# THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON THE DEVELOPMENT OF THE LUNG AS GAS EXCHANGER: THE PROTECTIVE EFFECT OF COPPER

**by**

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**ABSTRACT**

The influence of tobacco smoking on the developing fetus is well reported. Maternal nicotine exposure during pregnancy and lactation interferes with fetal and neonatal lung growth and development, rendering the lungs more susceptible to damage and disease. Interference with the normal developmental processes at any stage may compromise the gas exchange function of the lungs.

Maternal nicotine exposure during gestation and lactation causes a decrease in the elastic tissue content of the lungs, which may lead to the formation of emphysema-like lesions. Elastic tissue is catalysed by lysyl oxidase, an extracellular, copper dependent enzyme, from its soluble to its insoluble state. It is suggested that lesions occur in lungs exposed to nicotine because of a decrease in lung copper content, which will, in turn, cause decreased lysyl oxidase activity.

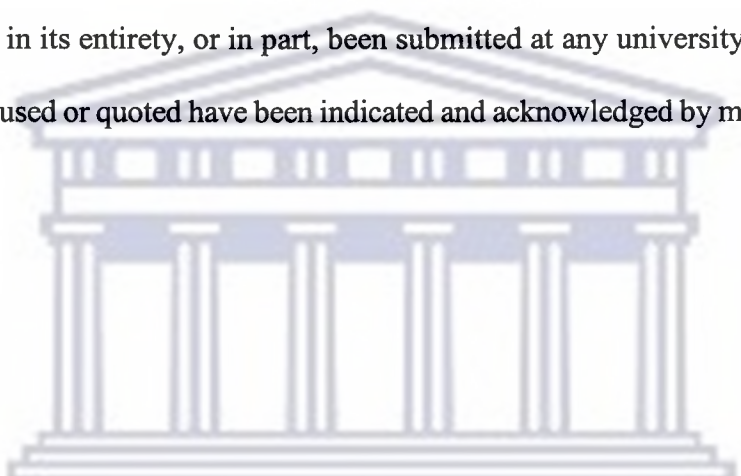
The aim of this study was thus to determine and quantify the effects of maternal nicotine exposure on the development of the fetal and neonatal rat lung as a gas exchanger and to establish whether copper supplementation will protect the lungs against these adverse effects of maternal nicotine exposure.

The pregnant rats were divided into four experimental groups. One group received nicotine (1mg/kg body weight/day) subcutaneously. Another received copper (1mg/kg body weight/day), while a third group received nicotine (1mg/kg body weight/day) in addition to copper (1mg/kg body weight/day). Control rats received sterile saline. These subcutaneous injections were given throughout gestation and lactation.

The results obtained showed that maternal nicotine exposure suppresses alveolarisation in the fetal and neonatal lung. The lungs of the offspring exposed to nicotine during gestation and lactation have a reduced radial alveolar count, and a decreased alveolar number. All this points to a reduced internal surface area available for gas exchange. Copper supplementation during gestation and lactation prevents the adverse effects of maternal nicotine exposure on development of the lungs of the offspring. Therefore, the lungs of the copper supplemented animals can develop to its full potential as gas exchangers.

## DECLARATION

I declare that ***The influence of maternal nicotine exposure on the development of the lung as gas exchanger: The protective effect of copper*** is my own work and has not previously in its entirety, or in part, been submitted at any university. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.



**H. L. Dennis**

**For my father**

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# TABLE OF CONTENTS

**LIST OF TABLES**

# CHAPTER 1

## LITERATURE REVIEW

The principal function of the lungs is that of gaseous exchange. The uptake of oxygen and removal of carbon dioxide from blood takes place in the respiratory units of the lung. Research has shown that exposure to tobacco smoke leads to abnormalities in the lung, impacting negatively on this vital process. The influence of tobacco smoking on the developing fetuses of pregnant women is well documented. The children of smoking parents are more disposed to general respiratory disorders and frequently have impaired lung function (Yarnell and St Leger, 1979). In order to understand the mechanisms of lung disease caused by cigarette smoking, a sound knowledge of normal lung development is required.

### 1.1 NORMAL LUNG DEVELOPMENT

Lung development in humans can be divided into: the embryonic phase, the pseudoglandular phase, the canalicular phase, the saccular phase, and the alveolar phase. Each phase is typified by characteristic histological features which makes identification possible. The relationship between these stages of development and stages of gestation varies between species (Harding, 1994).

In the fourth week, the precursor to the human lung, the laryngo-tracheal groove, appears as an evagination of the foregut. Caudal elongation leads to the formation of the tracheo-esophageal septum which separates the groove from the foregut. The laryngo-tracheal groove is converted into the laryngo-tracheal tube, and fusion of the

caudal ends of the tube give rise to the left and right lung buds. The tube is lined with epithelium which will eventually develop into the epithelial lining of the pulmonary airways (Johnson, 1988). By gestational day 34 the buds of the lobar bronchi have been formed. These primitive structures are lined with columnar epithelium. The surrounding splanchnic mesenchymal tissue will develop into non-epithelial structures such as blood and lymphatic vessels, cartilage, smooth muscle and connective tissue (Harding, 1994).

### 1.1.1 Pseudoglandular phase

During weeks 6 to 16, the major airways are formed and the pattern for the respiratory units of the lung, the acini, becomes apparent. Continuous growth and branching of the lung buds cause the lung to resemble a typical secretory gland. The appearance of bronchi and acini depends on the investing of the mesenchyme by the repeated branching of the periphery of the epithelial tubes. The simple columnar and cuboidal epithelium lining the tubes differentiates into goblet cells and mucous glands, and ciliated, flatter epithelium cells appear at weeks 11 to 13. Each of the major respiratory tubules is accompanied by a newly developed artery (Harding, 1994).



### 1.1.2 Canalicular phase

During weeks 16 to 24 of gestation, the airways undergo lengthening and widening. The mesenchyme becomes thinner because of repeated airway branching and widening of their terminations. Also characteristic of this phase is the formation of respiratory units. Mesenchymal capillaries invest the tubules and become closely associated with the epithelium, marking the development of the blood-air interface (Burri and Moschopulos, 1992).

### 1.1.3 Saccular phase

Weeks 24 to 40 sees the development of transient alveolar ducts from the terminal saccules. Progressive enlargement of the potential air spaces gives rise to the characteristic spongy appearance of adult lung tissue (Burri and Moschopulos, 1992). This is accompanied by an increase in the volume of lung fluid. The epithelial lining differentiates to form type I and type II pneumocytes; surfactant is subsequently formed by the lamellar bodies in the latter. Proliferation of the capillary network, and the thinning of the intersaccular septa leads to the defining of the blood-air barrier, which will in future facilitate gas exchange (Harding, 1994).

### 1.1.4 Alveolar Phase

In humans, the alveolar phase is between week 36 of gestation and the first two years after birth. During this phase of lung development, the newly formed alveoli mature by the formation of septal divisions in the terminal sacs. The alveolar epithelium becomes thinner, and is richly endowed with blood capillaries which facilitate the process of gas exchange (Harding, 1994).



Except for the length of gestation, the phases of lung development described above are the same for mice, rats and the Rhesus monkey (Ten Have-Opbroek, 1991).

## 1.2 POSTNATAL LUNG GROWTH

In the rat, postnatal lung growth can be divided into three distinct phases (Emery and Mithal, 1960; Burri, 1974; Kauffman *et al*, 1974; Vaccaro and Brody, 1978). The lung expansion phase is evident in rat lungs from days 1 to 4 after birth. During this phase

the lung volume increases primarily from an enlargement of the existing air spaces (Burri *et al*, 1974). The air space volume increases by as much as 87%, with only a moderate increase in the tissue volume (Kauffman *et al*, 1974).

From postnatal day 4 to day 13 in rats, a phase of tissue proliferation occurs (Burri *et al*, 1974). Characteristic of this phase of development is the formation and outgrowth of secondary septa which serve to subdivide the existing saccules. This leads to the marked increase in internal surface area available for gas exchange. Because the proliferation of tissue is followed by an increase in the number of capillaries, the lung is preparing itself for its gas exchange function (Kauffman *et al*, 1974).

Somewhere between postnatal days 13 and 21 a phase of equilibrated lung growth starts (Burri *et al*, 1974). Growth and maturation are concurrent in this phase. There is a decline in cell production and continuous increase in the alveolar and capillary surface area, which leads to thinning of the alveolar interstitium (Kauffman *et al,* 1974).



## 1.3 FACTORS AFFECTING LUNG DEVELOPMENT

According to the Barker hypothesis certain diseases such as high blood pressure and non-insulin dependent diabetes mellitus result from interference with "programming" whereby a stimulus or insult at a critical sensitive period of early life result in long term compromise of organ function (Barker, 1997). Evidence in the literature indeed shows that this is in fact true for lung development. Several studies showed that maternal smoking or exposure of the mother to nicotine during pregnancy (Maritz, 1997) interferes with lung growth and development, thereby rendering it more susceptible to

damage and disease. Some other factors impacting on lung growth and development are glucocorticoid treatment during pregnancy (Tschanz *et al*, 1995), poor nutrition (Harding, 1995), the size of the mother's lungs (Faridy *et al*, 1988), and maternal hypoxia and hyperoxia (Faridy *et al*, 1988).

### 1.3.1 Glucocorticoids

Administration of dexamethasone, which is frequently used in pre-term neonates, impairs alveolar septation (Tschanz *et al*, 1995). It inhibits cell proliferation during alveolarisation, leading to larger but fewer alveoli (Blanco and Frank, 1993). This impairment of septation persists well into adult life and the lung does not recuperate (Massaro and Massaro, 1996).

### 1.3.2 Maternal Exercise

Fetuses of mothers who exercise throughout the duration of the pregnancy have reduced body and lung size as compared with inactive women, or women who undergo short term exercise. The lung tissue of these fetuses has thickened gas exchange walls, and its type II cells have fewer lamellar bodies and abundant glycogen (Nagai *et al*, 1993). This indicates that maternal exercise throughout pregnancy induces a disturbance in the maturation of these cells and the alveolar walls.

### 1.3.3 Maternal Nutrition

Components such as collagen, laminin, fibronectin and proteoglycans all play a role in the alveolarisation and maturation processes of the lung. Nutrient restriction may lead to structural changes in these factors, which may in turn be reflected as lesions

characterised by a reduced internal surface area and an increased alveolar size (Edelman *et al*, 1986). This will reduce the area available for gaseous exchange.

Compromised maternal nutrition leads to restriction of fetal growth (Gaultier, 1991). Although fetal body weight may be unaltered, fetal lung weight is reduced. This impact on lung development during gestation suggests that the lungs are especially sensitive to nutrient intake of the mother and fetus (Harding, 1995).

From the above information it is clear that the developing lung is sensitive to various external factors and that interference with lung growth and development during the fetal stage may result in a less well developed lung which may then compromise the function of the lung as gas exchanger.

## 1.4 TOBACCO SMOKING AND DISEASE

Cigarette smoke contains more than 4 000 different components. Tobacco smoke is an aerosol comprising 2 to 5 billion particles per millilitre of smoke (Huber, 1989). Particles include carcinogens, irritants, and other tumour promoters (Sherwin and Gastwirth, 1990). Reports linking tobacco smoking with impaired health are numerous. Hammond and Garfunkel (1966) stated that cigarette smokers have the greatest risk of developing cancer of the oral cavity and throat. A number of respiratory diseases including emphysema and chronic bronchitis have been linked to the smoking habit (Huber, 1989). There is a marked reduction in birth weight of babies born to smoking mothers. Maternal smoking increases late fetal and neonatal mortality rates by up to 28% (Butler *et al*, 1972).

