The Influence of Maternal Nicotine Exposure on Selected Glycolytic and Cytochrome P450 Enzymes in Developing Neonatal Rat Lung

by

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DEDICATION

This thesis is dedicated to my wonderful husband
and my beautiful children,
the only inspiration I will ever need.

ABSTRACT

The structural and functional integrity of a developing and maturing fetal and neonatal lung is critically dependent on carbohydrate metabolism. The energy derived from carbohydrate metabolism is utilized during the processes of cell growth and development. It is reported that maternal nicotine exposure during pregnancy and lactation results in the irreversible inhibition of glycolysis, for which no mechanism is currently proposed and a significant increase in glucose turnover. The principal objectives of this thesis are (1) to investigate the isoezyme patterns and transcript levels of selected glycolytic enzymes: Hexokinase (HK), Phosphofrutokinase (PFK), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Lactate dehydrogenase (LDH) in control developing neonatal rat lung, (2) to investigate the transcript levels of selected cytochrome (CYP) P450 enzymes: CYP1A1, CYP2A3, and CYP2B1 in control developing neonatal rat lung and (3) to determine the influence of maternal nicotine exposure during gestation and lactation on the isoenzyme patterns and transcript levels of the selected enzymes in developing neonatal rat lung, in an attempt to elucidate the mechanism of inhibition of glycolysis observed.

Tissue samples were obtained from the lungs of 1, 7, 14, 21, and 49-day old pups, from both control lung tissue and lung tissue exposed to nicotine during gestation and lactation (1mg/kg body weight/day). Isoenzyme separation is achieved using polyacrylamide gel electrophoresis (PAGE) techniques and was principally based on their differences in molecular weights. The PAGE gels are densitometrically analyzed and expressed as % Density/mg protein of lung tissue. Transcript levels are analyzed with the use of dot blots followed by densitometry of the blots. The results obtained for the mRNA subunits are expressed relative to ß-actin. The final data of both isoenzyme patterns and mRNA levels are analyzed statistically using the Wilcoxon unpaired T-test, in which $P<0.05$ is designated as significant.

Results of this study show that:

(1)All three isozymes, HKI, HKII and HKIII are detectable at the mRNA level however the method used is only sensitive enough to detect HKII from postnatal day 14. HKI is the dominant isoenzyme in the lung of control as well as rats exposed to nicotine at both the transcriptional and post-translational levels during gestation and lactation. Furthermore, the developmental pattern of the isoenzymes as the lung matures follows the same trend in lung tissue of control and nicotine exposed offspring.

(2) The three PFK sub-types are transcribed in control neonatal lung. PFK-M mRNA levels are expressed at significantly higher levels in control developing lung than PFK-L and PFK-C. However, maternal nicotine exposure during gestation and lactation results in over-expression of PFK-M and PFK-L, but has no influence on PFK-C mRNA levels. PFK-M was affected by maternal nicotine exposure during gestation and lactation, even at postnatal day 49. This implies that the long-term effect of maternal nicotine exposure on total PFK activity can be attributed to changes in the PFK-M isoenzyme activity.

(3) GAPDH mRNA is over-expressed at postnatal days 14 and 21 in the lungs of rat pups exposed to maternal nicotine, however no difference is observed between control and experimental lung tissue at postnatal day 49. The expression of GAPDH mRNA of control lung increased gradually between postnatal days 1 and 49. On the other hand, GAPDH mRNA expression in lungs of nicotine exposed rat pups show a pronounced increase in expression after postnatal day 7 and reached a maximum at postnatal day 14.

(4) Expression of all the LDH isoenzymes in control developing lung, except LD-1 between day 7 and day 14, decreased. LD-4 and LD-5 (the homozygous LD-M isoenzyme), the glycolytic associated isoenzymes displayed the greatest decrease. All LDH isoenzymes as well as sub-units at the mRNA level are over-expressed from day 7 onwards in the lungs of rats exposed to nicotine during pregnancy and lactation. LD-

5 (the glycolytic sub-unit) and LD-M (mRNA homotetramer isoform) were particularly affected by maternal nicotine exposure. LD-1 is the dominantly expressed isoform at the transcriptional and post-transcriptional levels, in both control and nicotine-exposed lung at any age group analyzed.

(5) The human orthologs of rat CYP1A1, CYP2A3 and CYP2B1 are transcribed in lung tissue of control neonatal rats and all display a significant increase in expression from postnatal day 1 to postnatal day 49. CYP2A3 is dominantly expressed at the mRNA level in control neonatal rat lung, followed by CYP2B1 and the lowest levels shown by CYP1A1. Maternal nicotine exposure results in the induction of CYP2A3 and CYP2B1, however has no influence on CYP1A1 expression. Moreover, CYP2B1 in control lung tissue remained significantly lower than CYP2A3, however in the experimental lung there are no differences between the two by day 49.

It is concluded that the inhibition of glycolysis in lungs of rats exposed to nicotine during gestation and lactation is not due to changes in the HK or PFK isoenzyme levels in the lungs of the offspring. It is, however, evident that the maturing rat lung attempts to compensate for the glycolytic inhibition by over-expressing the isoforms at the mRNA level associated with glycolytic activity and gluconeogenic activity. The over-expression of GAPDH transcript levels in nicotine-exposed lung tissue takes place only when nicotine is present, and therefore maternal nicotine exposure has no long-term effects on GAPDH mRNA expression. Inhibition of the glycolytic pathway results in the over-expression of LDH at both the transcriptional and post-transcriptional levels and it is proposed that the over-expression is in an attempt to rectify the hindrance caused by maternal nicotine exposure. CYP1A1, CYP2A3 and CYP2B1 are transcribed in neonatal rat lung and induction of CYP2A3 and 2B1 in the lungs of the offspring by maternal nicotine exposure is irreversible and thus "programmed". Furthermore, it is proposed that if the increased transcript levels reflect increases in activity of the CYP enzymes, this may explain the increase in glucose turnover observed in the lungs of offspring exposed to nicotine during gestation and lactation.

DECLARATION

I declare that "*The Influence of Maternal Nicotine Exposure on Selected Glycolytic and Cytochrome P450 Enzymes in Developing Neonatal Rat Lung"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the resources I have or quoted have been indicated and acknowledged by complete references.

Kareemah Gamieldien February 2005

Signed

PUBLICATIONS ARISING FROM THIS THESIS

Gamieldien K. and Maritz G.S. (2004) **mRNA expression of cytochrome P450 1A1, 2A3 AND 2B1in developing rat lung: Influence of Maternal Nicotine Exposure**. Experimental Lung Research. 30:121-133

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APPENDIX I: LIST OF ABBREVIATIONS

A	absorbancy
ADP	adenosine diphosphate
AGE	advanced glycation end product
AhR	aromatic hydrocarbon receptor
ALP	alkaline phosphatase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
cDNA	complementary DNA
CYP	cytochrome
DEPC	diethylperoxycarbonate
DHAP	dihydroxyacetone phosphate
d-UTP	deoxyuridine 5'-triphosphate
ETII	alveolar epithelial type II cells
GAP	glyceraldehyde-3-phosphate
G-6-P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT	glucose transporter
HBV	hepatitis B virus
HK	hexokinase
H_2O_2	hydrogen peroxide
HMS	hexose monophosphate shunt
kDa	kilodalton
Km	Michealis-Menton constant
LDH	lactate dehydrogenase
M	molar concentration
mA	milli amperes

MCTP	monocrotaline pyrrole
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
MTT	methylthiazoletetrazolium
Na	alveolar number
Na_2HPO_4	sodium monohydrogen phosphate
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NDMA	*N*-nitrisodimethylamine
NNAL	4-(methylnitroamino)-1-(3-pyridyl)-1-butanol
NNK	(methylnitroamine)-1-(3-pyridyl)-1-butanone
NNN	*N'*-nitrisonornicotine
NO	Nitric oxide
O_2^-	superoxide
P	probability
PAGE	Polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PARP	poly (ADP-ribose) polymerase
PFK	phosphofructokinase
P_i	inorganic phosphate
PK	pyruvate kinase
pKa	index of ionic dissociation
PKC	protein kinase C
PMS	phenazine methosulphate
PN	peroxynitrite
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
ß	beta

SEM	standard error of the mean
SSC	sodium chloride, sodium citrate
SM	slow metabolizer
TBE	Tris borate EDTA
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N',N'-Tetramethylethylenediamine
UDG	uracil DNA glycosylase
UVP	Ultra violet products
V	volts

CHAPTER 1

1. INTRODUCTION

1.1 Lung Development

Understanding mechanisms of lung disease requires a general comprehension of the processes of normal lung development. During gestation the development of the human lung may be classified into four stages: embryonic, pseudoglandular, canalicular and alveolar (Haworth and Hislop, 2003). The timing of progression from stage to stage may vary in other species and in some cases, the alveolar phase occurs postnatally (Hall *et al.*, 2000; Hall *et al*., 2002).

During the embryonic phase in humans the lung bud appears as a ventral diverticulum of the foregut within the first four weeks of gestation, in which it divides within and into the surrounding mesenchyme. The two lungs are easily distinguishable by six weeks. The specialized epithelial cells of the lung will arise from the endoderm lining the lung buds and all other components of the airway walls stem from the mesenchyme. (Hall *et al.*, 2000; Hall *et al.*, 2002).

The pseudoglandular phase usually occurs between weeks five and seventeen during which continuing division of airway buds into the mesenchyme results in all preacinar airways. Thus by week seventeen, airways to the level of terminal bronchioli can be observed. The epithelium starts to differentiate and smooth muscle followed by cartilage, submucosal glands and connective tissue, develops in the recently formed airway wall. (Hall *et al.*, 2000; McCray, 1993; Richards *et al.*, 1991). Smooth muscle cells can be found in the trachea and lobar bronchi between week eight and ten and innervation of this tissue has been observed as early as eight weeks (Sparrow *et al.*, 1999).

The canalicular stage extends from week sixteen to week twenty seven of gestation during which the preacinar airways increase in size. Furthermore, peripheral airways continue to divide, forming the respiratory bronchioli (generations two and three) which ultimately gives rise to the alveolar ducts. Type I and type II alveolar epithelial cells are present in the lining of the saccular-shaped alveolar ducts by week twenty to twenty two (Haworth and Hislop, 2003). Lamellar bodies are produced by the type II epithelial cells at about prenatal week twenty four and surfactant can be detected in the amniotic fluid four to five weeks later. The canalicular phase sees the thinning of the epithelium at the lung periphery with underlying capillaries, leading to the formation of the blood-air barrier. Its thickness, similar to that of the adult is enough to sustain life in extremely premature infants (Haworth and Hislop, 2003).

At the initial stages of the alveolar phase at week twenty seven, unobtrusive bundles of elastin and muscle are contained within the edges of the saccules. These form tiny crests subdividing the walls of the saccule (Hislop *et al.*, 1986). Elongation of the crests during week twenty eight and thirty two forms the primitive alveoli that contain a double capillary supply with the mesenchymal tissue between the two layers of epithelial cells. Eventually the mature alveoli with a single capillary will line the elongated saccules and a section of the respiratory bronchioli. The alveolar numbers will continue to increase to term by which time approximately 150 million alveoli have formed, representing between one-third and one-half of the adult number (Hislop *et al.*, 1986). In rats, the appearance of elastic fibers of the connective tissue framework of the lung also precedes alveolarization (Emry, 1970; Amy *et al*., 1977). However, this phase occurs between postnatal day 4 and 13 in which alveolar sacs that have originated from the respiratory tracts rapidly divide into alveoli (Brody and Vaccaro, 1979), in comparison to its occurance *in utro* in humans.

The pulmonary arterial medial thickness drops to mature levels by the third month of postnatal growth. The alveoli will continue to proliferate with their accompanying vessels until the adult numbers are attained by two to three years of age after which the alveoli size and surface area continue to increase beyond adolescence (Haworth and Hislop, 2003). The initial increase in airway size is linear with antenatal growth, then slows after the first year (Hislop and Haworth, 1989).

It is therefore clear that lung development is "programmed" to go through various phases to ensure that the lung develops into an efficient gaseous exchanger and to maintain its structural and functional integrity. It is therefore concievable that intereference with these phases of development may have an adverse impact on lung development and maintanance of its structural and functional integrity. Intereference may be attributed to various factors including poor nutrition, exposure to foreign substances and pre-term birth.

1.2 Nicotine

1.2.1 The Uptake of Nicotine

Nicotine is an alkaloid tertiary amine consisting of a pyridine and pyrolidine ring. Of its two possible stereoisomeric forms, (S)-nicotine and (R)-nicotine, approximately 90% of nicotine in tobacco is found in its pharmacologically active form, (S)-nicotine (Pool *et al.* 1985). Primarily, only 15% of the total nicotine in most commercial cigarettes is found in the mainstream smoke, 25 to 45% is expelled to side-stream smoke, and 15 to 25% precipitates in the butt and filter tip. The remainder is pyrolyzed to nicotine decomposition products (Huber, 1989).

