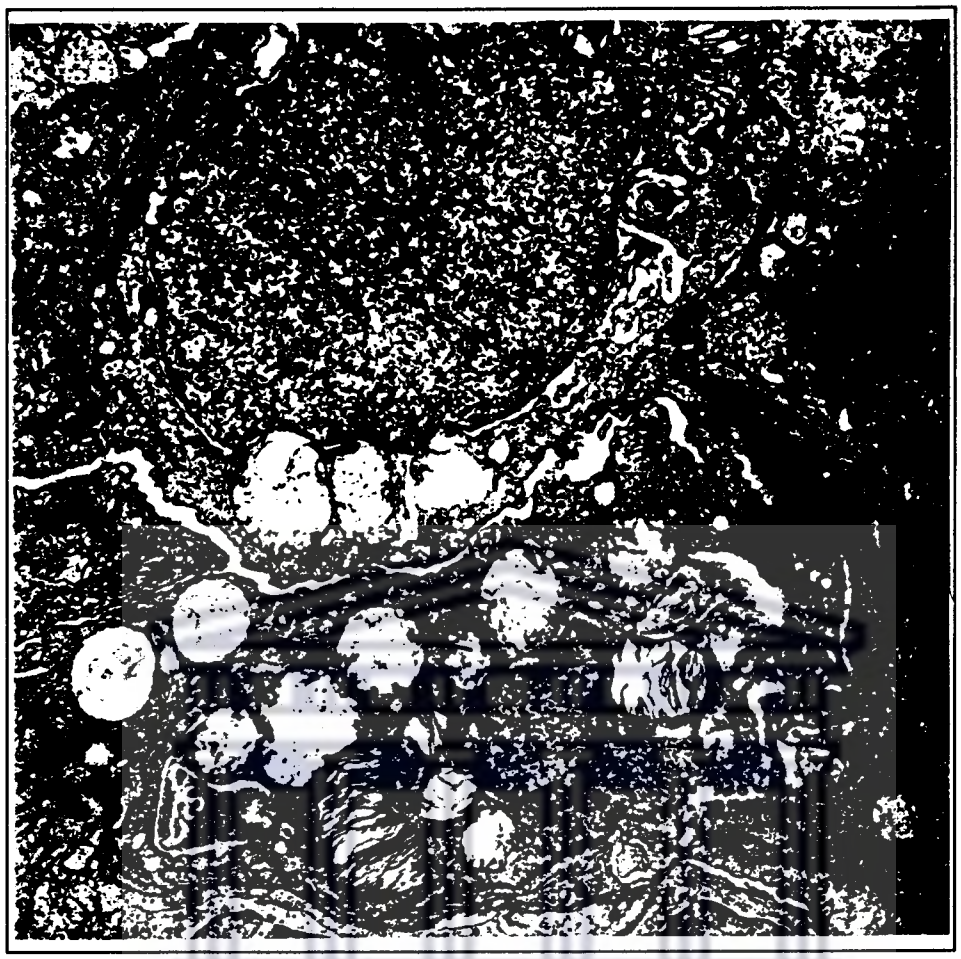

Figure 2: The influence of maternal nicotine exposure on the mitochondria of type II pneumocytes of the offspring. The mitochondria (thick arrows) are swollen with disrupted cristae (thin arrows) and membranous figures (double arrows) (x16 800).

control and nicotine exposed rat pups increased significantly ($P<0,001$) between days 1 and 7 after birth, whereafter it remained constant. On day 1 after birth the lamellar body volume of the type II cells of nicotine exposed neonatal lung was 87,5% higher ($P<0,001$) than in control lung. Further analysis of the data show that maternal nicotine exposure resulted in a higher lamellar body content in type II cells ($P<0,001$) of all age groups tested, than in lung tissue of control rat pups of the same age.

Transmission electron micrographs of type II cells of control pups of all the age groups show clear microvilli on the alveolar surface. Small dense mitochondria occur. No damage to the mitochondria are visible (fig. 1). However, the type II cells of the nicotine exposed rat pups of all age groups show swelling of mitochondria as well as disruption of the mitochondrial cristae. Membranous figures are often present in these mitochondria (fig. 2). No microvilli occur on the alveolar surface of the type II alveolar epithelial cells of nicotine exposed rat pups (fig. 2 and 3).