Evidence suggests that maternal smoking during pregnancy negatively affects fetal lung development and pulmonary function after birth (Harding, 1995). Colley *et al* (1973) link respiratory tract impairment in childhood to that in early adult life. Their study shows that childhood chest disease has a more profound effect on the increased frequency of respiratory disability in early adult life than social class or air pollution.

Maternal smoking reduces the expiratory flow rates in neonates and children, pointing to an impaired breathing capacity (Hanrahan *et al*, 1992). Maternal smoke inhalation in rats causes a reduction in the development of terminal saccules, resulting in the formation of fewer alveoli, and therefore a reduced gas exchange area (Collins *et al*, 1985).

## 1.5 NICOTINE

Nicotine, an alkaloid tertiary amine, is the primary addictive agent in cigarette smoke. Once a cigarette is burning, the pH of cigarette smoke is 6. At this level of acidity, most of the nicotine available from cigarette smoke is completely protonated. Protonated nicotine does not cross biological membranes in any notable amounts (Huber, 1989). Therefore, only small quantities of nicotine is absorbed in the mouth, pharynx, or upper respiratory passageway. In the lungs, the pH of 7.4 of the alveolar fluids buffers the nicotine droplets. There is a resultant increase in the index of dissociation, causing more than 30% of nicotine to be in the non-protonated state. This nicotine is now rapidly absorbed across the air-blood barrier in the lung. When entering the bloodstream, two thirds of nicotine is non-protonated (Benowitz *et al*, 1982). Nicotine levels measured in the lungs, brain and liver exceed that of levels in the plasma in adults (Wonnacott *et*

*al*, 1990). This is due to the high affinity these organs have for nicotine (Huber, 1989).

Research has shown that higher levels of nicotine are found in breast milk compared to the mother's blood after absorption. Nicotine is also found in nonlactating breast fluids (Luck and Nau, 1984). Nicotine freely crosses the placenta and is known to accumulate in the amniotic fluid. Nicotine and its metabolites, especially cotinine, are known to have a high affinity for the fetal lung (Turner *et al*, 1975).

Studies have also shown that the concentration of nicotine, with its half-life of 1 to 2 hours, markedly decreases in the mother's blood and breast milk over a period of 4 hours. However, the level of cotinine, which is a metabolite of nicotine, remains elevated (Luck and Nau, 1984; Dahlström *et al*, 1990), due to its half-life of 15 to 20 hours (Benowitz *et al*, 1982; Lambers and Clark, 1996).

After absorption into the blood and tissue, nicotine acts on sympathetic ganglia and the adrenal medulla to cause a release of acetylcholine, epinephrine and nor-epinephrine. These agents cause an increase in heart rate and cardiac output, a rise in blood pressure due to vasoconstriction of the peripheral blood vessels, an increase in blood glucose concentration, and the release of free fatty acids (Pirani, 1978).

In a study by Maritz and Woolward (1992b) it was furthermore found that maternal nicotine exposure leads to low elastic tissue content in the lungs of the offspring, to the extend where emphysema-like lesions are formed. It was suggested that the interference of nicotine with lung development and maturation may render the lungs of pups

exposed to maternal nicotine more susceptible to damage such as emphysema (Maritz *et al*, 1993).

Maternal nicotine exposure causes swelling of type II cell mitochondria of neonatal lung as well as the disruption of mitochondrial cristae (Maritz *et al*, 1994). This may compromise the role of the mitochondrion as energy generator of the type II cell and may eventually compromise type II cell function. It will also result in death of these cells. Nicotine also impacts on the status of the blood-air barrier in the lung. In 1994, Maritz and co-workers found that maternal nicotine exposure leads to thickening of the blood-air barrier. The number of blood capillaries found in the barrier is also reduced by maternal nicotine exposure (Maritz and Thomas, 1994), which will compromise the gas exchange function of the lungs of the offspring.

There is a decrease in the type I:type II cell ratio in the lung tissue of pups exposed to maternal nicotine (Maritz and Thomas, 1994). This change is due to type II cell proliferation in response to type I cell injury (Kauffman, 1980). Maritz and Thomas (1994) also found fewer alveoli in the lung tissue of these rat pups, which implies that a decreased area is available for gas exchange, thus impacting negatively on the principal function of the lungs.

Maritz and Thomas (1994) suggested that the reduced alveolar number was due to an interference with elastic tissue metabolism in the fetal and early neonatal lung by nicotine. This suggestion was based on the fact that it was not possible to stain for elastic tissue in early neonatal lung despite the fact that the total elastic tissue content

of the lungs of the nicotine exposed rat pups was normal. Since only the insoluble component of elastic tissue is stainable with Verhoeff's elastic tissue stain, it is conceivable that most of the elastic tissue in the lungs of the nicotine exposed rat pups was in the soluble form.

Conversion of the soluble elastic tissue to the insoluble form is catalysed by the copper dependent extracellular enzyme, lysyl oxidase (Harris *et al*, 1974). Formation of elastic tissue during the late fetal stage of lung development and during postnatal days 4 to 13 in neonatal rats is associated with rapid alveolarisation. This implies that lysyl oxidase plays an important role in alveolarisation and thus in the development of the lung into an effective gas exchanger. Since lysyl oxidase activity depends on $Cu^{2+}$, it is clear that $Cu^{2+}$ is also important for the functional development of the lung. Several studies have indeed shown that nutritional copper deficiency produces emphysema-like (O'Dell *et al*, 1978 and Soskel *et al*, 1982) or related lesions in lungs (Buckingham *et al*, 1981 and Lefevre *et al*, 1982). Studies by Maritz (1997) showed that maternal nicotine exposure also result in microscopic emphysema in the lungs of the offspring. It was furthermore illustrated by Maritz *et al* (1994) that the radial alveolar count and thus the number of alveoli per respiratory unit in the lungs of rat pups exposed to maternal nicotine during pregnancy and lactation was lower than in the lungs of control animals. It is therefore possible that the above impairments were induced by a decreased lysyl oxidase activity probably due to a reduced lung copper content. This is supported by the fact that cigarette smoke suppresses cross-linking of elastin *in vitro* and thus also lysyl oxidase activity (Laurent *et al*, 1983).

## 1.6 MOTIVATION FOR THIS STUDY

To function effectively as an organ of gas exchange, the lungs must have reached structural and metabolic maturity. It needs a large internal surface area in order to cope with the metabolic demands of the body. The developmental processes of the lungs are adversely affected by maternal cigarette smoking. Despite the vast amount of evidence pointing to impaired lung development and function, there is no reduction in the amount of people who smoke. Studies in the laboratory of the University of the Western Cape brought to light that maternal nicotine exposure may lead to development of emphysematous lesions (Maritz and Woolward, 1992a), thickening of the blood-air barrier, and a reduction of the number of alveoli (Maritz and Thomas, 1994). All these findings constitute an impairment in gas exchange. Since nicotine is at the centre of the controversy over lung injury, it was decided to investigate the influence of maternal nicotine exposure on the development of the lung as gas exchanger. In doing this, various morphologic and morphometric parameters will be analysed.

## 1.7 OBJECTIVES

1. To quantify the deleterious effects of maternal nicotine exposure on fetal and neonatal lungs by means of morphologic and morphometric analysis.
2. To investigate whether damage caused by maternal nicotine exposure is at all reversible.
3. To determine if copper supplementation will protect the lungs from the harmful effects of maternal nicotine exposure.
4. To ascertain if supplemented copper will accumulate in the lung tissue of these pups.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Experimental animals

The animals used in this study were virgin white rats from the Wistar strain and were bred in the animal laboratory of the Department of Physiological Sciences at the University of the Western Cape. Only animals free from visible signs of disease and ill-health were used in the study. Animals were fed a stock diet of Epol rat cubes, and they had fresh drinking water *ad libitum*. The rats were kept in a controlled environment to eliminate factors such as noise and unnecessary handling of the animals which might have resulted in experimental variations. The ambient temperature was maintained at 25 $\pm 1^0$C and a 12-hour light cycle was maintained.

The animals were mated overnight (12 hours) after which the sires were removed. The females were randomly divided into four experimental groups. The body weight of each female was recorded daily for the next 7 days. A significant mass increase over this time indicated that mating was successful. The pregnant rats were placed in individual straw lined cages for the duration of the study.

From gestational day 7 to day 21 post partum, the rats were given treatment as designated by the experimental group. Nicotine treated animals received 1 mg nicotine/kg body mass/day subcutaneously, whereas copper treated rats received 1 mg copper/kg body mass/day. The copper injection was prepared using $CuSO_4$ in distilled water. Control animals received a placebo of sterile saline to the dose of 1mg/kg body

mass per day. Finally, the last experimental group was subjected to the following procedure: an injection of 1 mg nicotine/kg body mass/day followed by an injection of 1 mg copper/kg body mass/day.

The dams received a dose of 1 mg nicotine/kg body mass/day subcutaneously. This dose lies within the range of intake of 0.16 to 1.8 mg/kg body mass/day of habitual smokers (Maritz and Woolward, 1992a).

The animals were injected daily between 9h00 and 10h00 with 1,0 ml plastic disposable syringes and needles. Care was taken during handling and injection of animals not to inflict pain and to prevent discomfort of the animals. Noise level in the animal laboratory was kept to a minimum.

## 2.2 Excision of lung tissue

Lung tissue for each of the experimental groups was obtained from the pups at postnatal days 14, 21, 35, and 42. In each of the age groups a total of between 5 and 13 lung samples were obtained.



Body weight was determined by weighing pups on a top loader laboratory balance (Sartorius 1475A). Animals were then sacrificed by intraperitoneal injection of an overdose of 0.5ml pentobarbitol. The trachea was ligated and the lungs were infused with 10% formaldehyde. Lungs were fixed at a pressure of 20mm Hg *in situ* for 30 minutes, after which it was removed *en bloc*. The other thoracic organs were removed, and the lungs were fixed in 10% buffered formalin until processing. All lung samples

remained in the fixative for a minimum of 24 hours before processing.

## 2.3 Embedding and processing

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

| | | |
|---|---|---|
| 1 | buffered formaldehyde | 1 hour |
| 2 | 50% ethanol | 30 minutes |
| 3 | 70% ethanol | 30 minutes |
| 4 | 80% ethanol | 30 minutes |
| 5 | 90% ethanol | 30 minutes |
| 6 | absolute ethanol | 1 hour |
| 7 | absolute ethanol | 1 hour |
| 8 | xylene | 1 hour |
| 9 | wax bath | 1 hour |
| 10 | wax bath | 1 hour |



An embedding system was used to embed the samples to form wax blocks, which were then sectioned to make microscope slides.

## 2.4 Microscope slide preparation

Wax blocks were cooled in a standard refrigerator for an hour prior to sectioning. A sliding microtome was used to make 5μm sections of these blocks. Sections were floated on a water bath from where it was picked up on clean, marked microscope

slides. Sections were fixed onto the glass slides in an incubator at 37$^0$C for an hour, after which it was stained with haematoxylin and eosin. If staining was not performed on the day, slides were stored, and prior to staining it was heated for an hour in the incubator at 37$^0$C.

## 2.5 Mayer's haematoxylin and eosin staining technique

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

### 2.5.1 Reagents

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



Xylene: use as supplied by manufacturer.

Ethanol: diluted to desired concentrations using distiled water.

Mayer's alum haematoxylin: 1 g of haematoxylin and 50 g of potassium ammonium was dissolved in a small volume of water. 0,2 g of sodium iodate was added, made up to a volume of 1 litre, and left overnight. 50 g of choral hydrate and 1 g of citric acid was added and it was boiled for 5 minutes.

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

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

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

DPX: used as supplied by manufacturer.

### 2.5.2 Procedure:

1. Sections were fixed onto glass slides for 30 minutes in a hot air oven at $80^0$C.
2. Glass slides with sections were then agitated in a xylene bath for 1 minute.

Dewaxing was completed in a second xylene bath.