The pH of mainstream smoke is acidic ranging between 5 and 6 (Brunnemann and Hoffmann, 1974). In an aqueous environment nicotine has an index of

ionic dissociation (pKa) of approximately 8.0 implying that nicotine present in most tobacco smoke is almost completely protonated. In a protonated form, nicotine cannot cross biological membranes in significant quantities and thus almost no nicotine is absorbed in the mouth, pharynx, or upper respiratory tracts (Gori *et al.*, 1986).

The deliverance of the cigarette smoke micro-droplets on the internal surface of the lung results in the instantaneous buffering of these micro-droplets to a physiologic pH of close to 7.4. The pH of 7.4 is close enough to the pKa and results in a significant amount of nonprotonated nicotine which now rapidly and effectively crosses the alveolar-blood barrier (Benowitz *et al.*, 1988). The metabolism of this xenobiotic in the lung will be discussed later in the chapter.

1.2.2 Physiological Effects of Nicotine

Of the estimated 3500 different compounds found within tobacco smoke (Zevin *et al.*, 1998), nicotine is arguably responsible for more adverse health consequences than any other single compound (Hecht *et al.*, 2000). It is known to physiologically impact on the central and peripheral nervous system, the cardiovascular system, and the endocrine system (Nakajima *et al.*, 1996). Nicotine is also implicated as the chemical responsible for the addiction observed in cigarette smokers (Benowitz, 1997).

McMartin and coworkers (2002) found the lung tissue of children who had died from sudden infant death syndrome to have a higher concentration of nicotine than control children, regardless of whether smoking was reported. A few conditions found to be associated with nicotine and gestation, include prematurity, intra-uterine growth restriction, premature rupture of the membranes, spontaneous abortion. (Lambers and Clark, 1996). Adverse effects observed in neonates include low birth weight, SIDS, asthma and other chronic lung diseases (Lambers and Clark, 1996).

1.2.3 Potential Impact of Pre- and Post-natal Nicotine Exposure on Developing Lungs

Type I pneumocytes comprise approximately 90% of the alveolar surface, the rest of the surface is lined by type II pneumoctes (Naimark, 1977). The former, are dependent on glycolysis for their integrity (Massaro *et al.*, 1975) since mitochondria are rarely found within these cells (Vijeyaratnam and Corrin, 1972). Studies strongly suggest that carbohydrate metabolism is vital for the structural (Tierney and Levy, 1976) and functional development and growth of fetal and neonatal lung tissue (Gilden *et al.*, 1977; Maniscalco *et al.*, 1978; Bourbon and Josh, 1982). Furthermore, interference with carbohydrate metabolism during late gestation results in retardation of fetal lung growth (Rhoades and Ryder, 1981).

Research shows that nicotine accumulates in the respiratory passageways of the fetus (Szüts *et al.*, 1978). Nicotine also has the potential to directly interact with nicotinic receptors on non-neuronal cells in the developing rat lung (Sekhon *et al.*, 1999) and has been implicated as the component in cigarette smoke that may contribute to respiratory illnesses (Maritz, 1988; Meyer *et al.*, 1971). In 1983, Maritz demonstrated that nicotine interferes with carbohydrate metabolism in adult rat lung. Further studies showed that maternal nicotine exposure led to a significant increase in total glucose turnover with a markedly lowered *in vitro* lactate production (Maritz, 1986). These findings suggested that exposure to nicotine during gestation and lactation results in the hindrance of glycolysis in developing neonatal lung, giving rise to the possibility that nicotine compromises the integrity of a developing lung. This was substantiated by subsequent studies showing that maternal nicotine exposure results in impaired elastic tissue synthesis (Maritz and Woolward 1992). Additional findings with regard to maternal nicotine exposure in neonatal rat lung showed that there was a significant increase in the total adenine nucleotide pool and the ATP/ADP and ATP/ADP+AMP ratios (Maritz and

Burger, 1992), which after 4 weeks of withdrawal remained significantly elevated.

Morphometric studies by Maritz and co-workers (1994) show that the lungs of 42-day old rat pups exposed to nicotine during gestation and lactation have fewer alveoli than the lungs of control rat pups. They also found that the rate of alveolar formation in the lungs of these nicotine-exposed pups is slower than in the control lungs, even after weaning (Maritz and Winvogel, 2003). They suggest that the Na in the lungs of the experimental animals would possibly never reach the same number as in the control lungs due to changes in the "programming" that controls the growth and maintanance of the lung.

They also observed fenestrations in the walls of the alveoli, which they described as emphysema-like lesions (Maritz *et al.*, 1994), supported by reports that suggest that these fenestrations can be related to the onset of emphysema (Boren, 1962, Cosio *et al.*, 1986, Khun and Tavascoli, 1976). Moreover, a significant increase in lactate dehydrogenase (LDH) content was observed in the alveolar fluid, an indication of alveolar cell damage (Maritz and Najaar, 1995). Thus their studies provide strong evidence to support the work of previous researchers, demonstrating that interference in carbohydrate metabolism markedly impacts the structural and functional integrity of a developing lung. It also demonstrates that pre- and postnatal nicotine exposure causes inhibition of the glycolytic pathway, ultimately resulting in damage to the fragile type I pneumocytes since these cells are so dependent on the derived ATP for their survival.

Injury to the neonatal lungs was furthermore, biochemically demonstrated by analyzing the activity of enzymes that serve as sensitive markers for damage in the bronchoalveolar lavage (BAL) of rat pups. Maternal nicotine exposure results in the significant increase in LDH activity, indicating general cell damage, since this ubiquitous protein will only be present in the BAL if cell

membranes have ruptured (Maritz and Najaar, 1995). This study also showed that the activities of alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH) are higher in the BAL of rat pups exposed to maternal nicotine than those of the control pups. These elevated activities are indicative of injury to type II pneumocytes (Eylar and Hagopian, 1971) and type I pneumocytes (Vijeyaratnam and Corrin, 1972 Khun, 1968), respectively.

Research performed on the lungs of rhesus monkeys demonstrated that expression of the most abundant nicotinic receptor (alpha-7) in lung tissue is altered as a result of exposure to nicotine (1mg/kg body weight/day) during pregnancy from day 26 to day 134 (Sekhon *et al.*, 1999). The results of this study showed that chronic nicotine exposure caused an up-regulation in alpha-7 nicotinic receptors and also permanently inhibited the functioning of many of the nicotinic cholinergic receptors (nACHRs). Immunostaining techniques using antibodies against the alpha sub-unit (since all nicotinic receptors contain the alpha-subunit) indisputably showed the dramatic increase in the receptors' expression. Their data strongly suggests that nicotine crosses the placenta and interacts with the nicotinic receptors.

Recent studies investigating the effects of chronic nicotine exposure in rat hearts revealed that the expression of three classes of genes encoding cellular energy metabolism enzymes, transmembrane receptors and intracellular kinase network members are all reduced by more than 2.5 fold (Hu *et al.*, 2002). cDNA microarrays were performed on the cardiac tissue of rats fed via gavage with nicotine (3mg/kg/day). The expression levels of 1081 genes were analysed and eleven genes were shown to be down-regulated. In this study they proposed that chronic nicotine exposure might have a profound effect on cardiac function since the expression of proteins involved in energy metabolism and signal transduction pathways are suppressed.

Transcription is often the main controlling step during terminal differentiation and the activation or repression of tissue-specific genes (Skidmore and Beebee, 1990). Research shows that chronic nicotine exposure may exert its influences during post-transcriptional as well as transcriptional events. It is possible therefore, that maternal nicotine exposure during pregnancy and lactation may alter the expression of the individual glycolytic isoforms at transcriptional and/or posttranscriptional levels in an attempt to compensate for the inhibition of glycolysis observed by Maritz (1986).

1.3 Lung Metabolism

The structural and functional integrity of a developing and maturing fetal and neonatal lung is critically dependent on carbohydrate metabolism (Tierney and Levy, 1976; Gilden *et al.,* 1977; Maniscalco *et al.,* 1978; Bourbon and Josh, 1982). The energy derived from carbohydrate metabolism is utilized during the processes of cell growth and development. The importance of the carbohydrate metabolism becomes clear with respect to the activities of the enzymes involved in the pathways.

Fetal lung has the ability to accumulate glycogen (Bhavnani *et al.,* 1990) and the metabolism of glycogen in this fetal tissue provides the lung cells with ATP and substrates needed for phospholipid biosynthesis (Bhavnani, 1983; Maniscalco *et al.,* 1978). Studies by Bhavnani, *et al.* (1990) demonstrated that fetal lung generates the ATP and (nicotinamide adenine dinucleotide) NAD^+ in the presence of phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH). The high rate of anaerobic cellular respiration results in significant accumulation of lactate that can be transferred back to glucose via gluconeogenesis (Bhavnani *et al.,* 1990). In humans and nonhuman primates the capacity for gluconeogenesis develops during the last trimester (Burd *et al.,* 1975; Battaglia and Meschia, 1978). However, this pathway is

quite limited in rodent fetal tissue and develops only after birth (Burch *et al.*, 1963b; Girard *et al.*, 1975).

During late gestation, glycogen and glucose is linked to surfactant production (Hamosh *et al.,* 1978; Bourbon *et al.*, 1982) a relationship also observed in adult lung (Salisbury-Murphy *et al.*, 1966). It has been shown from gestational day 19 to term, that glycogen disappearance in type II pneumocytes is associated with surfactant synthesis (Bourbon and Josh, 1982) in which it supplies precursors for the production of this phospholipid, on condition that the glycolytic pathway is efficiently active. Studies by Bhavnani and Wallace (1990) show that the key enzymes involved lactate formation: PFK, PK, and LDH are functioning before rodent fetal lung starts to synthesize surfactant.

In the hexose monophosphate shunt (HMS), glucose-6-phosphate dehydrogenase (G-6-PDH) is its key regulatory enzyme and catalyses the oxidation of glucose-6-phosphte to 6-phosphogluconolactone with the production of NAPDH (Ho, *et al.*, 2000). The reduced equivalents formed in the cells are used for reductive biosynthesis and maintenance of the redox status. G-6-PDH's activity has been linked with synthesis and repair processes in the cell (Lehninger, 1975). This enzyme is also thought to be involved in the maintenance and protection of the lung cells since it plays an important role in detoxification of foreign substances that may have entered the lung via inhaled air or blood (Naimark, 1977). Its activity is also viewed as indispensable with regards to detoxification of reactive oxygen species (ROS) (Pandolfi *et al.*, 1995).

Development of the lung and the mechanisms of impact that maternal nicotine exposure has on it, remain unclear. In an attempt to elucidate the pathophysiological mechanisms involved, this study investigates the mRNA expressions and isozyme distributions of selected glycolytic enzymes. Furthermore, the metabolic compounds produced after oxidation of nicotine in

lung (and other tissues) has come under immense scrutiny, particularly with respect to its contribution to cancer. Thus this study also reviews and investigates the mRNA expression of selected Cytochrome P450s (CYPs) in the developing lung.

1.3.1 Hexokinase (HK)

Glucose is the major source for energy production in fetal tissue and is also vital for logarithmic growth and accumulation of glycogen stores (Krebs, 1972; Girard and Ferre, 1982). Immediately after birth the neonate is entirely dependent upon endogenous glucose, a period that represents a complex balance between the synthesis and regulation of glucose and the energy needs of the individual organs (Griffin *et al.*, 1992). In the lung, glucose is the primary source of the a-glycerophosphate moiety for the production of surfactant (Felts, 1964; Salisbury-Murphy *et al.*, 1966). Also the oxidation of glucose via the pentose phosphate shunt provides NADPH, a compound that plays a critical role in protecting the lung against inhaled oxidants (Tierney *et al.*, 1973).

The phosphorylation of glucose to glucose-6-phosphate is a reaction that represents the first and one of the principal steps in glucose metabolism (Fig.1). This reaction utilizes ATP and is catalysed by hexokinase (HK) (Griffin *et al.*, 1992). Glucose-6-phosphate is necessary for pathways such as glycolysis, glycogen synthesis and the hexose monophosphate shunt.