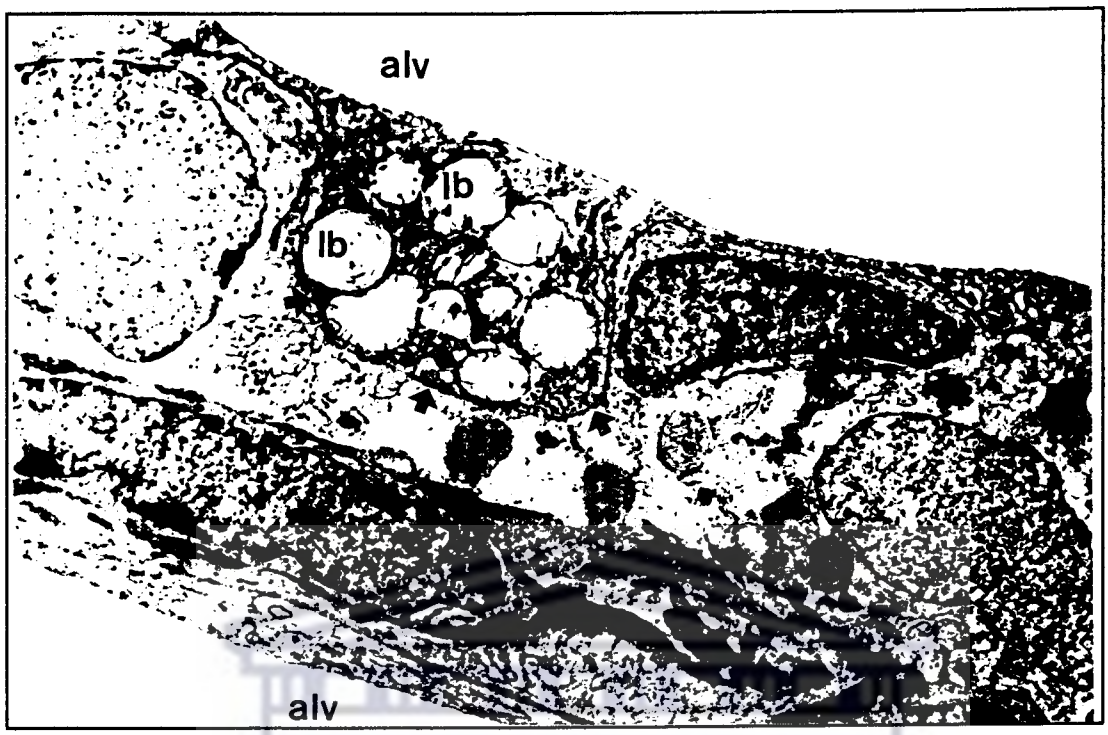

Figure 3: An alveolar septum illustrating a type II penumocyte (arrows) of lung tissue of rat pups exposed to nicotine via the placenta and mother's milk. Note the absence of microvilli on the alveolar surface and swollen mitochondria with disrupted cristae and membranous figures (x7 500). Alv. = alveolar space; L.B. = lamellar body; arrow heads = mitochondria showing disrupted cristae and membranous figures.

DISCUSSION

The alveolar type II pneumocyte is already present in the fetal rat lung on day 21 of gestation (O'Hare and Sheridan, 1978). It is recognized as the stem cell of the alveolar surface (Kauffman, 1978) where this surface acts as the primary site for gas exchange. Changes in the epithelial cell basement membranes is associated with type II pneumocyte differentiation and remodelling of the perinatal lung alveolar surface (Grant et al, 1984). Proliferation and differentiation of the type II cells and lung morphogenesis is also influenced by epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) in fetal lung. The type II cells are a source of EGF and TGF-α in fetal lung. The TGF-α and EGF may act in parallel in fetal rat lung morphogenesis and maturation (Strandjord et al, 1994).