3. Slides were agitated for 30 seconds in absolute ethanol.
4. Hydration of sections was continued by agitating slides for 30 seconds each in 95%, 80% and 70% ethanol.
5. Hydration was completed by rinsing slides under tap water for 1 minute.
6. Sections were stained in haematoxylin for 10 minutes.
7. Excess stain was rinsed under tap water.
8. Sections were differentiated in 1% acid alcohol, and rinsed under tap water.
9. Sections were then blued in Scott's tap water for 30 seconds, and rinsed under tap water.
10. Counterstaining in eosin took place for 1 minute, and excess stain was rinsed under tap water.
11. Sections were dehydrated for 30 seconds each in 70%, 80%, 90% and absolute alcohol.
13. Slides were cleared in two successive xylene baths, 30 seconds each.
14. Slides were mounted in DPX.

## 2.6 Morphometric Techniques

Various morphometric techniques can be used to assess abnormal lung development. Five of these techniques were used in this study to elucidate the effects of nicotine, copper, and nicotine + copper, injected during pregnancy and lactation. Used individually, the techniques quantify the effects of the above-mentioned agents. Used as a combination, it gives a global view of lung development, as well as the quantification of the ill effects of nicotine exposure. The parameters will also indicate whether copper supplementation can prevent or attenuate the alterations caused by nicotine. Haematoxylin and eosin stained slides free of cutting artefacts were used for these assessments. Three microscope slides of each animal sample were used. At least 6 fields per microscope slide were counted.

### 2.6.1 Lung Volume ($V_L$)

#### 2.6.1.1 Principle

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



#### 2.6.1.2 Method

After *in situ* fixation of the lungs with 10% buffered formaldehyde, lungs were carefully excised *en bloc*. Excess fluid on the lung surface was removed by gently dabbing with disposable tissue towels. Attached to a laboratory clamp by a length of cotton string, the lung was lowered into a beaker filled with buffered formaldehyde that was placed

on a laboratory balance. A weight was hooked onto the lung to ensure complete submersion of the lung. The reading on the balance was taken. This reading on the balance in grams corresponds to the volume of the organ in millilitres (Scherle, 1970). The mass displaced by the weight was determined, and the nett weight displaced by the lung, *ie* the lung volume ($V_L$), was then calculated. This procedure was repeated five times and the mean reading taken.

#### 2.6.1.3 Determination of the volume density of parenchymal tissue and gas exchange region

A graticule with 122 points was used to count the number of points falling on tissue intercepts on a microscope slide. The percentage of tissue space ($V_t$ %) was then calculated as follows:

$$\frac{\text{no. of intercepts}}{122} \times \frac{100}{1}$$

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



### 2.6.2 Mean Linear Intercept ($L_m$)

#### 2.6.2.1 Principle

The mean linear intercept is the average distance between alveolar walls. Therefore, the bigger the alveoli found in the lung, the greater the mean linear intercept (Dunnill, 1962). It may also be indicative of fewer alveoli in the lung.

### 2.6.2.2 Method

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

- Lungs were fixed at transpulmonary pressure of 20 mm formaldehyde.
- A X10 objective and X10 eyepiece were used.
- 6 fields per slide preparation were counted.

A hairline cross eyepiece micrometer (OC-M Olympus 10/100X 19m/m) was fitted into the 10X eyepiece to count the intercepts. The hairline was marked on either side of the centre intersection to gain lengths equal to half that of the vernier. The combined length of the vernier and marked off hairline was measured using a stage micrometer (S8, Graticules Ltd., England). This, at the given magnification, measured 2.04 mm.

The following structures were accepted as intercepts:

- All alveolar walls crossing the cross hairs.
- Alveolar walls touching the upper side of the horizontal line.
- Alveolar walls touching left side of vertical line.



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

$$L_m = \frac{N X L}{m}$$

where: N = the number of counted fields

L = the combined length of the cross hairs

m = the total number of intercepts counted.

### 2.6.3 Internal Surface Area (ISA)

#### 2.6.3.1 Principle

The internal surface area (ISA) of the lung is a measure of the potential area available for gas exchange (Butler, 1976). Alveolar walls are eroded in patients with emphysema, and Thurlbeck (1967) has shown that there is an inverse correlation between the internal surface area and emphysema. The ISA in fetuses of smoking females are markedly reduced (Collins *et al*, 1985).

#### 2.6.3.2 Method

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

$$ISA = \frac{4 \times V_L \times 1.09}{L_m}$$

where: $V_L$ = the lung volume

Lm = the mean linear intercept

1.09 = is a correlation factor for shrinkage during processing



### 2.6.4 Radial Alveolar Count (RAC)

#### 2.6.4.1 Principle

The radial alveolar count indicates the number of gas exchange units in the lungs.

#### 2.6.4.2 Method

The method developed by Emery and Mithal (1960) and modified by Cooney and Thurlbeck (1982), was used. The RAC is the number of alveolar spaces between a

respiratory bronchiole and the nearest connective tissue septum. A suitable respiratory bronchiole is one lined with epithelium in one part of the wall (Cooney and Thurlbeck, 1982). A 10X eyepiece that was fitted with a hairline, and 10X objective were used. The hairline was dropped from the centre of the particular bronchiole to the periphery of the acinus, and the number of alveolar spaces transected by the line was determined.

The following criteria as stipulated by Cooney and Thurlbeck (1982), were incorporated into the method of Emery and Mithal (1960) to facilitate reproducibility of results:

- In a symmetrical respiratory bronchiole, the geometric centre was used as starting point.
- In an irregular bronchiole, the starting point was halfway between the most proximal part (lined with epithelium) and the first point of branching.
- Should the hairline traverse the common wall of two adjacent alveoli, a count of one was made.



### 2.6.5 Destructive Index (DI)

#### 2.6.5.1 Principle

The destructive index represents the percentage of destroyed airspace as a fraction of the total air space in the lung. The destruction of alveolar walls is an important criterion of emphysema. It is more accurate in its prediction of emphysema than is the mean linear intercept. The destructive index indicates the measure of pulmonary tissue destruction even when the linear intercept is not yet afflicted (Saetta *et al*, 1985).

### 2.6.5.2 Method

The X10 eyepiece of a light microscope was fitted with a graticule (Weibel No 2, Graticules Ltd., England) containing 42 points. Alveolar and/or duct spaces falling under these points were assigned as normal (N) or destroyed (D). An alveolar space is defined as the space surrounded by an alveolar wall, whereas an alveolar duct is the air space surrounded by the openings of alveoli.

Alveolar spaces were counted as destroyed when the alveolar wall was broken in two or more places, or if there were two or more disruptions of contiguous alveoli that both opened onto a single duct system. Two or more isolated pieces of lung tissue in the lumen of a duct would result in it being counted as destroyed. Both alveolar and duct spaces were counted as destroyed when it was lined by thickened epithelium, or when a classic emphysematous lesion, *i.e.* permanent enlargement of the parenchyma, was present (Thurlbeck, 1973).

The destructive index was calculated as follows:

$$DI = \frac{D}{(D + N)} \times 100$$

where D = destroyed space

N = normal space

### 2.6.6 Determination of lung alveolar volume ($V_{alv}$) and alveolar number ($N_a$)

By using the Lm, the $V_{alv}$ was calculated by using the following equation (Boros *et al*, 1997):

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

Since the $V_L$, $V_a$ and $V_{alv}$ is known, the alveolar count of the lungs were calculated by using the formula of Blanco *et al* (1989):

$$N_a = V_L \times V_a/V_{alv}$$

## 2.7 Biochemical technique: Atomic absorption spectrophotometry

### 2.7.1 Sample preparation

From each harvested lung tissue sample, a section (0.2 to 0.4 g) of the inferior right lobe was cut off with a scalpel and weighed on a top loader laboratory balance. These tissue samples were placed in Eppendorf vials and stored in a freezer (Lasec Angelantoni Scientifica PR 340C) at -80$^0$C, until used for the determination of copper content.

To carry out the experiment, the tissue samples were thawed and then digested in Micro-Kjeldahl flasks with a sulphuric acid- hydrogen peroxide mixture in an aluminium digestion block. Initially the samples were heated gently and then the intensity was gradually increased up to 38$^0$C and maintained until the digest has cleared. The samples were then cooled, diluted with distilled-deionised water, filtered (No 44

paper), and quantitatively transferred into 10 ml volumetric flasks.

### 2.7.2 Digestion mixture

0.42 g Se and 14 g $LiSO_4$ was added to 350 ml $H_2O$. 420 ml $H_2SO_4$ was added slowly whilst the solution was being mixed and cooled (Allen, 1989).

### 2.7.3 Determination of copper content in digested samples

Initially the copper determinations were performed on a PYE UNICAM Flame Atomic Absorption Spectrophotometer. In many cases the digested samples were so small and the copper concentrations so low that the atomic absorption spectrophotometer was operating at its detection limit. The determinations were then repeated on the SPECTROFLAME ICP of the Peninsula Technicon. The copper content of lungs was determined at a setting of 324.8 nm. The results obtained with the ICP confirmed the results of the AA-spectrophotometer.

## 2.8 Statistical analysis

Results were recorded throughout as the mean ± standard error of the mean. Statistical analysis was performed using Medcalc. The unpaired student t-test was used for statistical analysis of the mean values of control and experimental groups. The probability level of $P<0.05$ was designated as being statistically significant.

# CHAPTER 3

## RESULTS

### 3.1 **Body Weight** (Tables 3.1 and 3.2)

On day 14 after birth, the body weight (BW) of control rat pups was at 34.26 ± 1.54 g higher ($P<0.05$) than that of the nicotine and copper exposed rat pups that was 25.38 ± 1.69 g and 25.36 ± 1.71 g respectively. The body weight of the control rat pups was also higher than the 28.74 ± 0.089 g of the nicotine + copper exposed rat pups ($P<0.05$). On postnatal day 21 the BW of the nicotine exposed rat pups was at 45.37 ± 1.98 g higher ($P<0.05$) than that of the other groups. On day 35 after birth the BW of the control, nicotine exposed and copper exposed rat pups were the same. However, the BW of the nicotine + copper exposed rats was at 127.34 ± 2.90 g markedly higher ($P<0.001$) than that of the other experimental groups. On postnatal day 42 the BW of the copper exposed group was at 171.10 ± 7.04 g 63.1g and 37.2 g respectively higher ($P<0.001$) than the 108.00 ± 3.80 g and 113.90 ± 4.69 g of the control and nicotine exposed rat pups. The BW of copper exposed animals was also higher ($P<0.01$) than the 152.01 ± 6.45 g of the nicotine + copper exposed rat pups.

Further investigation of the changes in BW as the animals matured showed that in control rat pups the BW increased by 0.73 g/day between postnatal days 14 and 21. Between postnatal days 21 and 35, and between days 35 and 42 after birth the BW increased by 3.50 and 2.81 g/day respectively. In the nicotine exposed animals the BW increased between postnatal days 14 to 21, 21 to 35 and 35 to 42 was 2.85, 2.68 and

2.99 g/day respectively. Rat pups exposed to copper during pregnancy showed a BW increase of 1.86 g/day from postnatal days 14 to 21. Between days 21 and 35 after birth the BW increased by 3.76 g/day which resembles the 3.5 g/day increase of control animals over the same time period. However, between days 35 and 42 after birth the BW of the copper exposed animals increased by 11.45 g/day which was about 4 times faster than in control and nicotine exposed rat pups.