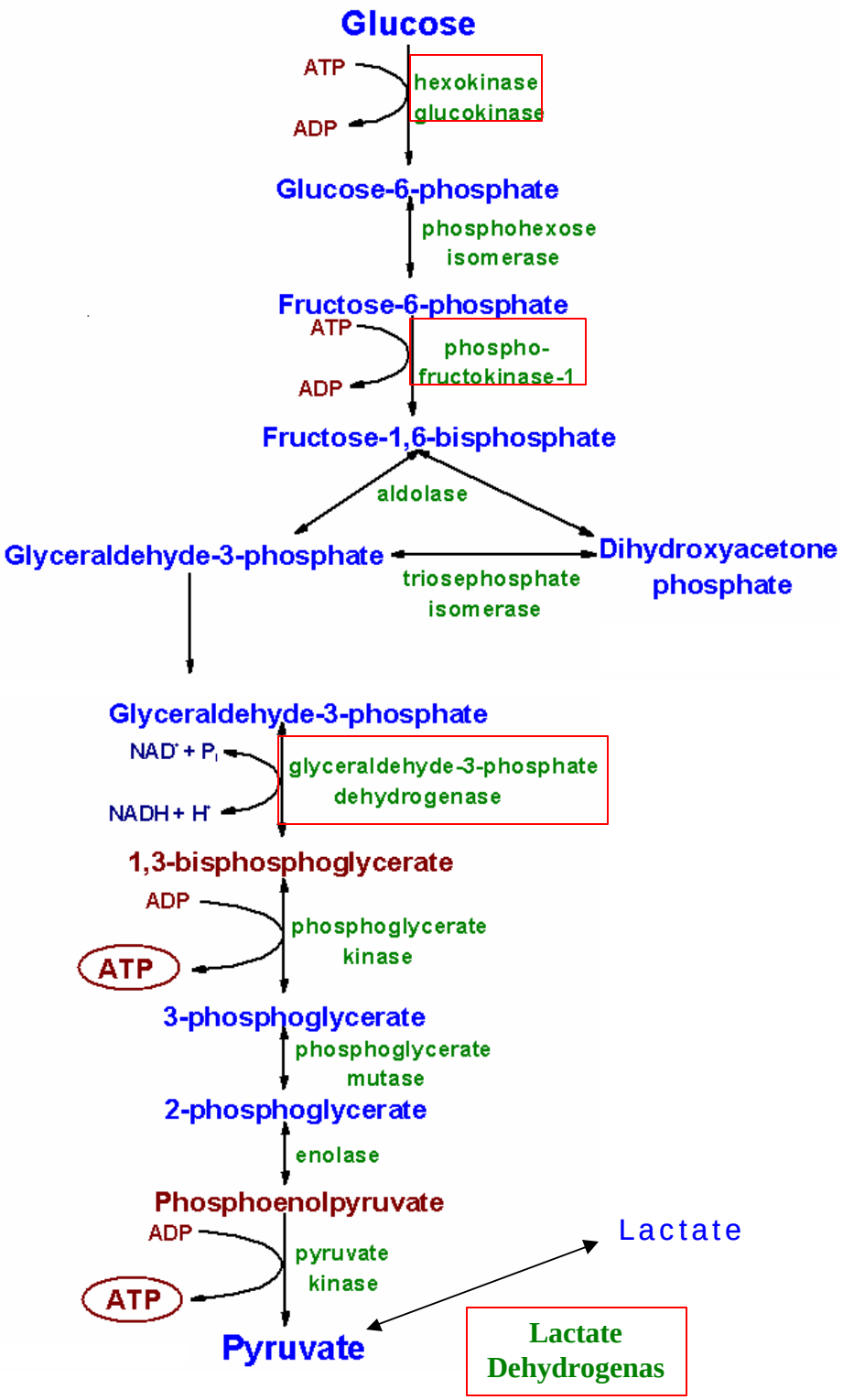


Fig.1. A schematic diagram of the glycolytic pathway.

HK exists in mammalian tissue as four isozymes encoded by a single-copy gene, each located on a different chromosome in the rat genomes (Sebastian *et al.*, 1997). HKI, II and III are single polypeptide chains with a molecular mass of 100kDa, and HKIV (glucokinase) has an approximate molecular mass of 50kDa (Katzen and Schimke, 1965). Their high relative affinity for glucose easily distinguishes HKI, II and III from glucokinase (Wilson, 1985). The metabolic state of an organism influences the tissue specific distribution of the isoforms (Rijksen and Staal, 1985; Adams *et al.*, 1991). HKI is predominantly found in tissues that rely almost entirely on glucose, such as brain and erythrocytes and has been detected in virtually all tissues except in the liver (Griffin *et al.*, 1992) and skeletal muscle tissue (Shinohara, *et al.*, 1998). A comparison between human and rat HKI shows very few differences between the species in which they have similar Ḿ, kinetic properties, and isoeletric points (Magnani, *et al.*, 1990). There is also strong evidence suggesting that HKI binds to mitochondria in brain (Wilson, 1980) and other tissues (Solatra and Singh, 1982, Parry and Pedersen, 1984, Katzen *et al.*, 1970). A molecular model of the HKI-mitochondrial membrane interaction has been put forward by Wilson (1980). At the surface of the mitochondria HKI putatively interacts with permeation pores composed of channel proteins, porin and adenylate translocase. These channel proteins span the outer and inner membrane of the mitochondria, respectively (Aleshin, *et al.*, 2000). This interaction between the membrane proteins and HKI allows for the direct exchange of adenine nucleotides between the mitochondrial matrix and the active site of HK (de Cerqueira and Wilson, 2002).

HKI is also the dominant isoform in the lung, however its expression levels are reported as considerably lower than activity data obtained from Bennett *et al.* (1978) and nearly equal quantities of HKII has also been reported (Salotra and Singh, 1982). HKI is inhibited by glucose-6-phosphate (Salotra and Singh, 1982) and it has been suggested that this isoform may be more dominantly expressed in aerobically active cells such as alveolar type II cells (Griffin *et*

al., 1992). In type II cells glucose is principally the precursor of dihydroxyacetone phosphate (DHAP) for surfactant formation (Rijksen *et al.*, 1985).

The lung cannot store adequate amounts of glycogen and is thus dependent on glucose as the main source for glycolysis. Moreover, the activities of HKI is best suited to meet the metabolic needs of the lung since it can be modulated according to the needs of the tissue by appropriately changing the intracellular concentrations of glucose-6-phosphate and inorganic phosphate (Salotra and Singh, 1982). HKI is thus important for surfactant production in the saccular phase and alveolar phase of neonatal lung development. It is suggested that regulation of HKI mRNA expression involves both transcriptional and post-translational mechanisms (Griffin *et al.* 1992).

HKII has been reported as the dominant isoform in muscle tissue (Reid and Masters, 1985) where its function has been described as mainly aiding in glycogen production (Easterby, *et al.*, 1981). This isoform also binds to the mitochondria but it is also strongly suggested that HKII binds to the sarcoplasmic membrane, the site where preparations are made to convert glucose to glycogen (Pette, 1975). Thus it seems that HKII in muscle may redistribute itself between the mitochondria and the sarcoplasmic membrane depending on whether the tissue is engaged in glycolysis or glycogen synthesis. During the early fetal period HK activity in liver is high since this tissue is largely dependent on glucose (Reid and Masters, 1985). Data from mouse indicates that HKII is dominant at this stage however this is not the case in rat. HK activity declines in the late postnatal period, a direct result of the liver's gluconeogenic role during this phase. This decrease in HK activity is accompanied by a noticeable increase in HKIII and HKIV (Ureta, 1982; Faulkner and Jones, 1976).

Immunohistochemical techniques utilized by Coerver and Gray (1998) show that HKIII is present in brain, heart, liver, kidney and skeletal muscle tissue of rat during gestation and postnatally, however its presence in spleen was only observed after birth. Furthermore, studies also implementing imunohistochemical analysis in adult rat report that HKIII is localized in the nuclear periphery of cells in the kidney, liver, spleen, lung and brain (Preller and Wilson, 1992). HKIII has the lowest K_m of the three isoforms and thus is active at substantially lower concentrations of glucose than the other forms (Coerver and Gray, 1998). Furthermore, HKIII in adult animals is inhibited at physiological concentrations of glucose *in vitro*, indicating that it can only function at very low intracellular glucose levels (Wilson, 1985 and 1995). This isozyme is also inhibited by low levels of glucose-6-phosphate (Grossbard and Schimke, 1966).

Individual HK isoenzymes are shown to be expressed in association with a particular glucose transporter (GLUT). Shinohara and co-workers (1998) re-affirmed that in normal rat tissue HKI is expressed with GLUT1, HKII with GLUT4 and HK IV with GLUT2. They also suggested that expression of both the HK and GLUT isoforms could be regulated by the same mechanisms. In malignant tumor cells however, the relationships between the GLUT and HK isoforms differed. GLUT1 was associated with HKII, suggesting that the transcriptional regulation of these isoforms were different from normal tissue (Shinohara, *et al.*, 1998).

In normal liver only small amounts of the low K_m isoenzymes (HKI-III) can be detected, whereas the high K_m isoform, HKIV is found in abundance (Rempel *et al.*, 1994). In liver tumor cells (ELD- and AH130) however, only HKI and HKII are exhibited. An increase in both activity and mRNA expression is observed, strongly indicating that regulation of HK expression is mainly at the transcriptional level. It is suspected that HKII plays a vital role in neoplastic transformations (Rempel *et al.*, 1994) stemming from the fact that HKI is

easily inhibited by glucose-6-phosphate, while HKII displays delayed inhibition (Wilson, 1985). Thus upon glucose supply, cells with elevated HKII content have the ability to build up large amounts of glucose-6-phosphate, signaling to the cell that glucose is available to the cell (Rempel *et al.*, 1994).

Studies by Berstein and Kipnis (1973) show that HKII in skeletal muscle and adipose tissue in rat progressively decline, reaching plateau levels after attaining body mass of 300g. HKII is predominantly supported by GLUT1 (Shinohara, *et al.*, 2001) thus enabling the cell to rapidly utilize glucose at a constant level throughout. Analysis of the mRNA expression of HKII in skeletal muscle tissue also displayed a decrease in the first two postnatal days and then gradually increased (Shimokawa *et al.*, 1998).

In lung tissue, in early gestation it is reported that all HK activity is confined to the cytosol (Rijksen *et al.*, 1985). However, 1 to 2 days prior to birth some mitochondrial binding is evident, reaching a significant total activity of approximately 30% at birth. Studies using cellulose acetate electrophoresis report that HKI and II are present in pre- and postnatal lung tissue, however HKIII is barely traceable in some postnatal samples (Rijksen, *et al.*, 1985). Densitometry on these cellulose acetate gels reveal that HKII shifts from a 50% distribution at gestational day 6 to about 10% shortly after birth, a value almost comparable to adult tissue. They also report that HKI remains relatively constant.

1.3.2 Phosphofructokinase (PFK)

The phosphorylation of fructose-6-phosphate to fructose 1,6-bisphosphate (Fig.1) is a critical regulatory step in the glycolytic pathway and is catalyzed by phosphofructokinase (PFK) (Dunaway, 1983). PFK is an oligomeric protein (Mhaskar and Dunaway, 1995) and in its catalytic state it is in its smallest active species, a tetramer that is subject to allosteric regulation (Kemp

and Foe, 1983; Dunaway and Kasten, 1987; Mhaskar, *et al.,* 2000). PFK activity is particularly inhibited by high concentrations of adenosine triphosphate (ATP), citrate or phosphocreatine and activated by fructose 2,6-bisphosphate, adenosine monophosphate (AMP), cyclic AMP, inorganic phosphate or fructose 1,6-bisphosphate (Mansour, 1979, van Schaftingen and Hers, 1980, Uyeda *et al.*, 1981; Hue and Rider, 1987).

Random association of available subunits results in the formation of three homo- or heterotetramers of differing allosteric and catalytic properties in human (Khan *et al.,* 1979, Dunaway and Kasten, 1988) and in rat (Dunaway and Kasten, 1985a,b). The PFK monomer is 80-85 kDa in size (Gunasekera and Kemp, 2000). The M-type subunit is the only isoform found in skeletal muscle, L-type subunit is the dominant isoform in liver and C-type subunit is found in high levels in brain and platelets (Foe and Kemp, 1984; Dunaway and Kasten, 1985a; Gekakis *et al.,* 1994). In humans, the genes encoding the individual isoenzymes are located on different chromosomes, namely chromosome 1, 21 and 10, respectively (Vora *et al.*, 1982, 1983; van Keuren *et al.*, 1986; Morrison *et al.*, 1992).

The M_4-homotetramer displays the highest affinity for fructose 6-phosphate, which promotes a capacity for a large and rapid increase in the glycolytic rate at lower levels of this substrate. The C-type subunit, a heterotetramer, however, exhibits the lowest affinity for this substrate. Furthermore, M_4 shows the lowest sensitivity to ATP inhibition, whereas L_4 and brain PFK display a similarly high sensitivity to this inhibition (Dunaway and Kasten, 1985a; Dunaway and Kasten, 1988). Moreover, the nature of the kinetic/regulatory properties of PFK seems to be largely controlled by the subunit composition of the PFK isoenzyme pools (Kasten, *et al.*, 1993; Dunaway and Kasten, 1989).

To date, information with regards to developmental changes in PFK isoenzyme patterns and mRNA expression in tissues of animals is scant, particularly in lung tissue. Most of the data pertaining to the activities of PFK is obtainable before the year 2000. Furthermore, although information on mRNA expression in developing heart, brain and muscle tissue has been reported, studies in developing lung tissue have not been documented as yet.