Type II cell function is dependent on metabolic energy. In the presence of sufficient quantities of oxygen, ATP energy for type II cell function and maintenance of type II cell viability is produced by the glycolytic pathways and especially during mitochondrial oxidative phosphorilation. However, under hypoxic conditions the type II cell derive ATP from mainly glycolysis and the pathway presumably plays an important role in maintaining the viability of this cell under hypoxic conditions (Simons et al, 1978). The above finding clearly illustrates the importance of glycolysis in not only maintaining the viability of the type II cell but also the importance of

this metabolic pathway in maintaining lung alveolar development and integrity.

Recent students showed that maternal nicotine exposure irreversibly inhibits the glycolytic pathway in neonatal lung (Maritz, 1987; Maritz and Burger, 1992) and thereby reduce the capacity of the cells of the lung to derive ATP from glycolysis. Furthermore, in the present investigation it was clearly illustrated that maternal nicotine exposure also induce swelling of the mitochondria of the type II cells. Moreover, disruption of the cristae, the site of oxidative phosphorilation in mitochondria, also occur. In addition to this, membranous structures also occur in the mitochondria, where the latter is an indication of irreversible mitochondrial damage (Laiho and Trump, 1974). It is therefore very likely that the mitochondrial ATP supply will be insufficient to maintain type II cell integrity. Furthermore, since glycolysis is also inhibited, these cells will be unable to sustain their role in maintaining alveolar integrity. It is therefore conceivable that the inhibition of ATP energy supply will result in a gradual deterioration of the type II cells. Also, since membranes are dependent on glycolysis for their integrity (Bohn et al, 1988), the inhibition thereof will also contribute to a loss of type II cell integrity as illustrated by the loss of surface microvilli.

The total cell cycle time of type II alveolar cells has been estimated at 21-23,1 hours. The transformation of the type II alveolar cells to type I alveolar cells appears to take another approximately 2 days (Witschi, 1976). This means that it takes about 3 days for a normal type II alveolar cell to divide and for 1 of the daughter cells to differentiate into a type I alveolar cell. It is known that damage to the type I cells serves as a stimulus for type II cell proliferation (Crapo et al, 1980). The increase in type II cell numbers and the decrease in type I cell numbers and consequently the decrease in the type I: type II cell ratio in lungs of rat pups exposed to nicotine clearly show type II cell proliferation in an effort to replace damaged type I cells. It is however clear that most of the proliferation took place before birth. After birth the rate of proliferation was actually very slow as indicated by the increase in type II cell numbers by only 4,8% over a period of 3 weeks. However, the fact that type I cell numbers decrease, and more importantly that rupturing of blood-air barriers occur in lungs of nicotine exposed rat pups (Maritz and Thomas 1994), suggest that the rate of type II cell proliferation and differentiation after birth was not quick enough to prevent a decrease in type I cell numbers and rupturing of the blood-air barriers.

This view is supported by the fact that glycogen and glucose is an important source of energy for mitosis (Bullough, 1952). Inhibition of energy metabolism will therefore reduce the rate of type II cell proliferation. Consequently, the integrity of the alveolar region of the lung will be adversely affected. It is therefore conceivable that interference with the development of this cell will result in retarded and even abnormal alveolar development. Due to the important role of this cell in maintaining alveolar integrity (Mason et al, 1977, Crapo et al, 1980), injury to this cell may render the lung more susceptible to disease related to exposure to the pollutants in the environment. Various studies indeed illustrated that exposure of rat lungs to cigarette smoke, induced focal alveolitis (Kendrick et al, 1975), alveolar cell metaplasia (Davis et al, 1975), distortion of pulmonary parenchyma as well as areas of collapse (Le Mesurier et al, 1981).