The BW increase of animals exposed to nicotine + copper between postnatal days 14 and 21 was at 0.77 g/day the same as for control animals. Between days 21 and 35 after birth the BW of these rat pups increased by 6.66 g/day which was about twice that of the control, the nicotine exposed and copper exposed pups. Between days 35 and 42 after birth, the rate of increase in BW of these rat pups decreased to 3.52 g/day, which was only slightly higher than in the control and nicotine exposed rat pups. It is interesting to note that the increase in body weight between days 14 and 21 after birth of control, copper exposed, and nicotine + copper exposed rat pups were slower than that between days 21 to 35 and days 35 to 42 after birth. It was also slower than in the nicotine exposed rat pups at the corresponding age groups. The BW increase of the nicotine exposed rat pups was remarkably constant over the postnatal period under investigation compared to the fluctuation in the other groups of rats.

Table 3.1: Comparison of body weight (g) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age (Days) | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 34.26 ± 1.538 | 25.38 ± 1.699 | 25.36 ± 1.707 | 28.743 ± 0.079 |
| 21 | 39.36 ± 1.838<br>P<0.05 * | 45.37 ± 1.980<br>P<0.001 * | 38.41 ± 2.628<br>P<0.0007 * | 34.145 ± 0.541<br>P<0.001 * |
| 35 | 88.29 ± 1.858<br>P<0.001 * | 82.97 ± 3.521<br>P<0.001 * | 90.98 ± 2.303<br>P<0.001 * | 127.342 ± 2.902<br>P<0.001 * |
| 42 | 108.00 ± 3.797<br>P<0.001 * | 113.90 ± 4.686<br>P<0.001 * | 171.10 ± 7.043<br>P<0.001 * | 152.008 ± 6.446<br>P<0.001 * |

Table 3.2: P values for comparison of body weight of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.001 * | 0.001 * | 0.009 * | 0.99 | 0.118 | 0.117 |
| 21 | 0.041 * | 0.780 | 0.011 * | 0.046 * | 0.001 * | 0.142 |
| 35 | 0.222 | 0.379 | 0.001 * | 0.076 | 0.001 * | 0.001 * |
| 42 | 0.291 | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.059 |

## 3.2 Lung Volume and BW/$V_L$ ratios

### 3.2.1 Lung Volume ($V_L$) (Tables 3.3 and 3.4)

There was a significant rise in the mean lung volumes of pups at all the age increments in all four experimental groups. This followed the same trend as the data for body weight. It would appear as though the lung volume increased with the increase in BW during maturation.

Between postnatal days 14 and 42 there was a 3.8 fold increase in lung volume of control animals from 0.879 ± 0.076 ml to 3.355 ± 0.123 ml, with that of nicotine exposed animals marginally higher with a 4.4 fold increase from 0.769 ± 0.066 ml to 3.419 ± 0.159 ml. The $V_L$ for copper and nicotine + copper treated rats increased 5.7 and 5.3 times respectively during the same period.

The mean lung volume for the different experimental groups at postnatal days 14 and 21 were the same as that of controls ($P>0.05$). Copper and nicotine + copper treated rats had significantly higher lung volumes than control rats at postnatal days 35 and 42. At day 42 after birth the $V_L$ of copper treated rats was 32% higher ($P<0.001$) than that of controls, and 30% higher ($P<0.001$) than that of nicotine exposed rats at the same age. Similarly, the $V_L$ of nicotine + copper exposed rats was 29% higher ($P<0.006$) than controls, and 27% higher ($P<0.014$) than that of rats exposed to maternal nicotine. This corresponded with the marked increase of the body weight of the two groups of copper supplemented rats at the given ages.

### 3.3.2 $BW/V_L$ ratios (Tables 3.5 and 3.6)

A comparison of the $BW/V_L$ ratios indicate that the ratio gradually decreased from 41.10 ± 3.13 on postnatal day 14 in control rat pups to 32.46 ± 1.17 on postnatal day 42 ($P<0.015$). This can be attributed to the fact that the lung volumes increased by 281.82% between postnatal days 14 and 42 while the body weight increased by 215.24%. For the nicotine exposed rats the $BW/V_L$ ratio remained unchanged ($P>0.05$) since the rate at which the body weight and lung volume increased was the same.

The $BW/V_L$ ratio of animals that were exposed to copper during pregnancy and lactation remained constant between postnatal days 14 and 35 ($P>0.05$). However, between postnatal days 35 and 42 there was an increase in the $BW/V_L$ ratio ($P<0.031$) since the body weight increase was much more than the increase in the lung volume.

In the case of the rat pups that were exposed to nicotine and copper simultaneously, the $BW/V_L$ ratio remained constant except for day 21 after birth. On day 21 after birth the $BW/V_L$ ratio of 25.98 ± 0.96 was smaller ($P<0.001$) than the 37.42 ± 3.34 on day 14 after birth. This was due to a 66.25% increase in $V_L$ from postnatal day 14 to day 21 against an increase of 18.87% in body weight.

The above data therefore indicate that the $BW/V_L$ ratio of only the control animals decreased as the animals matured, indicating that the increase in $V_L$ exceeded the increase in BW.

Table 3.3: Comparison of lung volume (ml) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 0.879 ± 0.076 | 0.769 ± 0.066 | 0.785 ± 0.076 | 0.803 ± 0.065 |
| 21 | 1.191 ± 0.056<br>P<0.0039 * | 1.345 ± 0.100<br>P<0.0002 * | 1.247 ± 0.111<br>P<0.0036 * | 1.333 ± 0.057<br>P<0.001 * |
| 35 | 2.79 ± 0.072<br>P<0.001 * | 2.674 ± 0.084<br>P<0.001 * | 2.801 ± 0.12<br>P<0.001 * | 3.785 ± 0.243<br>P<0.001 * |
| 42 | 3.355 ± 0.123<br>P<0.001 * | 3.419 ± 0.159<br>P<0.001 * | 4.458 ± 0.211<br>P<0.001 * | 4.331 ± 0.304<br>P<0.001 * |

Table 3.4: P values for comparison of lung volume of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.288 | 0.394 | 0.466 | 0.874 | 0.728 | 0.871 |
| 21 | 0.219 | 0.678 | 0.094 | 0.518 | 0.922 | 0.509 |
| 35 | 0.331 | 0.921 | 0.001 * | 0.388 | 0.001 * | 0.001 * |
| 42 | 0.752 | 0.001 * | 0.006 * | 0.001 * | 0.014 * | 0.744 |

Table 3.5: Comparison of body weight/ lung volume ratios of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 41.101 ± 3.131 | 33.980 ± 2.056 | 33.095 ± 1.390 | 37.416 ± 3.339 |
| 21 | 33.580 ± 2.029<br>P<0.06 | 35.783 ± 2.981<br>P<0.638 | 33.277 ± 4.051<br>P<0.969 | 25.989 ± 0.955<br>P<0.002 * |
| 35 | 31.748 ± 0.715<br>P<0.01 * | 31.033 ± 0.914<br>P<0.180 | 32.782 ± 0.915<br>P<0.851 | 34.018 ± 1.498<br>P<0.461 |
| 42 | 32.464 ± 1.165<br>P<0.015 * | 33.778 ± 1.443<br>P<0.935 | 38.959 ± 2.074<br>P<0.031 * | 36.173 ± 1.583<br>P<0.713 |

Table 3.6: P values for comparison of body weight/ lung volume ratios of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.073 | 0.031 * | 0.435 | 0.726 | 0.374 | 0.216 |
| 21 | 0.565 | 0.951 | 0.003 * | 0.623 | 0.007 * | 0.108 |
| 35 | 0.557 | 0.394 | 0.141 | 0.194 | 0.102 | 0.479 |
| 42 | 0.759 | 0.015 * | 0.128 | 0.049 * | 0.276 | 0.290 |

### 3.3 **Specific Lung Volume** (Tables 3.7 and 3.8)

Specific lung volume express the lung volume, as determined by fluid displacement (Scherle, 1970), per 100g of body weight in order to eliminate the influence of changing body weight on the lung volume.

The specific lung volume of control rats increased significantly ($P<0.009$) from 2.54 ± 0.17 ml/100g BW at postnatal day 14 to 3.13 ± 0.13 ml/100g BW at postnatal day 42, an increase of 23%. The greatest change took place between day 14 and 21 after birth, when the specific lung volume increased by 17%. The specific lung volumes of rats exposed to maternal nicotine did not change over the duration of the experiment, but remained constant from day 14 to 42 after birth($P>0.05$).

In animals subjected to copper treatment, the specific lung volume remained constant from postnatal day 14 until postnatal day 35. That of pups aged 42 days was significantly lower than the other ages ($P<0.037$). Specific $V_L$ of rats treated with nicotine + copper was higher at postnatal day 21 ($P<0.001$), but at day 42 after birth it was no different from values obtained at postnatal day 14.



There was no significant difference between the specific lung volumes of control and nicotine exposed rats at all the age increments. Copper ($P<0.013$) as well as nicotine + copper ($P<0.071$) treated rats had significantly smaller specific lung volumes than controls at day 42. The two groups receiving copper supplementation differed from each other only at postnatal day 21, where samples of the nicotine + copper group has a greater specific lung volume ($P<0.003$).

Table 3.7: Comparison of specific lung volume (ml/100g body weight) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 2.544 ± 0.165 | 3.041 ± 0.181 | 3.075 ± 0.142 | 2.787 ± 0.189 |
| 21 | 3.072 ± 0.175<br>P<0.041 * | 3.000 ± 0.232<br>P<0.896 | 3.286 ± 0.224<br>P<0.457 | 3.898 ± 0.139<br>P<0.001 * |
| 35 | 3.164 ± 0.069<br>P<0.003 * | 3.253 ± 0.094<br>P<0.289 | 3.075 ± 0.084<br>P<0.998 | 2.965 ± 0.143<br>P<0.52 |
| 42 | 3.134 ± 0.126<br>P<0.009 * | 3.017 ± 0.121<br>P<0.912 | 2.631 ± 0.136<br>P<0.037 * | 2.817 ± 0.109<br>P<0.887 |

Table 3.8: P values for comparison of specific lung volume of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.058 | 0.025 * | 0.345 | 0.883 | 0.352 | 0.232 |
| 21 | 0.813 | 0.474 | 0.001 * | 0.385 | 0.004 * | 0.033 * |
| 35 | 0.471 | 0.433 | 0.175 | 0.180 | 0.117 | 0.501 |
| 42 | 0.509 | 0.013 * | 0.071 * | 0.047 * | 0.234 | 0.294 |

### 3.4 Volume Density of Parenchymal Tissue and Gas Exchange Region of Neonatal Rat Lung (Table 3.9)

The data summarised in table 3.9 show that the volume density of the gas exchange region ($V_a$) of the 14 day old control, nicotine, and nicotine and copper exposed rats were the same. The $V_a$ of the 14 day old copper exposed rat pups was however significantly bigger ($P<0.05$) than that of the other groups of the same age. Further analysis of the data showed that the $V_a$ of lung tissue of the control and the nicotine exposed rat pups increased between postnatal days 14 and 35 ($P<0.01$). No further increases in $V_a$ occurred after postnatal day 35. The $V_a$ of the lung tissue of the rat pups exposed to only copper and to a combination of copper and nicotine, increased between postnatal days 14 and 21 ($P<0.001$). No further increases occurred after day 21 after birth. This implies that copper supplementation may have increased the rate at which the gas exchange region of the neonatal lung developed. However, from day 35 after birth the $V_a$ and thus the $V_t$ of all the rat pups of all the experimental groups were the same. Calculation of the $C_{42}/C_{14}$ ratios showed that the air volume of the gas exchange region of the control rat lung had increased by 4.22 times between postnatal days 14 and 42. During the same period the volumes of the gas exchange region of the nicotine, copper, and nicotine + copper exposed rats increased by 4.92, 6.23, and 5.86 times respectively. The tissue volume of the gas exchange area of the lung tissue of the control rat pups increased by 2.22 times versus the 2.47, 3.61, and 3.29 times of the nicotine exposed, copper exposed, and nicotine + copper exposed rat pups respectively. This implies that maternal nicotine exposure was not affecting tissue growth while copper and a combination of nicotine and copper increased the rate of tissue growth.