In 1968, Hommes and Wilmink reported on the developmental changes in rat PFK activity in brain. Their research shows that PFK activity decreased by approximately 50% from prepartum day 7 to 2 days, prepartum, after which its activity seem to stay the same. They did report however, that by 25 days postpartum total PFK activity had doubled (Hommes and Wilmink, 1968). Baquer *et al.* (1973) however, reported a four-fold increase in PFK activity from 20 days postpartum brain tissue compared to fetal tissue levels. In 1983, Dunaway reported an increase from 2.7units/g in fetal brain to 16.6units/g in adult brain. These finding and previous reports thus confirmed that total PFK activity increases to adult levels as the brain tissue matures after birth.

Studies performed in fetal rat liver show that PFK activity was nearly 3.5-fold greater than in that of adult liver (Burch *et al.*, 1963a). In addition, they found that the activity of PFK decreases more rapidly before birth than after birth with PFK values reaching adult levels by the 9th day after birth (Burch *et al.*, 1963). Similar studies by Pokrovskii and co-workers (1972) demonstrated PFK activity levels in near term liver 1.5-fold greater than in adult liver. Reports by Hommes and Wilmink (1968) described a 50% decline in PFK activity from five days prepartum to birth, after which it remained constant. Furthermore, Dunaway (1983) found that PFK activity in near term liver was four times that of the adult liver. These findings clearly show a distinct decline in PFK activity as the liver matures. In the same study, Dunaway (1983) also shows changes in the levels of the isoforms PFK-M and PFK-L in which their levels decrease rapidly within 24 hours of birth. Within the first week PFK-M

is not detectable and between week two and three the PFK-L levels are similar to that of adult tissue (Dunaway, 1983). To date, very little research has been performed on PFK-C levels in developing tissue.

Studies in skeletal and heart muscle tissue show that total PFK activity dramatically increases from 5.8units/g to adult levels of 31.6units/g in the skeletal muscle tissue (Thrasher *et al.*, 1981). Both PFK-M and PFK-L are present in fetal and neonatal skeletal muscle however PFK-L is not detectable in adult tissue. The increase in PFK activity is correlated with the rise in PFK-M levels and also with the increased contractile ability of the skeletal muscle tissue (Thrasher *et al.,* 1981). In fetal heart, PFK activity is estimated at being 56% of that of the adult PFK activity in which PFK-L is reported as the dominant isotype. Fetal heart is principally dependent on glycolysis for energy synthesis and is also quite tolerant to anoxic conditions and it is during this phase that PFK-L appears to be the dominant isoform (Dunaway, 1983). In the adult heart PFK-M and -L are present in a 70:30 ratio (Thrasher *et al.*, 1981). As with skeletal muscle, heart muscle PFK activity increases as the rat developed (Baldwin *et al.*, 1977) and is also primarily as a result of an increase in PFK-M. As the heart matures there is an increase in mitochondria allowing for an increased capability for oxidative phosphorylation. This period coincidentally is characterized by increased levels of PFK-M which are more sensitive to ATP and citrate inhibition than PFK-L (Hosey *et al.*, 1980). It is suggested that the presence of PFK-L, which is less sensitive to inhibition by ATP and citrate may provide a form of energy during anoxia via glycolysis. Thus the presence of PFK-L in fetal heart tissue and other tissue may be associated with increased tolerance to anoxia (Dunaway, 1983).

PFK's importance in lung carbohydrate metabolism and ultimately in maintaning lung integrity is clear with respect to its location in the metabolic pathway. It is the only regulatory enzyme of glycolysis situated between glycogen derived from glucose-6 phosphate and dihydroxyacetone phosphate,

the precusor of the glycerolphosphate moiety for surfactant production (Rijksen *et al.*, 1985).

Of the few documented studies on PFK in lung, according to the findings of Rijksen *et al.* (1985) the specific activities of PFK increases during fetal development, reaching its peak 1 to 2 days prior to birth. PFK activity is then reported to decline steeply ($P< 0.05$) until postnatal day 2, at which time the values are comparable to that in adult lung.

1.3.3 Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis (Fig.1) and is found in all human and other mammalian tissues (Edwards *et al.*, 1985; Reid and Masters, 1986; Ryzlak and Pietruszko, 1988). As one of the most abundant cellular proteins it accounts for no less than 15% of all soluble proteins (Scopes and Stoter, 1982).

During anaerobic glycolysis it catalyzes the reversible phosphorylating reaction of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate in which nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH (Habenicht, 1997). Furthermore, it also catalyzes the oxidation and subsequent phosphorylation of substrate aldehydes to acyl phosphates forming adenosine triphosphate (ATP) via the electron transport chain (Modziak *et al.*, 2003). It has been suggested that under certain circumstances, for example increased glycolytic rate, GAPDH may regulate the rate of flux in this pathway (Oguchi *et al.* 1973). This enzyme is strongly inhibited by adenine nucleotides (Constantinides and Deal, 1969; Yang and Deal, 1969), the mechanism of which entails the prevention of the binding of NAD^+ by adenosine triphosphate (ATP) and adenosine diphosphate (ADP) which also blocks the formation of enzyme-substrate intermediates. The degree of inhibition is

dependent on pH, inorganic phosphate (Pi) and magnesium (Mg^{2+}) (Oguchi *et al*., 1973).

Enzymes generally occur in multiple molecular forms and while multiple forms of GAPDH has been reported in invertebrates (Reid and Masters, 1986), this enzyme is, however, one of the few considered to be the exception to this rule (Ryzlak and Pietruszko, 1988). In vertebrates GAPDH exists as a tetramer composed of four identical subunits of 37 kDa (Ercolani *et al.*, 1988) and consists of a polypeptide chain of 330 amino acids (Sirover, 1999). GAPDH is encoded by a single functional gene, (Reid and Masters, 1986) located on the short arm of chromosome 12 (Bruns and Gerald, 1976). Thus unlike other tetrameric components of the glycolytic pathway, this enzyme is not present in multiple forms. GAPDH may also exist in vivo as a tetrameric or a monomer, but its glycolytic function is restricted to its tetrameric form (Mazzola and Sirover, 2003) of approximately 150 kDa (Sirover, 1999).

Full length cDNAs encoding GAPDH from rat and human have been isolated and sequenced and genomic library screening shows that the cDNAs in rat and human is 89% homologous in the coding region (Tso *et al.*, 1985). The sequences are highly conserved across the phylogenetic scale.

It is reported that GAPDH belongs to a large multiple gene family comprised of at least 150 GAPDH-like sequences of which a large number are processed pseudogenes (Tso *et al.*, 1985; Benham *et al.*, 1984). Thus the gene family is comprised of a single functioning gene and a variety of genes lacking introns (Sirover, 1999).

GAPDH has for many years been considered as a classical glycolytic enzyme that participates exclusively in cytosolic energy production. It is also commonly used as a "house-keeping" gene in methods involving gene expression analysis (Sirover, 1999). Furthermore, studies show that hypoxia

results in the transcriptional alteration of GAPDH in vascular endothelial cells (Graven *et al.*, 1998). More recently, studies investigating the effects of hypoxia in alveolar epithelial type II (ETII) cells in the lower respiratory tract reported that GAPDH is significantly induced in these hypoxic-tolerant cells (Escoubet *et al.*, 1999). They report a significant increase in GAPDH mRNA, protein synthesis and enzymatic activity. Transcriptional regulation of GAPDH and consequently the increase in activity is thought to be part of the mechanism involved in supporting the cell during high energy demands or when ATP production is severely impaired. It is this mechanism of induction that has been proposed as the reason for the survival of ETII cells *in vitro* of up to 48 hours in 0% oxygen (Escoubet *et al.*, 1999).

GAPDH has been shown to be particularly vulnerable to the effects of oxidants. A loss of activity has been reported when GAPDH is exposed to hydrogen peroxide (Brody and Reed, 1990; Vaidyanathan *et al.*, 1993; Janero *et al.*, 1994). In myocardial tissue, it plays a vital role in regulating glycolysis during anoxia (Williamson, 1966) and ischemia (Rovetto *et al.*, 1975). During myocardial reperfusion, superoxide anions, H_2O_2, hydroxyl radicals and nitric oxide are produced and their interaction with GAPDH renders it inactive (Knight *et al.*, 1996). It is this inhibition that then leads to the transient halting of glucose utilization discovered during early reperfusion in dogs and pigs (Buxton and Schelbert, 1991; McFalls *et al.*, 1994). More recently, Lei *et al.* (2004) showed that GAPDH activity significantly decreases by 25% in failing hearts compared to normal hearts and that its gene and protein expression also markedly decreases.

These findings are of particular interest to this investigation since Maritz (1983, 1986) showed that maternal nicotine exposure during pregnancy and lactation results in a significant increase in total glucose turnover despite the inhibition of glycolysis. Furthermore, it is reported that maternal nicotine exposure results in the inhibition of GAPDH activity (Maritz, 1997) during the

critical phase of alveolarization. Thus the above-mentioned studies may provide the mechanism by which glycolysis is inhibited. Furthermore, inhibition of GAPDH may also have an adverse effect on lung growth since it may result in a reduction in energy supply, at a stage when energy demand is high.

Recently, studies also revealed that GAPDH is multidimensional in function in which it displays a variety of discrete activities apart from its glycolytic function. Glaser and Gross (1995) reported on the fusogenic activity of GAPDH (ie. the ability to merge phospholipid bilayers) and thus, demonstrated the critical role it plays in normal cell function. Its role in membrane fusion was also demonstrated by Hessler *et al.* (1998) and thus non-glycolytic GAPDH may be involved in cell division, the immune response, synaptic transmission and the response to environmental change.

Studies by Kumagai and Sakai (1983) detected that GAPDH binds to microtubules. Investigations by Caswell and Corbett (1985) demonstrated that GAPDH catalyzes the formation of triad junctions from isolated transverse tubules and terminal cisternae. Their findings clearly add to the role of GAPDH in regulating cytoskeletal structure.

Several studies have also reported that GAPDH displays phosphotransferase/kinase activity. Initial studies show that GAPDH has autophosphorylating abilities (Kawamoto and Caswell, 1986). Furthermore, GAPDH may play a part in viral pathogenesis in human cells where by GAPDH interacts with hepatitis B virus (HBV) and also phosphorylates the viral proteins (Duclos-Vallee *et al.*, 1998).

It has been suggested that GAPDH may also be involved in nuclear RNA transport. This nuclear, non-glycolytic function is thought to play a role in apoptosis and it is reported that the appearance of nuclear GAPDH is

prevented when apoptosis is inhibited (Sawa *et al.*, 1997). GAPDH has in addition to all the other significant non-glycolytic functions also displayed DNA repair activity in which it exhibits uracil DNA glycosylase (UDG), the DNA repair enzyme, activity (Arenaz and Sirover, 1983; Vollberg *et al.*, 1987; Vollberg *et al.*, 1989). GAPDH also binds to sequences on mRNA essential for its modulation (Nagy and Rigby, 1995) and its tetrameric form is required for the binding (Schultze *et al.*, 1996). Recent studies indicate that oxidation of GAPDH enhances its binding to nucleic acids (Arutyunova *et al.*, 2003). Furthermore, this protein interacts with glutathione, as this interaction is thought to be a function of oxidative stress (Schuppe-Koistinen *et al.*, 1994).

For years, GAPDH has commonly been used as a control in gene expression studies. However, many studies are now demonstrating that this protein may not be a suitable standard. Studies performed in human cells, show changes in the gene expression and levels of this protein during cell proliferation (Meyer-Siegler *et al.*, 1992; Mansur *et al.*, 1993). Its mRNA expression is augmented under hypoxic conditions in alveolar epithelial type II cells (Escourbett *et al.*, 1999) and genes of human cervical carcinomas display an increase in the expression of GAPDH (Kim *et al.*, 1998). More recently, studies using post hatch chicks show that its expression varies with age and nutritional status (Modziak *et al.*, 2003), suggesting that GAPDH is not an efficient internal standard for studies using quantitative RNA analysis.