The areas of collapse in the lungs of smoke exposed rat lungs are associated with type II cells containing excessive numbers of lamellar bodies (Le Mesurier et al, 1981). It is known that a balance

exists between release of lamellar bodies and thus of surfactant into the alveolar space and the removal thereof in order to prevent alveolar collapse or accumulation of excessive amounts in the alveoli (Fisher and Chander, 1985). The fact that cigarette smoke exposure resulted in the accumulation of these lamellar bodies in the type II cells (Le Mesurier et al, 1981) implies that a substance or substances in the inhaled cigarette smoke interfered with the control of synthesis and/or release of surfactant from these cells. In the present investigation it was shown that maternal nicotine exposure during pregnancy and lactation also resulted in the accumulation of lamellar bodies in the type II cells. From the data available it is plausible that nicotine in cigarette smoke induce the accumulation of lamellar bodies in type II cells. The mechanism whereby nicotine exposure induce this accumulation is not known. However, from the literature it is clear that surfactant secretion is an active process involving ATP, ATP-generating pathways, and cAMP. The microfilament and microtubular system of the type II cells are also playing a role (Marino and Rooney, 1980). Secretagogues that elevate cytosolic cAMP levels in isolated type II cells also activate cAMP-dependent protein kinase, supporting the hypothesis that protein kinase activation and subsequent protein phosphorilation is involved in secretion of surfactant by type II cells (Rice et al, 1985). Studies by Rice and co-workers (1984) demonstrated that cytochalasins, which disrupt microfilaments, enhanced ^{3}H-phosphatidyl choline release in association with alteration of filamentous actin around the lamellar bodies. Actin was also noted to be a substrate of cAMP-dependent protein kinase in Type II cell cytosol, and this actin phosphorilation is clearly ontogenically regulated, appearing in the perinatal period in the rat in association with enhanced surfactant synthesis and release (Whitsett and Lessard, 1984). In recent studies it was shown that maternal nicotine exposure result in an accumulation of ATP in the lungs of the offspring. It is highly likely that this accumulation is due to inhibition of ATP utilization since maternal nicotine exposure was not stimulating the ATP generating pathways in the cytoplasm and mitochondria (Maritz, 1986 and 1987). It is therefore conceivable that protein phosphorilation, which is required for surfactant release, will be suppressed. This may then result in the observed accumulation of lamellar bodies in type II cells of the nicotine exposed rat pups. This may also explain the accumulation of lamellar bodies in type II cells of smoke exposed rats (Le Mesurier, 1981).

Another factor which needs to be considered is the surface characteristics of the type II cell membrane. For secretagogues such as ß-adrenergic agonists and catecholamines to activate adenyl cyclase with the subsequent formation of cAMP from ATP, the presence of specific receptor sites are required on the cell membrane. In addition to the intracellular ATP, the ATP in the bronchoalveolar fluid of the lung is also involved in the control of surfactant by binding to the P_2-purinoceptors on the surface of the type II cells (Rice and Singleton, 1986; Gilfillan and Rooney, 1987). In this study it was also shown that the alveolar surface of the type II cells of the nicotine exposed lungs were lacking the characteristic microvilli suggesting a change in the functional characteristics of this membrane. The quick disappearance of the cationic binding sites from the alveolar epithelial cell surface from birth to day 21 after birth (Scott, 1994) is further evidence that maternal nicotine exposure induced changes to the surface characteristics of these cells. As a consequence the cells may be unable to respond effectively to secretagogues involved in the control of surfactant release. Further studies to

investigate these theories are presently undertaken.

Another important observation is that all the changes induced in the type II cell in neonatal lung as a result of maternal nicotine exposure during pregnancy and lactation, occur during the fetal and early postnatal stage of lung development since after day 7 after birth, these changes are only maintained and are not becoming worse. This shows that the interference by nicotine during this period of rapid lung modelling and growth may render these lungs more sensitive to the harmful effects of foreign substances which the lung is exposed to. This may render the lung more susceptible to disease. It is therefore conceivable that nicotine, the most important alkaloid in tobacco smoke, contributes to the higher incidence of respiratory disease in the offspring of smokers.

In conclusion, it is clear that maternal nicotine exposure induce lesions in type II cells of lung tissue of neonatal rats which resembles that caused by cigarette smoke. It is therefore conceivable that maternal smoking during pregnancy and lactation will interfere with type II cell integrity in the developing lung which again may result in the pathogenesis of progressive alveolar collapse and even pulmonary emphysema.

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