Table 3.9 Comparison of volume density of different experimental groups.

$V_L$ is lung volume; $V_a$% percentage are filled space; $V_t$% percentage tissue; $L_{Va}$ volume of air space; $L_{Vt}$ volume of tissue.

| | Control | | Nicotine | | Copper | | Nicotine + Copper | |
|---|---|---|---|---|---|---|---|---|
| Age (days) | 14 | 42 | 14 | 42 | 14 | 42 | 14 | 42 |
| $V_L$ (ml) | 0.88 ± 0.08 | 3.36 ± 0.12 | 0.77 ± 0.67 | 3.42 ± 0.16 | 0.79 ± 0.08 | 4.46 ± 0.21 | 0.80 ± 0.07 | 4.33 ± 0.30 |
| $V_a$ (%) | 79.81 ± 0.96 | 88.12 ± 0.33 | 80.00 ± 0.69 | 89.18 ± 0.36 | 84.13 ± 0.99 | 89.45 ± 0.47 | 82.67 ± 0.90 | 89.48 ± 0.36 |
| $V_t$ (%) | 20.18 ± 0.96 | 11.89 ± 0.33 | 20.00 ± 0.69 | 10.82 ± 0.36 | 15.18 ± 0.99 | 19.55 ± 0.47 | 17.31 ± 0.90 | 10.52 ± 0.36 |
| $L_{Va}$ (ml) | 0.70 | 2.96 | 0.62 | 3.05 | 0.66 | 3.99 | 0.66 | 3.87 |
| $L_{Vt}$ (ml) | 0.18 | 0.40 | 0.15 | 0.37 | 0.13 | 0.47 | 0.14 | 0.46 |
| $C_{42}/C_{14}$ ($L_{Va}$) | 4.22 | | 4.92 | | 6.23 | | 5.86 | |
| $C_{42}/C_{14}$ ($L_{Vt}$) | 2.22 | | 2.47 | | 3.61 | | 3.29 | |

The data in table 3.10 show that the $V_{alv}$ of the control rats of all ages were smaller ($P<0.05$) than the $V_{alv}$ of the nicotine exposed rat pups. The $V_{alv}$ of the lung tissue of the copper exposed as well as the animals exposed to both nicotine and copper, was significantly lower ($P<0.001$) than that of the control as well as nicotine exposed rat pups.

Calculation of the alveolar number ($N_a$) (table 3.12) showed that the $N_a$ of lung tissue of 14 day old nicotine exposed rats were 30% less ($P<0.005$) than that of the control rat pups of the same age. On day 42 after birth the $N_a$ of lung tissue of control rat pups was at 3.99 ± 0.36 million, 39.6% higher ($P<0.001$) than the 2.41 ± 0.22 million of the nicotine exposed rat pups. On the other hand, the $N_a$ of animals exposed to copper was about 20% higher ($P<0.05$) than the $N_a$ of control lung of the 14 to 35 day old rat pups. On day 42 after birth the difference was 44.7%.

The $N_a$ of the lung tissue of the 14 day old animals exposed to both nicotine and copper was 17.4% higher than in the lung tissue of the control rats of the same age. The difference in $N_a$ increased to 29.1% on day 21 after birth and to 51.9% on postnatal day 42. This clearly shows that copper supplementation during pregnancy and lactation prevents the adverse effect of maternal nicotine exposure on alveolarisation of fetal and neonatal lung.

Table 3.10 Comparison of the alveolar volume ($\mu m^3 \times 10^4$) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age (Days) | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 10.99 ± 0.35 | 11.42 ± 0.90 | 4.60 ± 0.098 | 4.94 ± 0.25 |
| 21 | 10.81 ± 0.54<br>P>0.05 | 14.01 ± 1.33<br>P>0.05 | 5.42 ± 0.16<br>P<0.001 * | 5.16 ± 0.10<br>P>0.05 |
| 35 | 9.57 ± 0.28<br>P>0.05 | 14.09 ± 1.69<br>P>0.05 | 5.63 ± 0.16<br>P<0.001 * | 4.65 ± 0.09<br>P>0.05 |
| 42 | 9.44 ± 0.74<br>P>0.05 | 13.87 ± 1.35<br>P>0.05 | 5.69 ± 0.09<br>P<0.001 * | 4.62 ± 0.13<br>P>0.05 |

Table 3.11 P values for comparison of alveolar volume of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Copper vs Nicotine + Copper | Nicotine vs Nicotine + Copper |
|---|---|---|---|---|
| 14 | P>0.05 | P<0.001 * | P<0.001 * | P<0.001 * |
| 21 | P<0.05 | P<0.001 * | P<0.001 * | P<0.001 * |
| 35 | P<0.01 * | P<0.001 * | P<0.001 * | P<0.001 * |
| 42 | P<0.005 * | P<0.001 * | P<0.001 * | P<0.001 * |

Table 3.12 Comparison of the alveolar number (x $10^6$) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age (Days) | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 0.70 ± 0.05 | 0.49 ± 0.03 | 1.47 ± 0.21 | 1.36 ± 0.13 |
| 21 | 1.29 ± 0.17<br>P<0.005 * | 0.83 ± 0.08<br>P<0.005 * | 1.82 ± 0.20<br>P>0.05 | 2.30 ± 0.13<br>P<0.001 * |
| 35 | 2.70 ± 0.12<br>P<0.001 * | 1.33 ± 0.08<br>P<0.001 * | 4.46 ± 0.25<br>P<0.001 * | 6.92 ± 0.42<br>P<0.001 * |
| 42 | 3.99 ± 0.36<br>P<0.001 * | 2.41 ± 0.22<br>P<0.001 * | 7.02 ± 0.35<br>P<0.001 * | 8.60 ± 0.57<br>P<0.001 * |

Table 3.13 P values for comparison of neonatal lung alveolar number of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nicotine+ Copper | Nicotine vs Nicotine + Copper | Copper vs Nicotine + Copper |
|---|---|---|---|---|---|
| 14 | P<0.005 * | P<0.005 * | P<0.001 * | P<0.001 * | P>0.05 |
| 21 | P<0.05 * | P<0.05 * | P<0.001 * | P<0.001 * | P>0.05 |
| 35 | P<0.001 * | P<0.001 * | P<0.001 * | P<0.001 * | P<0.001 * |
| 42 | P<0.001 * | P<0.001 * | P<0.001 * | P<0.001 * | P<0.05 * |

### 3.5 **Internal Surface Area (ISA)** (Tables 3.14 and 3.15)

There was a significant increase in internal surface area available for gas exchange in all the experimental groups from postnatal day 14 through to day 42 after birth. However, the rate of increase in ISA differed between the various treatment groups. ISA of control animals increased by 4.5 $mm^2$ per day over the four weeks from postnatal day 14 to 42 ($P<0.001$), whilst that of nicotine exposed animals increased by 3.7 $mm^2$ per day ($P<0.001$). The gain in ISA for copper and nicotine + copper supplemented rats was more profound than that of control and nicotine exposed rats. From postnatal days 14 to 42 there was a 6.8 $mm^2$ daily rise in ISA of copper supplemented rats, while the ISA of rats exposed to maternal nicotine + copper supplementation increased by 7.2 $mm^2$ per day from postnatal days 14 to 42. These results suggest that the increase in ISA of control animals per day exceeded that of the nicotine exposed animals by 1.0 $mm^2$. Those animals that were exposed to copper during pregnancy and lactation gained 2 $mm^2$ per day more than control animals and 3 $mm^2$ more than the nicotine exposed rats.

The difference between internal surface area of control and nicotine exposed rats became more pronounced as the animals matured, since the rate at which the ISA increased in control lung exceeded that of the nicotine exposed rat pups. The internal surface area of nicotine exposed rats on postnatal day 35 was at 110.352 ± 3.892 $mm^2$,12% lower ($P<0.007$) than control rats, and at day 42 it was 20% less than that of control animals of the same age. The two copper supplemented groups showed only marginal differences between the surface area available for gas exchange at postnatal day 14 (copper treated animals has greater ISA) and 35 (copper treated animals has smaller ISA). However, at day 21 after birth, rats of these two groups have developed a significantly greater surface area available for gas exchange than the nicotine treated rats.

Table 3.14: Comparison of internal surface area ($mm^2$) of different experimental groups. All p values are in comparison with day 14 in the specific treatment group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 38.884 ± 3.423 | 31.44 ± 2.168 | 49.195 ±5.534 | 48.794 ± 4.844 |
| 21 | 54.858 ± 3.517<br>P<0.004 * | 61.482 ± 5.126<br>P<0.001 * | 68.803 ± 5.824<br>P<0.003 * | 73.981 ± 3.079<br>P<0.001 * |
| 35 | 126.083 ± 3.379<br>P<0.001 * | 110.353 ± 3.892<br>P<0.001 * | 149.935 ± 6.792<br>P<0.001 * | 216.602 ± 13.781<br>P<0.001 * |
| 42 | 173.312 ±10.166<br>P<0.001 * | 138.589 ± 6.353<br>P<0.001 * | 238.903 ± 11.362<br>P<0.001 * | 248.378 ± 17.249<br>P<0.001 * |

Table 3.15: P values for comparison of internal surface area of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * |
| 21 | 0.325 | 0.153 | 0.001 * | 0.564 | 0.057 | 0.304 |
| 35 | 0.007 * | 0.005 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * |
| 42 | 0.009 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.665 |

### 3.6 **Specific ISA** (Tables 3.16 and 3.17)

The internal surface area expresses the internal surface area per 100g of body weight. The internal surface area of control animals increased by 44% from 112.844 ± 8.203 $mm^2$ at postnatal day 14 to 162.782 ± 11.489 $mm^2$ at postnatal day 42, where 14% of the increase took place between postnatal day 35 and day 42 after birth ($P<0.004$). The specific ISA of animals exposed to nicotine during pregnancy and lactation did not change over the period of the experiment. This implies that the specific ISA of control animals increased by 1.78 $mm^2$/day while no increase was observed for nicotine exposed animals.

The specific internal surface area of rats receiving copper supplementation did not change from postnatal days 14 to 35, but there was a marked reduction of 21% in the parameter from postnatal day 14 to day 42 after birth ($P<0.009$). This implies that the specific ISA of copper exposed animals decreased by 1.37 $mm^2$ per day from day 14 after birth. Despite this decrease in specific ISA, especially after day 21 after birth, the specific ISA of the copper exposed rat pups were higher than that of control animals up to day 35 after birth. No difference was recorded on postnatal day 42 between control and copper exposed rats. The specific ISA of copper exposed rat pups were always higher ($P<0.05$) than that of nicotine exposed rats.

A comparison of the specific ISA of control animals with that of animals exposed to both nicotine and copper showed that the specific ISA of nicotine + copper exposed animals was always higher than that of control animals except on postnatal day 42. This could be ascribed to the decrease in the specific ISA of the latter group of animals of

2.61 $mm^2$/day from postnatal day 21 while that of the control animals increased. At postnatal day 21 animals who received nicotine + copper showed significantly higher ($P<0.008$) specific internal surface area compared to the values on postnatal day 14. The difference was, however, cancelled when the animals matured to postnatal day 42.

The specific ISA of control rats differed from nicotine exposed rats only at postnatal day 42, where the former was 25% greater than that of animals subjected to maternal nicotine exposure ($P<0.004$). The specific ISA of control animals were significantly lower than copper as well as nicotine + copper treated rats at postnatal days 14, 21 and 35. Nicotine treated rats showed consistently lower values for specific internal surface area than copper and nicotine + copper treated rats at all the age increments ($P<0.05$). The two copper supplemented experimental groups had similar values for specific ISA.