1.3.4 Lactate Dehydrogenase (LDH)

An enzyme extensively studied in vertebrates is lactate dehydrogenase (LDH). This cytoplasmic enzyme catalyses the interconversion of L-lactate and pyruvate (Fig.1) with nicotinamide adenine dinucleotide (NAD^+) in the glycolytic pathway (Tsoi *et al.*, 2001; Li, 1998). In mammals, two LDH sub-units (H-type and M-type) are expressed at varying levels in most tissues and each are coded for by individual genes (Tsoi *et al.*, 2001; LaPlace-Marieze *et*

al., 1994). Sub-unit expressions are specifically regulated by the physiological need of the tissue (Marker *et al.*, 1975; Marker, 1982). A third gene exists that codes for the C-type sub-unit exclusively found in male germinal cells (Skidmore and Beebee, 1990). LD-M is best adapted for pyruvate reduction in anaerobic tissues such as muscle, whereas LD-H is more suited for L-lactate oxidation in aerobic tissues such as heart (Li, 1998). Generally, five LDH isoenzymes exist as a result of the random association of the H and M sub-units (LaPlace-Marieze *et al.*, 1994). The isoenzymes exists as tetramers and are designated LD-1 (H_4), LD-2 (H_3M), LD-3 (H_2M_2), LD-4 (HM_3) and LD-5 (M_4) in order of their decreasing anodal mobility in an alkaline medium (Drent *et al.*, 1996). LD-M expression can be induced by estrogen (17ß-estradiol) and/or cAMP (Hou and Li, 1987). LD-H in contrast is not inducible by cAMP since it lacks the cAMP responsive element on its promoter sequence (Li, 1998). Studies show however, that H and M polypeptide levels are not controlled by mRNA abundance in a tissue (Skidmore and Beebee, 1990). Furthermore, studies performed on rabbit muscle indicate that mRNA abundance shows no correlation with LDH activity and seems to depend on the muscle type and age of the tissue (LaPlace Marieze *et al.*, 1994).

Tissue levels of LDH are approximately 500 fold greater than those normally observed in serum. Any damage to even a small mass of tissue will cause leakage of this cytoplasmic enzyme and significantly increase the serum LDH levels (Drent et al., 1996). Several pulmonary disorders such as emphysema, pulmonary embolism and tuberculosis, have been associated with elevated serum LDH levels in humans (Drent et al., 1996). The common factor observed amongst these diseases, is that cell damage and/or inflammation has occurred (Matusiewicz et al., 1993; Hoffman and Rogers, 1991).

Generally, an increase in airway LDH activity may be as a result of a diverse number of sources (Roth, 1981). Firstly, the rupturing of alveolar epithelial

cells and alveolar macrophages could increase serum LDH levels. When the air-blood barrier have been rendered more permeable than usual, as in the case of edema and hemorrhage, this may also increase serum levels. An increase in systemic plasma levels may cause a greater influx of LDH from the plasma across the air-blood barrier of a normal lung (Roth, 1981).

The factors mentioned above may, thus impact on the airway serum LDH levels and therefore assessment of serum LDH may provide non-specific information with regards to the lung status. Research shows that pattern determination of the LDH isoenzymes may prove more accurate in serum.

Many clinical conditions such as cancer, myocardial infarction and liver disorders have been associated with changes in expression of the individual isoenzymes (Kopperschlager and Kirchberger, 1996). As diagnostic tools, LDH isoenzyme patterns have been evaluated in serum (Hawkins and Whyley, 1966; Hoffman and Rogers, 1991), bronchoalveolar lavage fluid (BALF) (Henderson *et al.*, 1979; Henderson, 1988, Tsai *et al.*, 2003), saliva (Nagler *et al.*, 2001), cerebrospinal fluid (Lampl *et al.*, 1990) and in homogenates of placenta (Tsoi *et al.*, 2001), muscle, kidney, brain, liver (Reid and Masters, 1985; Yasuda *et al.*, 1989; Sylvén *et al.*, 1989), and lung tissue (Vergnon *et al.*, 1984; Schultze *et al.*, 1994).

Analysis of cerebrospinal fluid of patients with bacterial meningitis (Beaty and Oppenheimer, 1968), and carcinomatous meningitis shows an elevation in LD-5 (Wasserstrom *et al.*, 1982). LD-1 increases within patients suffering from bacterial meningitis with severe brain damage and also in cases of viral meningitis. Thus, this demonstrates that these isoforms may be used as biological markers for the above-mentioned disease.

A study performed on patients with benign and malignant pleural effusions show that no significant differences are found in total serum LDH between the two groups analyzed (Paavonen *et al.*, 1991). However, serum LDH in normal controls is significantly lower than within patients with malignant effusions. Analysis of the isoenzyme patterns in pleural fluid shows that LD-4 and LD-5 increases in both patient groups, with the simultaneous decrease in LD-1 and LD-2. This research indicates that regardless of whether the effusion is benign or malignant in origin, the pleural epithelial cells synthesizes and secretes more anaerobic isoenzyme types (Paavonen *et al.*, 1991). These results further support the theory that isoenzyme analysis provides valuable information with regards to tissue oxygen utilization and the tissue's need to use anaerobic glycolysis. Analyzing the LDH isoenzyme patterns in lung homogenate of developing neonatal rat lung will provide essential detail with regard to the tissues metabolic need as it matures.

Maternal nicotine exposure during pregnancy and lactation results in the inhibition of glycolysis even four weeks after nicotine withdrawal in the lungs of neonatal rat pups (Maritz, 1988). Analysis of total LDH activity in the BALF of the nicotine-exposed lung shows that LDH activity significantly increases (Maritz and Najaar, 1995), indicating that general cell damage has occurred. Furthermore, the study shows an increase in BALF alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH), which reflected type-II cell (Eylar and Hagopian, 1971) and type-I cell (Vijeyratnam and Corrin, 1972) injury. Maritz (1988) proposed that maternal nicotine exposure causes the marked hindrance of glycolysis and that this interference then renders the type-I cells more vulnerable to injury with a concomitant cell death and release of LDH into the BALF.

Since LD-1 is linked to oxidative processes and LD-5 is associated with glycolytic processes, electrophoretic separation based on the difference in molecular weight, of non-denatured LDH isoenzymes (from the supernatant of lung tissue homogenates), and the densitometrical scanning of the fragments might aid in understanding the mechanism of injury and also help in determining the response of the lung tissue to the damage inflicted. Furthermore, analysis at the mRNA level is critical since maternal nicotine exposure may alter expression of the LDH isozymes at the transcriptional levels.

1.3.5 Cytochromes P450 (CYP P450) Enzymes

The lung is the primary target for all inhaled toxicants. Although it contains many enzymatic pathways with the ability to metabolize xenobiotics, general consensus is that the cytochrome (CYP) P450 superfamily of enzymes is its dominant means of metabolizing these exogenous substances (Hukkanen *et al.*, 2001). Upon entering the lung, many of the chemicals are not hazardous as such, but are frequently biotransformed by the CYP enzymes into reactive intermediates (Nelson *et al.*, 1996). Therefore, a critical factor contributing to the aetiology or modification of respiratory disease is whether the lung tissue has the ability to activate or efficiently inactivate chemicals (Raunio *et al.*, 1999).

In addition to exposure to air-borne substances, the respiratory system is also exposed to chemicals via the systemic circulation. This is particularly true during gestation when the developing fetal lungs are exposed to chemicals transferred from the maternal circulation across placental tissue into fetal circulation (Lee *et al.*, 2000) and during lactation when compounds are conveyed via the mother's milk (Luck and Nau, 1984).

Nicotine is an ideal example of a chemical that freely crosses the placenta (Van Gilder, 1997) because it is lipid-soluble (Lambers and Clark, 1996) and has been found in significant quantities in the milk of smoking mothers (Luck and Nau, 1984). Thus this substance can freely interact with the developing fetus and neonate.

In an effort to understand the possible pathological impact nicotine may have on the lungs of developing neonates, this study also focuses on the critical aspect of metabolism of nicotine by CYP P450 enzymes.

The CYP superfamily is ubiquitously expressed in a diversity of life forms (Nelson *et al.*, 1996). These enzymes seem essential to eukaryotic species but not prokaryotes since some bacteria have displayed a lack of CYP enzymes (Nelson, 1999). Their presence is crucial for oxidative, peroxidative and reductive metabolism of a variety of compounds. These compounds include endobiotics such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes, and xenobiotics comprising of most therapeutic drugs and environmental pollutants (Bertz and Grannemann, 1997).

Eukaryotic CYP enzymes are membrane-bound, predominantly localized to the endoplasmic reticulum, but are also found in the mitochondrial inner membranes and they require an electron transfer chain in order to perform their critical function. In the endoplasmic reticulum, electrons are transferred by NADPH-cytochrome P450 reductase (Omura, 1999) and in the mitochondria, redoxin reductase provides the electrons to the CYPs (Gonzales, 1990).

Humans are estimated to have 53 different CYP genes and 24 pseudogenes. A standard nomenclature system has been developed to catagorize the gene families and subfamilies of the CYP enzymes (Nelson *et al.*, 1996). The family members are at least 40% identical and their enzymes within a given subfamily have a greater than 55% sequence homology.

Xenobiotic metabolism can generally be divided into two types: functional (Phase I) and conjugation (Phase II) reactions. The former is rate-limiting for the metabolism of xenobiotics and is primarily involved in the toxicity of chemicals. The latter catalyses the reactions of chemicals or their metabolites with endogenous substances such as glutathione or glycine, but seldom activate the chemicals (Nakajima, 1997). CYP P450 enzymes are the main group of enzymes involved in Phase I reactions (Lu and West, 1980). Although the primary purpose during Phase I reactions is detoxification, many of their substrates are transformed into carcinogenic, mutagenic and reactive intermediates that are far more toxic than their parent compounds (Oinenon and Lindros, 1998). Thus, functional reactions constitute an enzymatic interface between humans and a wide variety of chemicals.

The CYP enzymes predominantly involved in xenobiotic metabolism belong to the families designated CYP1, CYP2, and CYP3. The other families have essential roles in the metabolism of endogenous substances (Nelson, 1999). CYP 1-3 families metabolize a wide variety of xenobiotics, but some also possess the ability to metabolize endogenous compounds such as steroid hormones and arachidonic acid (Gonzalez, 1992; Capdevila *et al.*, 2000). Approximately half of the 53 human CYP forms belong to the CYP 1-3 families (Nelson, 1999).

The liver has been shown to express the majority of CYPs both quantitatively and by CYP diversity (Oinonen and Lindros, 1998). Some extrahepatic tissues also express them but on a smaller scale (Raunio *et al.*, 1995a), these include tissues such as lung, intestine, brain, skin, placenta, etc. (Oinonen and Lindros, 1998). Recent studies profiling 40 CYP P450 genes using reverse-transcriptase polymerase chain reaction-based (RT-PCR) assays in mouse (Choudhary *et al.*, 2003) shows that while the liver and kidney expresses most of them, the lung expressed a significant 24 of these genes. Since the liver acts as a port of entry for all ingested substances, the high concentration and diversity of CYPs

present in relation to its function is self-explanatory. Tissues such as lung and skin also play critical roles as the first lines of defense against exogenous substances, and this partly explains the presence of the CYP enzymes, albeit at lower levels.

Nakajima (1997) reports that expression of many CYPs during fetal development are low in most animals but increases swiftly after birth. Also certain CYPs, for example CYP2C11 are regulated developmentally in a sex-specific fashion (Waxman, 1988). It has been reported that levels of CYP2E1 and CYP2C11/6 are slightly lower during pregnancy (Nakajima, 1997). Other factors such as sex (Thomas *et al.*, 1987), nutrition (Nakajima and Sato, 1984) and species differences (Kato, 1979) also influence the level at which certain P450 isoforms are expressed.

1.3.5.1 Cytochrome P450 2A6 (CYP2A6)

Cytochrome P450 2A6 (CYP2A6) belongs to one of 10CYP2 subfamilies found in humans (Maurice *et al.*, 1991; Fernandez-Salguero and Gonzalez, 1995). In rat CYP2A3 gene (rat ortholog) is expressed in lung but it is not expressed in liver tissue (Ueno and Gonzalez, 1990). Its most potently known inducer is coumarin (Pelkonen *et al.*, 2002), but it also metabolizes toxic exogenous substances such as nicotine (Nakajima *et al.*, 1996), 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK) (Yamazaki *et al.*, 1992), *N*-nitrosodiethylamine (NDEA) (Yamazaki *et al.*, 1992), and alkoxyethers (Hong *et al.*, 1999; Le Gal *et al.*, 2001). Recent studies by Le Gal *et al.* (2003) shows that coumarin and nicotine can effectively be used as prototype probes for CYP2A6 phenotyping since both these substrates displayed very high affinities for this enzyme.

CYP2A6 is a genetically polymorphic enzyme in which 17 allelic variants have been identified and characterized *in vitro* and *in vivo*, to date (Sellers *et*

al. 2003). These variants may result in individuals having altered phenotypes with either a reduced or elevated ability to metabolize nicotine (Xu *et al.*, 2002). In ethnic and interethnic groups, the frequency of its allelic variants has been shown to vary as well (Xu, *et al.*, 2002; Tyndale, *et al.*, 2002). For example, African Americans have a noticeably lower clearance rate of cotinine and metabolic clearance of nicotine to cotinine than Caucasians (Benowitz *et al.*, 1999; Perez-Stable *et al.*, 1998). Research also shows that the metabolic ratio of cotinine to nicotine is higher in the Korean population than in that of the Japanese (Kwon *et al.*, 2001). The alterations in the rate at which CYP2A6 metabolizes its substrates might thus affect smoking behavior, incident rate of tobacco-related cancers and affectivity of nicotine replacement therapy (Sellers *et al.*, 2003).