### 3.7 **Body Weight/ISA ratio** (Tables 3.18 and 3.19)

The ratio of body weight to internal surface area of control animals decreased significantly from postnatal day 14 to postnatal day 42 ($P<0.002$). This means that, as the pups mature, the internal surface area of the lungs increased at a faster rate compared to the increase in body weight over the same period. The ratio of rats exposed to maternal nicotine did not change from postnatal day 14 to day 42 after birth. The values for control and nicotine exposed rats were similar from postnatal day 14 to day 35 after birth, but the ratio of nicotine treated rats was significantly higher than that of control rats at postnatal day 42 ($P<0.003$).

The body weight/ISA ratio of rats treated with copper did not change from postnatal day 14 to day 35 after birth, but at postnatal day 42 the value was at 0.728 ± 0.040, 27% higher than the 0.572 ± 0.029 recorded at day postnatal 14 ($P<0.006$). The body weight/ISA ratio of nicotine + copper exposed rats decreased by 25% between postnatal days 14 and 21($P<0.012$). However, from postnatal day 21 the BW/ISA ratio increased again from 0.47 ± 0.02 to 0.59 ± 0.03 on postnatal day 35 and 0.63 ± 0.03 on day 42 after birth.

The control group differed from the copper treated group on postnatal days 14 and 35 where it had higher reported values. Compared to the nicotine + copper group, control rats had higher BW/ISA ratios at postnatal days 14, 21 and 35. Throughout the experiment, nicotine exposed rats had higher ratios than the two copper supplemented groups, except at postnatal day 42 where it had similar values than that of the group receiving only copper. There was no significant difference between rats subjected to copper and nicotine + copper treatment, except at postnatal day 42 where the latter had a lower value ($P<0.048$).

Table 3.16: Comparison of specific internal surface area ($mm^2/100g$ body weight) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 112.844 ± 8.203 | 126.466 ± 9.032 | 179.430 ± 10.699 | 169.432 ± 15.296 |
| 21 | 142.400 ± 12.125<br>P<0.059 | 137.187 ± 11.739<br>P<0.492 | 181.833 ± 11.600<br>P<0.882 | 216.380 ± 7.389<br>P<0.008 * |
| 35 | 143.015 ± 3.451<br>P<0.004 * | 134.798 ± 6.155<br>P<0.443 | 164.489 ± 4.658<br>P<0.206 | 169.651 ± 8.063<br>P<0.992 |
| 42 | 162.782 ± 11.489<br>P<0.004 * | 121.963 ± 4.369<br>P<0.641 | 141.056 ± 7.494<br>P<0.009 * | 161.643 ± 6.131<br>P<0.597 |

Table 3.17: P values for comparison of specific internal surface area for the different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.279 | 0.001 * | 0.004 * | 0.002 * | 0.022 * | 0.279 |
| 21 | 0.762 | 0.030 * | 0.001 * | 0.013 * | 0.001 * | 0.762 |
| 35 | 0.284 | 0.003 * | 0.004 * | 0.002 * | 0.006 * | 0.284 |
| 42 | 0.004 * | 0.154 | 0.933 | 0.033 * | 0.001 * | 0.004 * |

Table 3.18: Comparison of body weight/internal surface area ratio of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 0.933 ± 0.073 | 0.825 ± 0.054 | 0.572 ± 0.029 | 0.627 ± 0.062 |
| 21 | 0.745 ± 0.057<br>P<0.06 | 0.798 ± 0.076<br>P<0.815 | 0.598 ± 0.072<br>P<0.764 | 0.468 ± 0.017<br>P<0.012 * |
| 35 | 0.703 ± 0.018<br>P<0.007 * | 0.758 ± 0.032<br>P<0.268 | 0.613 ± 0.017<br>P<0.231 | 0.594 ± 0.026<br>P<0.691 |
| 42 | 0.646 ± 0.039<br>P<0.002 * | 0.832 ± 0.032<br>P<0.923 | 0.728 ± 0.040<br>P<0.006 * | 0.630 ± 0.027<br>P<0.986 |

Table 3.19: P values for comparison of body weight/internal surface area for the different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.267 | 0.001 * | 0.007 * | 0.001 * | 0.029 * | 0.395 |
| 21 | 0.552 | 0.137 | 0.001 * | 0.024 * | 0.001 * | 0.106 |
| 35 | 0.173 | 0.001 * | 0.004 * | 0.001 * | 0.007 * | 0.566 |
| 42 | 0.002 * | 0.161 | 0.737 | 0.051 | 0.001 * | 0.048 * |

### 3.8 **Radial Alveolar Count (RAC)** (Tables 3.20 and 3.21)

As control animals matured, the radial alveolar count decreased markedly with each age increment, from 5.44 ± 0.14 at postnatal day 14 to 4.45 ± 0.10 at day 42 after birth, a decrease of 18.2% ($P<0.001$). The radial alveolar count of nicotine exposed rats remained unchanged between postnatal days 14 to 42. The alveolar count in lungs of rats exposed to copper and nicotine + copper treatment decreased significantly from days 14 to 42, by 16.4% ($P<0.001$) and 19.9% ($P<0.001$) respectively.

Nicotine exposed animals showed consistently lower ($P<0.001$) radial alveolar counts than control rats at all the age groups (table 3.18). Animals exposed to copper supplementation differed from control animals only at postnatal day 21, where it had a higher RAC of 5.35 ± 0.08 versus the 4.63 ± 0.13 of the control rat pups ($P<0.001$). The rest of the copper supplemented animals as well as all the rats in the group receiving nicotine + copper had radial alveolar counts similar to that of control rats ($P>0.05$).



Animals that were exposed to copper and both nicotine and copper during gestation and lactation showed significantly higher radial alveolar counts than that counted in lung tissue of animals that were exposed to only nicotine throughout the duration of the experiment, while the two copper supplemented experimental groups differed from each other only at postnatal day 21 where the RAC of copper treated rats was 5.35 ± 0.084, exceeding that of the nicotine + copper group at 4.66 ± 0.093 by 13% ($P<0.001$). The 4.66 ± 0.09 of the nicotine + copper group was the same as the 4.63 ± 0.13 of the control rat pups of the same age.

Table 3.20: Comparison of radial alveolar count of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 5.44 ± 0.139 | 4.16 ± 0.104 | 5.06 ± 0.14 | 5.43 ± 0.182 |
| 21 | 4.63 ± 0.127<br>P<0.001 * | 4.07 ± 0.061<br>P<0.44 | 5.35 ± 0.084<br>P<0.076 | 4.66 ± 0.093<br>P<0.008 * |
| 35 | 4.49 ± 0.104<br>P<0.001 * | 4.051 ± 0.077<br>P<0.41 | 4.52 ± 0.107<br>P<0.006 | 4.62 ± 0.05<br>P<0.006 |
| 42 | 4.45 ± 0.1<br>P<0.001 * | 3.86 ± 0.107<br>P<0.06 | 4.23 ± 0.136<br>P<0.001 * | 4.35 ± 0.088<br>P<0.001 * |

Table 3.21: P values for comparison of radial alveolar count of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.001 * | 0.068 | 0.937 | 0.001 * | 0.001 * | 0.124 |
| 21 | 0.001 * | 0.001 * | 0.833 | 0.001 * | 0.001 * | 0.001 * |
| 35 | 0.002 * | 0.841 | 0.425 | 0.001 * | 0.001 * | 0.545 |
| 42 | 0.001 * | 0.374 | 0.460 | 0.018 * | 0.002 * | 0. |

### 3.9 **Mean Linear Intercept (Lm)** (Table 3.22 and 3.23)

There was no change in the mean linear intercept of control animals from postnatal day 14 to day 35 after birth. However, at postnatal day 42 the distance between the alveolar walls of control rats decreased from 0.099 ± 0.002 mm to 0.091 ± 0.003 mm ($P<0.05$) on postnatal day 14. The mean linear intercepts of rats subjected to maternal nicotine exposure remained unaffected over the duration of the experiment ($P>0.05$). The Lm of rats receiving copper supplementation increased from 0.076 ± 0.001mm on postnatal day 14 to 0.082 ± 0.001mm on day 42 after birth ($P<P<0001$). This change, however, occurred between postnatal days 14 and 35, after which the linear intercept showed no further change. The distance between alveolar walls of rats exposed to nicotine + copper remained unaltered at 0.078 ± 0.001 mm as the rats matured.

The Lm of lung tissue was significantly higher in animals exposed to nicotine as compared to control animals at postnatal days 35 and 42. The distance between alveolar walls of animals treated with copper and nicotine + copper were lower ($P<0.001$) than that of control animals in all the age groups, the alveoli of the former therefore being smaller with probably a greater surface area. The Lm of lung tissue of rats exposed to maternal nicotine was significantly higher than that of copper treated and nicotine + copper treated rats at all the age increments. At postnatal day 42 the Lm of nicotine treated rats was 24% higher ($P<0.001$) than copper treated, and 30% higher ($P<0.001$) than nicotine + copper exposed animals. At postnatal days 35 and 42 there was a significant difference in the distance between alveolar walls of the two copper supplemented groups. The Lm of copper treated rats showed an 7.9% increase ($P<0.001$) over the duration of the experiment, while that of nicotine + copper treated rats remained unchanged.

Table 3.22: Comparison of mean linear intercept (mm) of different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 0.099 ± 0.002 | 0.107 ± 0.005 | 0.076 ± 0.001 | 0.078 ± 0.001 |
| 21 | 0.168 ± 0.003<br>P<0.47 | 0.104 ± 0.005<br>P<0.51 | 0.080 ± 0.001<br>P<0.003 * | 0.079 ± 0.001<br>P<0.41 |
| 35 | 0.097 ± 0.001<br>P<0.35 | 0.107 ± 0.003<br>P<0.67 | 0.082 ± 0.001<br>P<0.001 * | 0.076 ± 0.001<br>P<0.36 |
| 42 | 0.091 ± 0.003<br>P<0.04 * | 0.108 ± 0.004<br>P<0.90 | 0.082 ± 0.000<br>P<0.001 * | 0.076 ± 0.001<br>P<0.23 |

Table 3.23: P values for comparison of mean linear intercept of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.115 | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.201 |
| 21 | 0.200 | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.242 |
| 35 | 0.020 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.002 * |
| 42 | 0.001 * | 0.008 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * |

### 3.10 **Destructive Index (DI)** (Tables 3.24 and 3.25)

Control rats showed a significant decrease in destructive index as the pups matured. There was a reduction of 35.7% in the DI from postnatal day 14 to day 42 after birth in this group of animals ($P<0.001$). This change took place between postnatal days 21 and 35 when there was a 37% decrease in DI. Except for a significant decrease of 8.5% at postnatal day 21 ($P<0.036$), the destructive index of nicotine exposed animals remained constant over time.

The DI of copper treated rats increased by 51% from $2.96 \pm 0.108$ at postnatal day 14 to $4.483 \pm 0.217$ at day 42 after birth ($P<0.001$). That of nicotine + copper supplemented rats increased from $2.973 \pm 0.168$ at postnatal day 14 to $4.568 \pm 0.086$ at day 42 after birth. This represents an increase of 53% ($P<0.001$).

At postnatal days 14 and 21 the destructive index of control rats was significantly higher than that of nicotine exposed rats. However, at days 35 and 42 after birth the DI of these animals were the same. The same trend prevailed when comparing control rats to copper and nicotine + copper treated rats. Where the destructive index of control rats at day 14 was 59% greater than that of copper as well as nicotine + copper exposed rats, there was no difference at day 42.

The destructive index of rats exposed to maternal nicotine was notably increased at postnatal days 14 and 21 when compared to that of the two groups supplemented with copper. There was no difference between the destructive indices of rats treated with copper and those given a combination of nicotine and copper.