It has been hypothesized that individuals who are carriers of null or defective CYP2A6 allele(s) and thus have a lower rate at which they metabolize nicotine (slow metabolizers [SM]) have a decreased risk of becoming tobacco-dependent (Sellers *et al.*, 2003). Since the SM also maintain constant levels of nicotine longer than extensive metabolizers (subjects with two functional copies of the CYP2A6 [EM]), they would probably smoke less than fast metabolizers (FM) (Sellers *et al.*, 2003). Furthermore, individuals carrying one or more of the defective CYP2A6 alleles may be at a lower risk of getting lung cancer since they firstly have the characteristics described above and they have a decreased ability to biotransform certain tobacco-specific pro-carcinogens (Sellers *et al.*, 2003).

1.3.5.2 Cytochrome P450 2B6 (CYP2B6)

CYP2B6 belongs to the CYP2B family that metabolizes various drugs, xenobiotics and steroids (Lewis and Lake, 1997). In rat liver phenobarbital induces CYP2B1 (rat ortholog) by 50-fold (Waxman and Azaroff, 1992).

CYP2B1 is also known to extensively metabolize nicotine (Hammond *et al.*, 1991; Miksys *et al.*, 2000). However, this induction is found to occur in rat brain but not in rat liver (Miksys *et al.,* 2000) in which the induction is not only brain region specific but cell specific too. In humans, CYP2B6 is predominantly expressed, extrahepatically and has the ability to metabolize nicotine (Flammang *et al.*, 1992). Results obtained in rats using R-T PCR, immunocytochemistry and immunostaining suggests that brain CYP2B1 is regulated at the mRNA level (Miksys *et al.* 2000). However, the molecular events involving the activation of transcription are not clear (Oinonen and Lindros, 1998).

Metabolic reactions of both CYP2B1 and CYP2B2 can cause the biotransformation of tobacco-specific nitrosamines *N*-nitrosodimethylamine (NDMA) (Stiborova *et al.,* 1996) and 4-(methylnitros-amino)-1-(3-pyridyl)-1-butanone (NNK) (Code *et al.*, 1997). In humans, nicotine is extensively metabolized via C-oxidation to cotinine (Benowitz and Jacob III, 1987). This alkaloid is metabolized mainly in the liver and to a lesser extent in other tissues such as the lung (Van Gilder *et al.*, 1997). 70-80% of the nicotine is typically converted to cotinine and then consequently converted to trans-3'-hydroxycotinine (Benowitz and Jacob III, 1994).

Nicotine C-oxidation is predominantly catalyzed by CYP enzymes in which the 5' position is hydroxylated to yield an unstable intermediate, 5'-hydroxynicotine that exists in equilibrium with nicotine-1'(5')-iminium ion (Benowitz and Jacob III, 1994). Cytosolic aldehyde oxidase then catalyzes the oxidation of the intermediate to cotinine (Brandlänge and Lindblom, 1979).

In the 1960s, McKennis and co-workers (KcKennis, 1965; McKennis *et al.*, 1964) proposed that further metabolism of cotinine, via 5'-hydroxylation of nicotine would yield 4-oxo-4-(3-pyridyl) butanoic acid (keto acid) and 4-hydroxy-4-(3-pyridyl) butanoic acid (hydroxy acid). However, recent analysis

of the urine of smokers indicated that the keto acid and hydroxy acid only accounted for 10-15% of urinary nicotine metabolites (Hecht *et al.*, 1999) in comparison to the 70-80% nicotine to cotinine conversion. Furthermore, it was also determined that keto acid and hydroxy acid in total only accounted for less than 0.5% in the urine of non-smokers dosed with cotinine (Hecht *et al.*, 2000).

Hecht and co-workers (2000) hypothesized that the actual source of keto acid and hydroxy acid was via 2'-hydroxylation of nicotine. This pathway would yield 2'-hydroxynicotine which then spontaneously forms nicotine-1'(2') iminium ion and 4-(methylamino)-1-(3-pyridyl)-1-butanone (amino-ketone). Amino-ketone could ultimately be converted to keto acid by keto aldehyde.

Amino-ketone is the direct precursor to the tobacco-specific lung carcinogen 4-(methylnitros-amino)-1-(3-pyridyl)-1-butanone (NNK) which can be formed by simple nitrosation (Caldwell *et al.*, 1993). These findings, although quantitatively less important than 5'-hydroxylation provide a potentially significant link between nicotine metabolism and lung cancer in mammals.
Tobacco smoke analysis has revealed the presence of several carcinogenic chemicals that have been implicated in respiratory diseases, including lung cancer (Hecht, 1996). The nitrosamines reported to cause cancer include 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK), 4-(methylnitroamino) 1-(3-pyridyl)-1-butanol (NNAL) and N'-nitrosonornicotine (NNN) (Hoffman and Hecht, 1985; Hecht and Hoffman, 1988; Hecht, 1996). In order for these chemicals to evoke their carcinogenic potentials in mammals they require metabolic activation by P450 enzymes.

1.3.5.3 Metabolism of Nicotine by CYP2A6 and CYP2B6

In humans, 5'-hydroxylation of nicotine is predominantly catalyzed by CYP2A6 (Messina *et al.*, 1997). CYP2B6 has also shown to be involved in

nicotine C-oxidation (Yamazaki *et al.*, 1999), particularly at high substrate concentration. Recently Hecht *et al.* (2000) showed that CYP2A6 also catalyzes 2'-hydroxylation of nicotine to yield substantial amounts of aminoketone (a lung cancer precursor). Both CYP2A6 and CYP2B6 play a major role in the activation of NNK (Hecht, 1998; Code *et al.*, 1997) and a reduced risk of lung cancer has been associated with deletions of its gene (Miyamoto *et al.*, 1999). It has been suggested that these two CYP2 isoforms are coordinately regulated in *Homo sapiens*, particularly since their respective genes occupy similar chromosomal locations (Gonzalez, 1992). CYP2A6 and CYP2B6 are expressed in lung tissue and are suspected of playing critical roles in tobacco-related biological effects in humans (Yamazaki *et al.*, 1999).

1.3.5.4 Cytochrome P450 1A1 (CYP1A1)

Also of interest to this investigation is a dominant extrahepatic CYP enzyme called CYP1A1 (Raunio *et al.*, 1995) involved in the toxicity of a variety of carcinogens, particularly polycyclic aromatic hydrocarbons (PAHs) (Shimada *et al.*, 1996). CYP1A1 is one of two members of the CYP1A family essentially expressed at low levels in extrahepatic tissues, but induction by aromatic (aryl) hydrocarbon receptor (AhR) ligands has been demonstrated in tissues such as lung, lymphocytes, mammary glands and placenta (Raunio *et al.*, 1995a). The CYP1A1 gene displays low constitutive expression and high inducibility. Toxicological analysis of the environmental toxicant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) revealed that this chemical is its most potently known inducer (Whitlock, 1999).

Two genetic polymorphisms has been detected, in which the first mutation was observed at the 3' flanking region of the gene (Kawajiri *et al.*, 1990) and a second located in exon 7 where isoleucine amino acid is substituted for valine amino acid (Hayashi *et al.*, 1991). Both these variant alleles are more common

in Asians than in Caucasians, suggesting that the former have a higher risk of lung cancer than the latter.

Induction of CYP1A1 is mediated by two regulatory proteins, AhR and AhR nuclear translocator (Arnt). The induction process is rapid and the regulatory proteins pre-exist within the cell. Mechanism of induction basically entails the binding of the inducer to the AhR which then enters the cell nucleus and heterodimerizes with Arnt to produce a DNA-binding transcription factor. The AhR/Arnt heterodimer then binds to the enhancer chromatin. An initation complex forms at the promoter, and transcription follows (Whitlock, 1999).

CYP1A1 is also highly inducible by cigarette smoke (Anttile *et al.*, 1991; Whitlock, 1999). Oxygenation by CYP1A1 induced by PAHs in tobacco smoke generates arene oxide that can create mutations leading to neoplastic tranformation. Thus polymorphisms in CYP1A1 activity may be linked with different susceptibilities to smoking-induced lung cancer. The polymorphisms could entail changes in gene expression or enzyme activity (Whitlock, 1999).

In lung, CYP 1A1 appears to be expressed predominantly at the epithelium of the peripheral airways and does not continue any further than the epithelium of bronchi greater than 1mm in diameter (Anttile *et al.*, 1991). Studies indicate that this CYP is not expressed in alveolar macrophages (Anttila *et al.*, 1991; Piipari *et al.*, 2000). Immunohistochemical studies show that induced CYP1A1 is also detected at low levels in type II pneumocytes (Forkert *et al.*, 1996)

Studies by Wei *et al.* (2002) and Iba *et al.* (1998) report that CYP1A1 is induced by nicotine. In contrast to their findings, Le Gal *et al.* (2003) and Nakayama *et al.* (1993) have found that nicotine does not induce this cytochrome.

1.3.5.5. Formation of Reactive Species by CYP P450 Functional Reactions

Although the primary purpose of functional reactions is detoxification, many of their substrates are transformed into reactive intermediates that may have far more structural and functional adverse effects than their parent compounds (Oinenon and Lindros, 1998). A proposed reaction pathway that may contribute to a significant increase in reactive species involves CYP P450-catalyzed hydroxylation pathways that result in the formation of multiple electrophilic oxidant species produced in the natural course of CYP P450 reactions (Newcomb *et al.*, 2003). The pathway of reactions starts with the reversible binding of the CYP to the substrate, resulting in the lowering of the iron (II)-enzyme's reduction potential. P450 reductase then transfers an electron to the iron (II) species and subsequently oxygen reversibly binds to produce a superoxide-iron species. A second reduction step occurs to give the peroxo-iron species where oxygen is in the formal oxidation-state of hydrogen peroxide. The reactions following the formation of the second intermediate is fast and intermediates do not accumulate under natural conditions.

The peroxo-iron species is protonated on the oxygen and produces the hydroperoxo-iron intermediate. Alternatively, protonation on the proximal oxygen forms iron-complexed hydrogen peroxide, which can dissociate. The latter reaction is reversible, and the P450 enzymes can be shunted with hydrogen peroxide to give an active oxidant (Nordblom *et al.*, 1976, Coon, 2003). Thus oxygen reduction not only leads to substrate hydroxylation but also results in the release of superoxide and hydrogen peroxide (Coon, 2003).

Nicotine is metabolized by CYP2A6 (Messina *et al.*, 1997) and 2B6 (Yamazaki *et al.*, 1999) and these hydroxylation reactions produce superoxides and hydrogen peroxides (Coon, 2003). It is, therefore plausible that maternal nicotine exposure during pregnancy and lactation results in the induction of rat orthologs CYP2A3 and 2B1. Moreover, hydroxylation

reactions may result in the formation of reactive oxidant species that could contribute significantly to damage at both the structural as well as genetic level in the lungs of developing neonates.

1.4 Research Approach and Rationale

Maternal nicotine exposure during pregnancy and lactation results in many adverse structural and metabolic changes of developing neonatal rat lung.. Structural alterations include fewer alveoli than in the lungs of control rat pups with fenestrations in the walls of the alveoli, described as emphysema-like lesions (Maritz *et al.,* 1994). Also thickening of the walls of type 1 pneumocytes and the endothelial cells are observed, thus demonstrating thickening of the alveolar-blood barrier and degeneration of type I pneumocytes (Maritz *et al.,* 1994). Maternal nicotine exposure also results in impaired elastic tissue synthesis (Maritz and Woolward 1992). Studies by Maritz and Windvogel (2003) indicate a retarded rate of secondary septal formation and that alveolar formation in the lungs of these nicotine-exposed pups is slower than in the control lungs (Maritz *et al.*, 2000).

The metabolic alterations demonstrated in 1983 by Maritz, show that nicotine interferes with carbohydrate metabolism in adult rat lung. Further studies show that maternal nicotine exposure leads to a significant increase in total glucose turnover with a markedly lowered *in vitro* lactate production (Maritz, 1986). These findings suggest that exposure of nicotine to developing lung during gestation and lactation results in the hindrance of glycolysis in developing neonatal lung, giving rise to the possibility that nicotine compromises the integrity of a developing lung. Additional findings show that there is a significant increase in the total adenine nucleotide pool and the ATP/ADP and ATP/ADP+AMP ratios (Maritz and Burger, 1992), which after 4 weeks of withdrawal still remained significantly elevated. Furthermore, Maritz (1997) shows that maternal nicotine exposure, results in the inhibition of activity of

the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GADPH), an oxidant sensitive protein, during the critical phase of alveolarization.