Table 3.24: Comparison of destructive index of the different experimental groups. All p values are in comparison with day 14 in the specific experimental group. Significant difference is indicated with (*).

| Age Days | Control | Nicotine | Copper | Nicotine + Copper |
|---|---|---|---|---|
| 14 | 7.304 ± 0.254 | 4.79 ± 0.174 | 2.96 ± 0.108 | 2.973 ± 0.168 |
| 21 | 7.001 ± 0.177<br>P<0.34 | 4.38 ± 0.083<br>P<0.036 * | 3.97 ± 0.148<br>P<0.001 * | 3.77 ± 0.203<br>P<0.01 * |
| 35 | 4.431 ± 0.06<br>P<0.001 * | 4.574 ± 0.051<br>P<0.21 | 4.47 ± 0.147<br>P<0.001 * | 4.461 ± 0.371<br>P<0.016 * |
| 42 | 4.696 ± 0.053<br>P<0.001 * | 4.645 ± .0156<br>P<0.54 | 4.483 ± 0.217<br>P<0.001 * | 4.568 ± 0.086<br>p<0.001 * |

Table 3.25: P values for comparison of destructive index of different experimental groups. Significant difference is indicated with (*).

| Age (Days) | Control vs Nicotine | Control vs Copper | Control vs Nic + Cu | Nicotine vs Copper | Nicotine vs Nic + Cu | Copper vs Nic + Cu |
|---|---|---|---|---|---|---|
| 14 | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.001 * | 0.971 |
| 21 | 0.001 * | 0.001 * | 0.001 * | 0.022 * | 0.009 * | 0.436 |
| 35 | 0.092 | 0.826 | 0.913 | 0.513 | 0.648 | 0.980 |
| 42 | 0.753 | 0.294 | 0.209 | 0.540 | 0.670 | 0.699 |

### 3.11 **Copper Content of Lung Tissue** (Table 3.26)

The amount of copper found in lung tissue of control rats decreased significantly ($P<0.002$) by 59% over the period under investigation from 12.61 ± 1.87 µg/g at postnatal day 14 to 5.1 ± 0.60 µg/g at postnatal day 42. This change did, however, take place after day 14 after birth ($P<0.002$). After this time, the copper concentration of lung tissue of these pups remained constant. The copper concentration in the lung tissue of rat pups exposed to nicotine during gestation and lactation also decreased significantly ($P<0.001$) from postnatal day 14 to 42, from 8.06 ± 0.62 µg/g at day 14 after birth to 4.22 ± 0.45 µg/g at day 42 after birth, a change of 48%. The decrease in lung copper content of nicotine exposed rats was spread evenly over the time period under investigation.

Table 3.26: Comparison of lung tissue copper content (μg/g) of the different experimental groups. Significant difference is indicated with (*).

| Age<br>Days | Control | Nicotine | Control vs<br>Nicotine |
|---|---|---|---|
| 14 | 12.61 ± 1.87 | 8.06 ± 0.62 | P<0.02 * |
| 21 | 5.62 ± 0.77<br>P<0.002 * | 6.38 ± 1.16<br>P>0.05 | P>0.05 |
| 42 | 5.18 ± 0.60<br>P<0.002 * | 4.22 ± 0.45<br>P<0.001 * | P>0.05 |

# CHAPTER 4

# DISCUSSION

## 4.1 Maternal Nicotine Exposure and the Development of the Neonatal Rat Lung as a Gas Exchanger

In order to function effectively as an organ of gas exchange at birth, the lung must have reached a high level of structural and metabolic maturity, and it must be able to function in a way that it has never functioned before. To operate effectively as an organ of gas exchange at birth, the lung must have developed a large internal surface area by the formation of millions of thin-walled alveoli and a dense capillary network must have formed close to the lining of the alveoli (Harding and Hooper, 1993). It was also demonstrated that elastic tissue formation by the fibroblasts in the alveolar septa plays an important role in alveolarisation and thus the expansion of the gas exchange area of the lung (Emery, 1970). Experiments by Maritz and Woolward (1992b) showed that maternal nicotine exposure during pregnancy and lactation interferes with elastogenesis in the lungs of the offspring. In a follow-up study Maritz and Dolley (1996) illustrated that, as a consequence of this interference, the three dimensional structure of the lungs of these neonatal rats were compromised.



It is therefore conceivable that interference with lung development in the fetus and neonate will have an adverse effect on the gas-exchange function of the lung. In a study by Vidic *et al* (1989) it was shown that maternal chronic exposure to whole cigarette smoke induced a slower pace of septal growth and thus of alveolarisation in the lungs of the offspring. Research studies showed that maternal nicotine exposure during pregnancy resulted in an irreversible inhibition of glycolysis (Maritz, 1988), where

glycolysis is important for lung growth (Sorokin, 1961). In addition, maternal nicotine exposure also resulted in an increase in alveolar septal and blood-air barrier thickness in the lungs of the offspring (Maritz *et al*, 1993). It is therefore clear that exposure to nicotine, the major alkaloid in tobacco smoke, during pregnancy, interferes with neonatal lung development. This will in all likelihood also interfere with the development of the neonatal lung into an effective gas exchanger.

The main function of the lung is to supply the organism with oxygen and to remove carbon dioxide. The exchange of these two gasses takes place in the alveoli. Each alveolus is surrounded by a dense capillary meshwork. This enables the two gasses to be exchanged over a large and complex three-dimensional surface area (Gehr and Crapo, 1988). During lung growth and development, alveolarisation occur which increases the surface area available for gas exchange (Burri, 1974). In addition, thinning of the septa and respiratory membranes occur to improve the diffusion capacity of the lung, the $O_2$ supply to the blood and the removal of $CO_2$ (Weibel and Knight, 1964).



In the rat, postnatal lung growth can be divided into three phases namely, lung expansion up to day 4 after birth, tissue proliferation from postnatal days 4 to 13, and equilibrated lung growth from postnatal days 13 to 21. During this first phase of rapid lung expansion, the lung volume increased almost exclusively from an enlargement of existing air spaces. From day 4 to 13 after birth the bulk of the alveoli are formed by a rapid outgrowth of secondary septa from the primary septa present at birth (Burri, 1974). The formation of the secondary septa and the lengthening thereof will result in more alveoli and an increase in the ISA available for gas exchange. The capillary density also increase rapidly during this phase of lung growth (Maritz and Thomas,

1994) thereby increasing the effectivity of the lung as a gas-exchanger.

Interference with alveolarisation and capillary formation will thus not only reduce the number of alveoli and the ISA, but the gas-exchange function will also be compromised. In a recent study it was shown that maternal nicotine exposure suppresses alveolarisation in the offspring (Maritz and Van Wyk, 1997). These findings were confirmed in the present study. In addition to this, the depressed RAC (table 3.20) and therefore the reduced number of alveoli per lung acinus and thus in the whole lung was maintained into adulthood.

The destructive index is an early and sensitive parameter for determining alveolar damage. The fact that the DI (table 3.24) of nicotine exposed rat pups were lower than that of the control animals on postnatal days 14 and 21, and not different from the control rat pups on postnatal days 35 and 42, is an indication that nicotine had not resulted in alveolar wall damage. The lower RAC of nicotine exposed rat pups when compared to control animals, was therefore not due to alveolar wall damage, but rather due to suppression of alveolarisation.



During lung development and growth, the $V_L$ and ISA available for gas exchange must increase in such a manner that it will meet the requirements of the body during resting conditions as well as during strenuous physical activity during which the demand for oxygen increases. This implies that the $V_L$ and in particular the ISA must develop a reserve capacity to meet the increased demand of the body during strenuous physical activity. The fact that the $BW/V_L$ ratio (table 3.5) was the same for both the control and the nicotine exposed animals, indicate that the increase in $V_L$ (table 3.3) of both groups

was proportional to the increase in BW. However, since the RAC of the lungs of the nicotine exposed rat pups were smaller than that of the control animals, the deduction can be made that the alveoli of the nicotine exposed rat pups must be larger than that in lung tissue of the control animals. This was indeed the case. This fact is illustrated by the increased Lm (table 3.22) and bigger alveolar volume (table 3.10) of the nicotine exposed rat pups. Since alveolarisation during this phase of lung development is due to the formation of secondary septa (Burri *et al*, 1973), it is conceivable that the smaller alveolar number in lungs of nicotine exposed rat pups was due to inhibition of the formation and growth of secondary septa. As a consequence of this interference with alveolarisation, it can be expected that the ISA of lung tissue of nicotine exposed rat pups will be smaller than that of lung tissue of control rats of the same age. The present investigation indeed showed that the ISA of the nicotine exposed rat pups were smaller than that of the control animals of the same age despite the fact that the lung volumes were the same for control and nicotine exposed animals (table 3.3). However, the fact that the ISA of the lungs of the control and nicotine exposed rats both increased 4,2 times between days 14 and 42 after birth, indicates that the adverse effects of nicotine on alveolarisation and thus of expansion of the ISA was during the early phases of lung development. This coincides with the phase of rapid alveolarisation which occurs from days 4 and 13 after birth.

In a study by Burri *et al* (1973) it was found that somewhere between days 14 and 21 after birth a period of equilibrated lung growth starts. During this phase, the newly formed respiratory units are progressively enlarged. Replication of existing alveoli also occurs (Blanco, 1995). In the present study it was found that the lung volume and ISA of the control rat pups increased 3,6 and 4,2 times respectively between days 14 and 42

after birth. The lung volume and ISA of nicotine exposed rat pups increased 4,5 and 4,2 times respectively. Further analysis of the data shows that the rate at which the lung volume and ISA increased was highest after postnatal day 21 for all the experimental groups. The fact that the ISA of lung tissue of control animals increased at a faster rate than the lung volume, while the ISA and lung volume of nicotine exposed rat pups increased at the same rate, is further proof that the interference with structural modelling in order to increase the surface area for gas exchange, was suppressed in the lungs of the nicotine exposed rat pups. This finding suggests that maternal nicotine exposure also suppressed equilibrated lung growth of the terminal respiratory units of the lung. The terminal respiratory unit is defined as that mass of airspace distal to a respiratory bronchiole (Emery and Mithal, 1960; Karlinsky and Snider, 1978), or that portion of the lung parenchyma that is connected to the first-order respiratory bronchiole (Weibel, 1973) to constitute a lung acinus.

As a consequence of the suppression of structural modelling to develop the lung into an effective gas-exchanger, the BW/ISA ratio of nicotine exposed rats remained unaltered. In contrast to this, the BW/ISA ratio of control animals decreased as the animals matured and the lungs developed into efficient gas-exchangers (table 3.18). This was due to the fact that the rate at which the ISA increased exceeded the rate at which the BW increased. This implies that the lungs of the control animals were better equipped to match the growing metabolic needs of the animal. This is further supported by the fact that the specific ISA of the control animals gradually increased as the animal matured. On the other hand, the specific ISA of the nicotine exposed animals remained constant after weaning on day 21 after birth, indicating that the lungs of these animals were not developing according to the demands of the animal.

According to Weibel (1979), the pulmonary diffusing capacity in growing rats increased proportionally to body weight. The diffusion capacity of the respiratory gasses depends on the surface area available for gas exchange, the septal capillary density as well as the harmonic mean total barrier thickness of the path along which $O_2$ and $CO_2$ must diffuse. In a study by Maritz *et al* (1994) it was found that, in addition to the smaller ISA, the alveolar septa and the blood-air barrier was thicker in the lungs of rats exposed to nicotine during pregnancy and lactation than in the lungs of the control rats. It is therefore conceivable that the diffusion capacity of $O_2$ and $CO_2$ could be adversely affected in the lungs of animals exposed to nicotine via the placenta and mother's milk. This furthermore implies that the pulmonary diffusing capacity in growing rats exposed to nicotine via the placenta and mother's milk will not increase proportionally to body weight.