Studies suggest that nicotine metabolism by CYP P450 enzymes may result in significant levels of reactive oxygen species to which GAPDH is extremely sensitive to (Coon, 2003). Thus, nicotine metabolism itself may contribute substantially to the inhibition of the glycolytic pathway observed in the lungs of rat pups exposed during gestation and lactation. Moreover, Maritz (1986) shows that maternal nicotine exposure during gestation and lactation results in an increase in glucose turnover. There is a possibility that maternal nicotine exposure results in increased CYP P450 activity in the lungs of the offspring and thus this tissue would require more glucose utilization and consequently NADPH formation via the Hexose Monophosphate Pathway to support the increased CYP P450 activity (Fig.2). If this is true, it will explain the increase in glucose turnover in the nicotine-exposed lungs, albeit the inhibition of glycolysis.

Since it is strongly suggested that carbohydrate metabolism is vital for the structural (Tierney and Levy, 1976) and functional development and growth of fetal and neonatal lung tissue (Gilden *et al.*, 1977; Maniscalco *et al.*, 1978; Bourbon and Josh, 1982), interference with carbohydrate metabolism will undoubtedly affect the integrity of the developing tissue.

In an attempt to elucidate the mechamisms involved in the increased glucose turnover despite the inhibition of the glycolytic pathway (Maritz, 1983; 1986) and the inhibition of GAPDH activity (Maritz, 1997), selected enzymes in the glycolytic pathway and in the zenobiotic metabolism of nicotine were analysed during neonatal lung development.

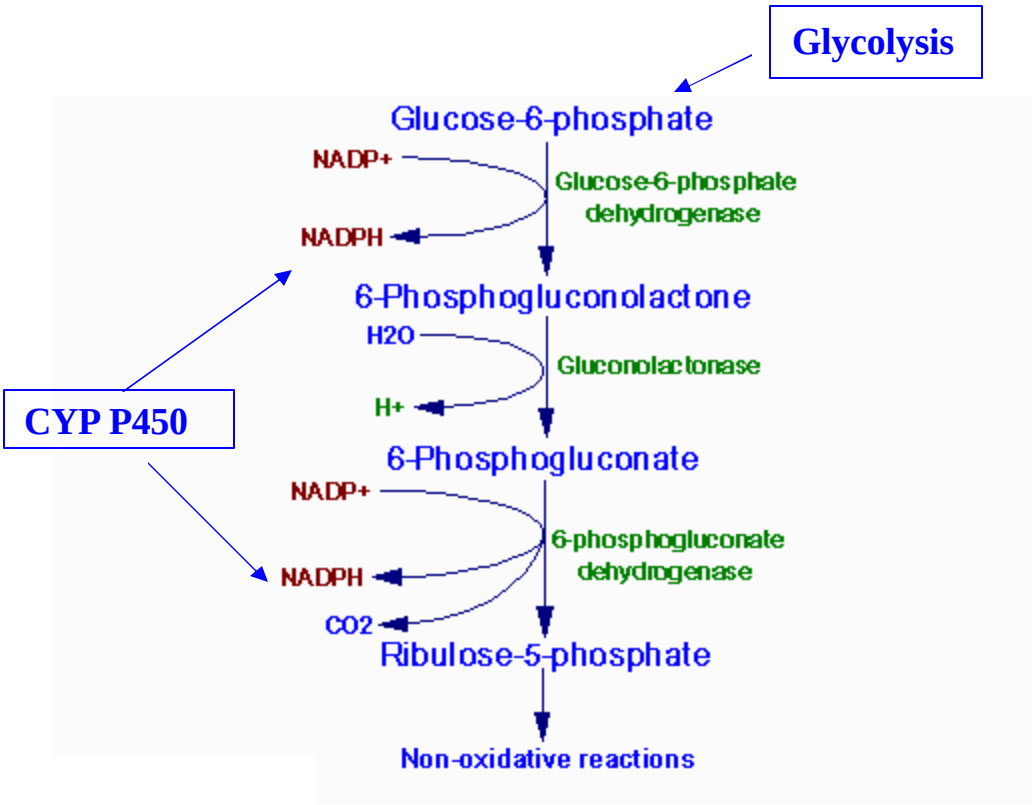


Fig.2. Schematic diagram of the Hexose monophosphate Pathway.

This study has two main areas of investigation to:

(1) Establish the isoenzyme patterns and mRNA expression of glycolytic enzymes HK, PFK, GAPDH and LDH in (a) normal developing rat lung. This entails the quantitative analysis of the isoforms of the selected glycolytic enzymes, and quantifying the expression of these enzymes at the mRNA level and (b) determine the effects that maternal nicotine exposure during gestation and lactation may have on these selected glycolytic enzymes in the lungs of the offspring.

(2) Investigate the expression of rat orthologs CYP1A1, CYP2A3 and CYP2B1 of the human CYP1A1, 2A6 and 2B6 at the mRNA level in, (a) the developing lungs of normal postnatal rats, (b) determine the effect that mater

nal nicotine exposure during gestation and lactation has on the expression of these CYPs and the possible implications. This may provide a better understanding for the inhibition of GAPDH during the critical phase of lung development and the increase in glucose turnover despite a reduced glycolytic activity.

To date, no data with regards to development of the isoenzyme patterns and transcript levels of glycolytic enzymes (HK, GAPDH, LDH, PFK) and mRNA expression of CYP P450 enzymes (CYP2A6, 2B6, 1A1) in normal postnatal rat lung has been reported. Moreover, the influence of maternal nicotine exposure during pregnancy and lactation on expression of these enzymes have not been documented either.

CHAPTER 2

2. Materials and Methods

2.1 Sample Selection and Management

Virgin female rats (Wistar descendents) of 200-250 g are selected. Throughout the experiment their diet is comprised of Epol rat cubes and they receive their food and water as required. A day-night cycle of 12 hours is maintained and the room temperature is kept at 22ºC.

A breeding program maintained by the Department of Physiological Sciences, UWC is employed. The animals are allowed to copulate overnight and the length of gestation is averaged at 22.5 days. The animals remained undisturbed at least up to the fourth day after mating, to ensure blastocyte implantation and initial embryonic growth. After which the rats are subcutaneously injected with nicotine (1mg/kg body weight/ day).

2.2 Determination of Isoenzyme Patterns in Selected Glycolytic Enzymes

2.2.1 *Tissue Preparations*

Tissue samples weighing approximately 20 mg are obtained from the lungs of 1, 7, 14, 21, and 49-day old pups, from both control and experimental group. The 1 and 7 day old pups are sacrificed by means of decapitation and the 14, 21 and 49-day old pups are overdosed with saggital (sodium pentobarbitone) by intraperitoneal injection (1mg/kg body weight/day).The tissue is immediately stored in 1.5 ml polypropylene tubes at –70ºC until required for analysis.

The frozen tissue is homogenized using a small glass rod in 100 µl of extraction buffer (0.1 M Tris-Cl, 0.001 M NaEDTA, pH 7.0, 0.25% Triton-X 100). The tissue is manually homogenized for 1 minute and allowed to stand for 30 minutes on ice. This ensures that an assayable quantity of enzyme is exposed. The homogenate is centrifuged for 3 minutes at 15 000g, using the Eppendorf Centrifuge 5414S. The supernatant is removed and placed in a fresh tube and stored at -70ºC for protein and isoenzyme analysis.

2.2.2 Protein Analysis

The final data is expressed in % Density/mg Protein. A direct spectrophometric technique for the protein analysis is used. 5 µl of supernatant is added to 1 ml of distilled water (1:200 dilution). The procedure is based on the Warburg-Christian Technique (Layne, 1957):

$$1.55\ (A_{280}) - 0.755\ (A_{260}) = \text{mg protein / ml}$$

2.2.3 Polyacrylamide Gel Electrophoresis (PAGE) Technique

The separation of isozymes using PAGE techniques is achieved principally because the isoforms differ in molecular weight. It is common to first extract and purify the enzyme of interest. Their isoforms are then separated on an (sodium dodecylsulphate) SDS-polyacrylamide gel, using one of many staining methods, for example silver staining which produces bands of high resolution and are easily quantified. The SDS however, denatures the enzymes and the proteins analyzed in this investigation should remain viable. Thus a technique using SDS is not suitable for this study.

In this investigation the isoenzymes separated on PAGE gels are extracted from crude tissue, which allows the researcher to analyze the individual patterns of non-degraded enzymes. Based on the reaction catalyzed by the enzyme isoforms, staining techniques are then designed. The separation of

phosphofructokinase (PFK) isoforms proved difficult since working with crude extract makes separating the PFK bands from other proteins in the crude tissue nearly impossible and it diminishes the quality and the efficiency with which the bands are separated. Thus separating PFK in crude extract on polyacrylamide gels did not present with bands that are quantifiable in this study. This is also documented by Zeitschel *et al.* (1996) when they reported that it was impossible for them to separate the isoenzyme bands in crude tissue, because they could not separate the bands efficiently enough from other proteins even when using silver stained PAGE gels.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not present in multiple forms in rat and exists in vivo as a dimer or a monomer, but its glycolytic function is restricted to its tetrameric form (Mazzola and Sirover, 2003). Since it only exists as a single isoform and its activity has been analyzed in developing neonatal rat lung and the influence of maternal nicotine exposure during pregnancy and lactation has also been investigated in the developing lung (Maritz, 1997), PAGE analysis of this enzyme was therefore not performed.

2.2.3.1 Lactate Dehydrogenase (LDH) isoenzyme patterns analysis

Since LD-1 is cathodic and LD- 2, 3, 4 and 5 are anodic, two vertical acrylamide gels are prepared per sample group. LD-1 is electrophoresed on a 4% gel, in which the polarity of the terminals is reversed. LD- 2, 3, 4 and 5 are electrophoresed on a 5% gel (Fig.3). The gel is prepared to a volume of 40 ml (40% Acrylamide/Bisacrylamide solution 29:1, 10X (tris, borate, EDTA) TBE, distilled water, N,N,N',N'-Tetramethylethylenediamine (TEMED), 10% Ammonium persulfate). 1X TBE (pH8.3) is used as running buffer.

The samples are kept on ice at all times before being loaded into the gel wells. 10 µl of loading buffer (30% glycerol, 4 mg/ml Napthol blue-black) is added to the supernatant and pulse spun.

8 µl of sample is loaded into the wells of the 4% gel and electrophoresed overnight at 8 mA. 20 µl of sample is loaded into the wells of the 5% gel and electrophoresed for 3 hours at 15 mA.

The LDH isoenzyme staining recipe is based on the revised work of Grant (1994): 0.1 M Tris-Cl pH 8, 0.01 M nicotinamide adenine dinucleotide (NAD^+), 10% Lactate pH 7.5, 1.0% methylthiazoletetrazolium (MTT), 1.0% phenazine methosulphate (PMS). The gel with its liquid overlay is incubated for 30 minutes in the dark at 37ºC.

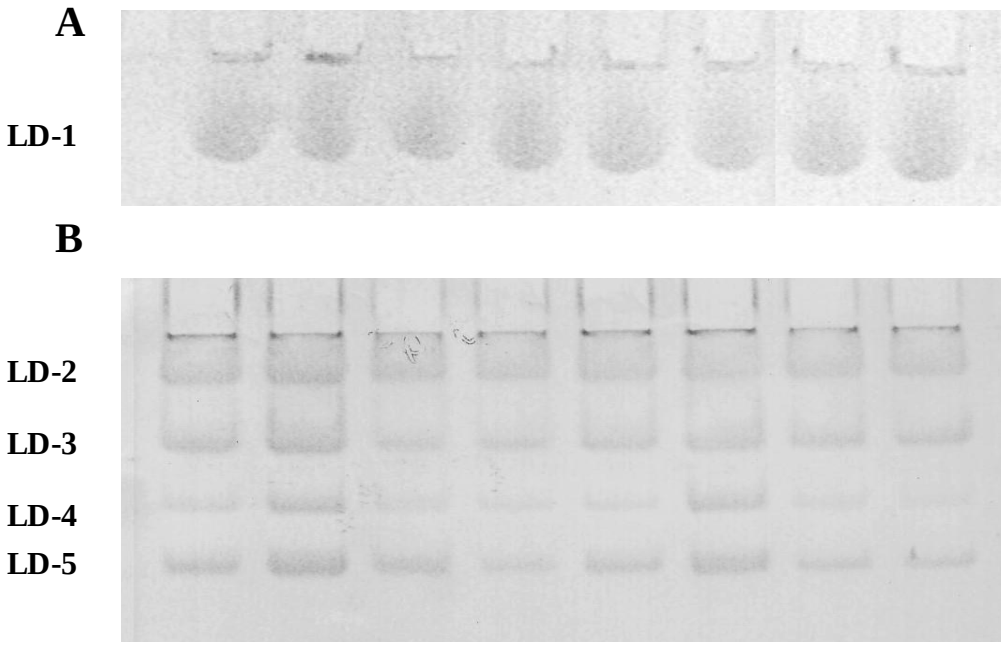


Fig.3. LDH isoenzymes of a 49 day old control rat lung run on a polyacralamide gel. LD-1 is run on a 4% gel (A) and LD-2, 3, 4, and 5 is run on a 5% gel (B).