The size of the pulmonary gas exchange apparatus is a critical and rate-limiting factor of the respiratory system whose function it is to effectively supply an adequate amount of oxygen to the cells of the body (Weibel, 1979). It is also hypothesized that the pulmonary diffusing capacity is increased as the $O_2$ needs of the organism rise. Research illustrated that if the oxygen needs of the organism are increased due to augmented physical activity, the structural determined pulmonary diffusing capacity is increased (Weibel, 1979). Convincing evidence exists that the growing lung can adapt the size of its gas exchange apparatus to make it commensurate to the requirements of the organism and to the conditions prevailing, and that it does so by regulating the lung's growth (Burri and Weibel, 1977). It is therefore clear that maternal nicotine exposure interferes with this ability of lung to adapt to the requirements of the lungs of the neonate. Another physiological implication of the adverse effect of maternal nicotine

exposure on the development of the gas exchange region of the lungs of the offspring and thus the diffusion capacity may therefore be a reduced capacity to perform physically, especially during strenuous and prolonged muscle activity. Studies to investigate this are presently in progress.

It is important to note that the fetal and neonatal rats were exposed to nicotine via the placenta and mother's milk. After weaning on day 21 after birth, the offspring were no longer exposed to nicotine. Despite this, the difference in ISA of lung tissue between control and nicotine exposed rat pups increased. It therefore appears that the adverse effects of maternal nicotine exposure on early fetal and neonatal lung development are irreversible. It is therefore also unlikely that the changes observed after postnatal day 14 were due to inadequate food intake since the increase in the BW of nicotine exposed animals were not suppressed.

In conclusion, it is clear that maternal nicotine exposure during pregnancy and lactation reduces the effectivity of the lungs of the offspring as gas-exchangers. Furthermore, the adverse effects of maternal nicotine exposure on lung development in the offspring were irreversible. In addition to the smaller ISA, less capillaries were found in the septa of the nicotine exposed rat pups (Maritz and Thomas, 1994). Taken together, it implies that maternal nicotine exposure during pregnancy and lactation compromised the gas exchange function of the offspring and that these detrimental effects persists into adulthood.

### 4.2 Copper Supplementation During Maternal Nicotine Exposure

In this study it was shown that the lung copper content of the 14 day old neonatal rats exposed to nicotine via the placenta and mother's milk was 1.6 times less than that of the control rats of the same age (table 3.26). Research has shown that copper deficiency result in distinct structural changes in the lung cells of guinea pigs. The cells most affected are the type II cells in the alveoli, Clara cells in the bronchioli and the residual macrophages in the capillary (Richmond and Chi, 1993). All three types of cells have some specialised function related to lipid metabolism. Characteristic of the changes in the type II cells and Clara cells of the copper deficient guinea pig was the increase in organellae related to packaging and storage of phospholipids in the lamellar bodies. It was suggested that the copper deficiency may have altered the synthesis or degradation of lipids and protein components or prevented their normal secretion into the airways or extracellular spaces (Richmond and Chi, 1993).

In a study by O'Dell *et al* (1978) it was found that copper deficiency resulted in low levels of hepatic cytochrome oxidase, growth retardation as well as an increase in alveolar size. It is well known that elastic tissue plays an important role in alveolar formation (Maritz and Dolley, 1996). Data from the lungs of copper deficient rats suggest that when the cross-linking of connective tissue proteins especially elastin is defective, the lungs develop fewer alveolar ducts and alveoli, resulting in abnormally large alveoli (Fisk and Kuhn, 1976).

Copper is an essential component of lysyl oxidase (Harris *et al*, 1974), the enzyme which catalyses the oxidative deamination of lysine residues in the precursor of elastin. Lysyl oxidase plays a key role in the cross-linking of elastin and the conversion of

soluble elastin into insoluble elastin. A deficiency of copper could then be expected to result in a metabolic defect such as an increase in the soluble component of the protein and a decrease in the insoluble component. Studies by Buckingham *et al* (1981) indeed showed that the soluble elastic component increased 2-4 fold in the lung of copper deficient chicks. Alterations in the staining properties of elastin, as observed when lysyl oxidase is deficient, also occurred (O'Dell *et al*, 1978). Cross-linking of elastin may be crucial in further partitioning of the primitive alveolar sacs during alveolarisation.

It is important to note that the copper content of the lungs of the control rat pups was high (table 3.26) during the phase of lung development which is associated with rapid alveolarisation and cell multiplication. During this phase of lung development in the rat the rate of elastin synthesis is maximal, about 5-8 times the rate in the adult rat lung (Myers *et al*, 1983). It is therefore conceivable that the reduction in the lung copper content of the nicotine rat pups resulted in a decreased lysyl oxidase activity during the phase of rapid alveolarisation. Studies by Maritz and Dolley (1996) also illustrated changes in the staining properties of the lung tissue of 7 to 14 day old nicotine exposed rat pups. These changes were in all likelihood due to a reduced lysyl oxidase activity in the lungs of these rat pups. After postnatal day 14 the lung copper content decreased to levels which equalled that of the lung tissue of nicotine exposed rat pups. This phase corresponds with the phase of equilibrated lung growth during which formation of alveoli is slow when compared with the phase of rapid alveolarisation between postnatal days 4 and 13. It is therefore expected that nicotine will not affect lung development at this stage.

Calculation of the alveolar number of the rat pups exposed to nicotine during gestation

and lactation clearly showed that maternal nicotine exposure suppressed alveolarisation (table 3.12). It also showed that the alveolar volume was increased (table 3.10). The bigger alveolar volume will result in a smaller internal surface area available for gas exchange (table 3.14) since fewer septae occur. However, copper supplementation during gestation and lactation prevented the adverse effects of maternal nicotine exposure on alveolarisation. It is therefore conceivable that the mechanism whereby nicotine interferes with alveolarisation is by reducing lung copper content and therefore lysyl oxidase activity. As a consequence of this, the rate of cross-linking of lysine side chains will be suppressed and thus also the formation of insoluble elastin. This has previously been shown to result in slower alveolar formation (Dubick *et al*, 1985).

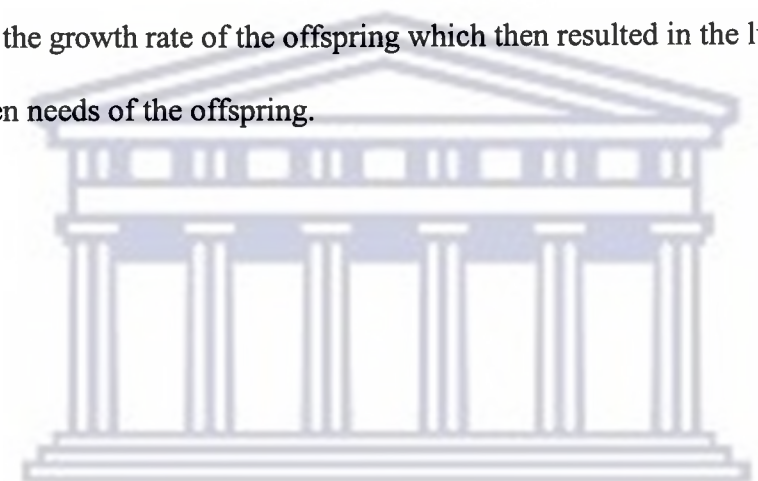
Although the septa of the lung tissue of the 14 day old nicotine exposed rat pups were thicker than that of the control, copper exposed, and nicotine and copper exposed animals, it returned to normal as the lung tissue matured. However, copper supplementation prevented thickening of the septa (table 3.9). Further analysis of the data showed that maternal nicotine exposure had no effect on the tissue volume ($V_v$) of the nicotine exposed rat pups. This is an indication that nicotine was not affecting tissue formation in the developing lung. Copper supplementation increased the rate at which the septa thinned as the lungs matured, but at day 42 after birth no difference in the tissue volume density between the control rat pups and the rat pups exposed to nicotine, copper, and to both copper and nicotine, was evident.

The morphometric data clearly proved that copper supplementation during gestation and lactation prevented the adverse effects of maternal nicotine exposure during gestation and lactation on growth and development of the lungs of the offspring. However, apart

from preventing the adverse effects of nicotine on lung development, copper also affected growth and development, resulting in smaller alveolar volumes (table 3.10). This was also reflected in a smaller linear intercept length (Lm) of lung tissue of animals exposed to copper as well as for those animals exposed to a combination of nicotine and copper (table 3.22). It is interesting to note that the alveolar volumes of only the copper exposed animals increased after postnatal day 14. The reason for this is not known. The bigger surface area available for gas exchange of copper exposed and animals exposed to both nicotine and copper, as compared to that of the control and nicotine exposed animals, can be attributed to the increased alveolar number of the lung tissue of these rats. This increase in the alveolar number of the lung tissue also contributes to the higher lung volumes found in copper exposed rat pups on postnatal day 42. Apart from the higher lung volumes of the copper exposed animals on especially day 42 after birth (table 3.3), the BW of these animals were also substantially higher than that of the control and nicotine exposed rat pups. In studies by Weibel (1979) it was illustrated that the lung is capable of adapting the size of its gas exchange apparatus to the needs of the organism. If the oxygen needs of the organism are increased due to augmented physical activity (Weibel, 1979), or when rats are raised on high altitude, where the oxygen partial pressure of the inspired air is reduced, thus reducing the pressure across the blood air barrier, the lung also adapts itself to meet the needs of the body (Hugennaud *et al*, 1977). If rats are raised in an oxygen-rich environment (40% $O_2$) they develop a reduced capacity for gas exchange (Burri and Weibel, 1971). This is strong evidence that the growing lung can adapt the size of its gas exchange apparatus to make it commensurate to the requirements of the organism, and that it does so by regulating the lung's growth rate (Burri and Weibel, 1971). It is therefore conceivable that the increase in the alveolar number, the internal surface area available for gas exchange, as well as the increase in

lung volume, can be attributed to adaptation of the growing lungs to satisfy the increased oxygen requirement of the bigger animals.

In conclusion, it is clear that maternal copper supplementation during pregnancy and lactation prevented the harmful effects of maternal nicotine exposure during gestation and lactation on lung development in the neonate. A direct consequence of this supplementation may be that copper could be stimulating the lungs of these animals to develop to its full genetic potential as gas exchangers. Copper supplementation also increased the growth rate of the offspring which then resulted in the lungs adjusting to the oxygen needs of the offspring.



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# APPENDIX

| PRODUCT | SUPPLIER/MANUFACTURER |
|---|---|
| Atomic absorption spectrophotometer | Unicam |
| Automatic tissue processor | Shandon Histokinette |
| Copper sulphate | Sigma |
| Coverslips | Marienfield |
| Disodiumhydrogen phosphate | Merck |
| DPX | Merck |
| Ethanol | Merck |
| Formaldehyde | Merck |
| Freezer ($-80^0$) | Lasec Angelantoni Scientifica PR340C |
| Graticules | Graticules Ltd. |
| Hydrochloric acid | Sigma |
| Hydrogen peroxide | Merck |
| Hydrogen sulphate | Merck |
| Hypodermic needles | Promex |
| Incubator | Heraeus T5028 |
| Laboratory balance | Sartorius 1475A |
| Lithium sulphate | Sigma |
| Magnesium sulphate | Sigma |
| Microscope | Zeiss research type |
| Microscope slides | Chance Propper |
| Microtome knives | Leica; Microm |
| Nicotine | Merck |

| | |
|---|---|
| Paraffin section mounting bath | Electrothermal MH8513 |
| Pentobarbitol | Rhône-Poulenc |
| Plastic cassettes | Anglia Scientific |
| Potassium bicarbonate | Merck |
| Rats | Medical Research Council |
| Refrigerator | Fuchsware domestic type |
| Selenium | Merck |
| Sliding microtome | Reichert-Jung |
| Sodium chloride | Merck |
| Sodiumdihydrogen phosphate | Merck |
| Stage micrometer | Graticules Ltd. |
| Syringes | Promex |
| Xylene | Merck |