2.2.3.2 Hexokinase (HK) isoenzyme patterns analysis

HKI, II, and III are very similar in moleculer weights, about 100 KDa. Separating these isoforms is easy when using a stacking-PAGE gel. Usually a stacking gel has two layers with the top layer having a lower gel percentage than the bottom layer. This protocol however, utilized a reversed stack gel in which the top layer has the higher gel percentage than the bottom. A single PAGE gel is thus made that contains to layers of differing percentages. The topmost layer is an 8% gel and the lower layer is a 6% gel. This ensures that the first isoform to reach the 6% layer during electrophoresis would then distinctly separate from the other two forms, since the isoform in the 6% gel would move at a faster pace that it would in the 8% gel (Fig.4).

The top PAGE gel is prepared to a volume of 10 ml and the bottom gel to a volume of 40 ml (40% Acrylamide/Bisacrylamide solution 29:1, 10X TBE, distilled water, TEMED, 10% Ammonium persulfate). 1X TBE -Na_2HPO_4 (pH 8.4) is used as running buffer.

The samples are kept on ice at all times. 10 µl of loading buffer (30% glycerol, 4 mg/ml Napthol blue-black) is added to the supernatant and pulse spun. 15 µl of sample is loaded into the wells of the top-layered 8% gel and electrophoresed for 3 hours at 40 mA (160 V).

The staining protocol is based on studies by Allen *et al.* (1998): 0.1 M Tris (pH 8), 0.5 mM EDTA, 10 mM $MgCl_2$, 20 mM ATP, 2 mM NAD^+, 3.2 U G6PDH, 1.0% MTT, 1.0% PMS. In their staining method, Allen *et al.* (1998) uses either 0.5 mM or 100 mM glucose. In this study we used 50 mM as used by (Reid and Masters, 1985). The gel with its liquid overlay is incubated overnight in the dark at 37ºC.

HKI

HKII

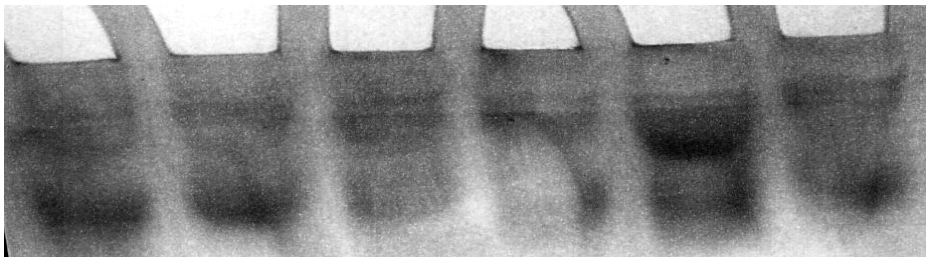


Fig.4. HKI and HKI of a 49-day old rat run on a reverse stacking PAGE gel.

2.2.4 Scoring of Polyacrylamide (PAGE) gels and Statistical Analysis

All the PAGE gels are scored on a white illuminator (Ultra violet products (UVP) Inc).

The isoenzymes are expressed in % density / mg protein. In order to normalize for loading inconsistency, the % density is expressed relative to the individual sample loaded and to the total protein content which is analogous to using an internal control in a northern hybridization. Densitometry is performed by utilizing the UVP system's Image Store 5000 and final calculations are as follows:

$$\% \text{ Density/mg Protein} = \frac{\text{Average \% of Band Density}}{[\text{Amount of Sample Loaded (ml)}][\text{Total Protein (mg/ml)}]}$$

Statistical outliers are determined by using the Box and Whisker plots and then removed from the final data. The final data is analyzed statistically using the Wilcoxin unpaired T-test, in which $P<0.05$ is designated as significant.

2.3 Analysis of mRNA expression

2.3.1 RNA Isolation Procedure

A ready-to-use reagent, TRIZOL LS Reagent (U.S. Patent No. 5,346,994) provides a simplistic method for the total extraction of RNA from rat lung tissue. The technique performs well when using small quantities of tissue (50-100 mg) and allows for the simultaneous processing of a large number of samples.

The following reagents (not provided) are also required for the procedure:

- Chloroform
- Isopropanol
- 75% Ethanol (in 0.01% (diethylperoxycarbonate) DEPC-treated water)
- 0.5% sodium dodecyl sulphate (SDS) solution (in 0.01% DEPC-treated water)

Protocol:

The procedure is carried out at 15 to 30°C (room temperature).

Homogenization:

Tissue samples (50-100 mg) are homogenized in 0.75 ml TRIZOL LS Reagent in 1.5 ml polypropylene tubes.

a. Phase Separation

The homogenate is incubated for 5 minutes at room temperature allowing for the complete dissociation of nucleoprotein complexes). 0.4 ml chloroform is then added. The tubes are securely closed and vigorously shaken. The samples are allowed to incubate for 10 minutes at room temperature and then centrifuged at no more than 12 000g for 15 minutes at 2 to 8°C. (The addition of

chloroform, followed by centrifugation, separates the solution into a lower phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The RNA is found exclusively in the aqueous phase).

b. RNA Precipitation

The aqueous phase (approximately 70% of the original volume) is transferred to a clean tube and 0.4 ml chloroform is again added. The solution is centrifuged at no more the 12 000g for 2 minutes at room temperature. The upper aqueous phase is again removed and placed in a clean tube. Addition of another, 0.4 ml chloroform ensures the removal of residual phenol in the aqueous phase followed by centrifugation. 0.5 ml isopropanol is added to the aqueous phase, to precipitate the RNA. The solution is incubated for 15 minutes at room temperature and then centrifuged at no more than 12 000g for 10 minutes at 2 to 8°C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

c. RNA Wash

The isopropanol is carefully poured off, leaving the RNA pellet sticking to the bottom of the tube. 1 ml of 75% ethanol is added and vigorously shaken to remove salts. The samples are then centrifuged at no more than 7 500g for 5 minutes at 2 to 8°C. The 75% ethanol is then carefully poured off.

***d.* Re-dissolving the RNA**

The RNA pellets are air-dried for 10 minutes. Taking care not to dry out the pellets completely as this will significantly decrease its solubility). The pellet is dissolved in 0.2 ml 0.5% SDS solution. Samples are lightly shaken and then incubated for 10 minutes at 55-60°C to ensure complete dissolution of the RNA.

e. Precautions for Prevention of RNAse Contamination

Since RNAses can easily be introduced into the RNA preparations and since their activities are difficult to inhibit, the following precautionary measures are taken:

- Powder-free, disposable gloves are always worn. (Bacteria and molds are present in skin, which may lead to contamination).
- Sterile, disposable plastic-ware is used and automatic pipettes are reserved for RNA work to prevent cross contamination.
- Glass items are baked at 150°C for 4 hours.
- Plastic items are soaked in 0.5 M (sodium hydroxide) NaOH, rinsed and autoclaved.

2.3.2 Spectrophometric Analysis of mRNA

10 µl of the RNA preparation is added to 0.99 ml distilled water. Readings are taken at A_{260}. The RNA (µg/ml) is quantified using the following equation:

$$\text{Reading at } A_{260} \times 40 \times 100$$

(Laboratory Practical Manual, Department of Biochemistry, UWC)

2.3.3 Concentrating the RNA to 1 mg/ml

250 µl isopropanol is added to the 190 µl SDS suspension. The solution is incubated for 20 minutes at -70°C. It is then centrifuged for 10 minutes at 12 000 g, resulting in the formation of the RNA pellet. The isopropanol is carefully poured off. The pellets are washed with 1 ml 75% ethanol and then centrifuged for 5 minutes at 7 500 g. The RNA is re-suspended in the appropriate volume of 0.5% SDS.

To obtain a final concentration of 1 µg/µl, the following calculations are made:

$$\frac{[190\ \mu l\ (\text{Remaining volume})]\ [\ \mu g/ml\ (\text{Original concentration})]}{1000}$$

The solutions are then incubated at 65°C for 10 minutes. To ensure dissolution of RNA.

2.3.4 Determination of Intact RNA

To ensure that the RNA used in the study is intact, 1 µg RNA is loaded onto a 0.1% agarose gel stained with 3 µl ethidium bromide and run for 30 minutes at 200 V. The gel is viewed on UVP system's Image Store 5000 to ensure the presence of the 18S and 28S bands.

2.3.5 Oligonucleotides used for Hybridization

The probes' sequences are summarized in Table 1 and Table 2. ß-actin is used as the internal standard.

Table 1: Glycolytic Enzyme Probes used for hybridization

Gene	Oligonucleotide Sequence	Genebank Accession #
		Rat
ß-ACTIN	5'CATGGCTGGGGTGTTGAAGGTCTCAAACAT'3	NP_1124 04
HKI	5' CCTCTTCACCGCATCCCTCAGTAAGGAGGC 3'	NM_012734
HKII	5' GCTCCGTGAATAAGCAGGCGATCATATGCG3'	NM_012735
HKIII	5' AGCCATGCACACGCGCTGCACGAGCTCGA 3'	NM_022179
PFK-M	5' CCTCCCAGCTGGAGCATCATGGACACGCTC 3'	NM_013715
PFK-L	5' CCACGGAGCTGGATGGCCTCGTCAAACCTC 3'	NM_013190
PFK-C	5' TCATTTCTGAGGACAAGGCCCCTCTGGATG 3'	L25387
GAPDH	5' GCTGGCATTGCTCTCAATGACAACTTTGTG 3'	NM_017008
LDH-M	5' GACGCTGAGGAAGACATCCTCCTTGATTCC 3'	NM_017025
LDH-H	5' ATGACTTCATAGGCACTGTCCACCACCATC 3'	NM_012595

The mRNA sequences used to design the oligonucleotide probes are extracted from the following web site:http://www.ncbi.nlm.nih.gov/

Table 2: Cytochrome (CYP) P450 Probes used for hybridization

Gene	Oligonucleotide Sequence	Genebank Accession #
		Rat
ß-ACTIN	5'CATGGCTGGGGTGTTGAAGGTCTCAAACAT'3	NP_1124 04
CYP1A1	5'GAGGCTATGGAGAAACTCTTCAGCG 3'	M26129
CYP2A6	5'ACCAAGTTCTTCATGTAGAACTCAG 3'	M33190
CYP2B6	5'GCAGATGATGTTGGCTGTGATGCAC 3'	M26129

CYP2A3 and CYP2B1 expressed in rat is closely related to CYP2A6 and CYP2B6 respectively, as expressed in humans (Murphy *et al.*, 2000; Czekaj *et al.*, 2000).

2.3.6 Oligonucleotide Labelling

All of the probes are labeled using the Gene Images ECL 3' -oligolabelling and detection system (Amersham, RPN 2130 and RPN 5770). Oligonucleotide probes are labeled at the 3'-end with flourescein-dUTP catalyzed by terminal deoxynucleotidyl transferase and the procedure is followed according to the standard protocol provided.

Protocol:

All components required for labelling are kept on ice. The following labeling reaction reagents are added to a 1.5ml polypropylene tube in this specific order:

Oligonucleotide ($100x10^{-12}$ moles)	x µl
Fluorescein-11-dUTP	5 µl
Cacodylate buffer	8 µl
Water	y µl

Terminal transferase	8 µl
Total	80 µl

The volumes corresponding to x and y are adjusted so that the total reaction volume is 80 µl.

The solution is gently mixed by pipetting up and down in the pipette tip. The reaction mixture is then incubated for 90 minutes at 37°C. The mixture is then placed on ice for 5 minutes after which it is stored at -20°C until required.

2.3.7 Membrane Blotting

To ensure an even diameter of the blots, an apparatus was devised to help reduce and equalize the blot sizes. The box consists of equal sized holes to which a piece of piping is connected. Using a vacuum system, the appropriate size membrane is placed on this box. When the suction system is operational, the membrane automatically adheres to the box and the location in which to blot is clearly shown as a result of the indentations caused by the vacuum system at the specific holes.

The smaller the blots, the more accurate the results. 4 µl of mRNA is blotted (2 mg/µl)

2.3.8 Hybridization

Rapid-hyb buffer (Amersham, RPN 1636) provides a time efficient method of hybridizing. Oligonucleotides only require 60 minutes of hybridization at 42°C.

The membrane is placed into 20 ml pre-heated hybridization buffer. The volume of the buffer is equivalent to 0.25 ml/cm^2 of the nylon membrane. Prehybridization for 30 minutes at 42°C is carried out in the Techne HB-1D

Hybridizer. 16 µl of labelled probe, equivalent to 5 ng per ml of hybridizing buffer is added. It is then further hybridized for another 30 minutes at 42°C.

2.3.9 Washing the Membrane

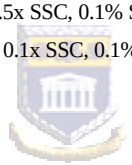
The following reagents are required:

a. 20x SSC: Na_3 citrate 0.3 M
NaCl 3 M

b. Sodium dodecylsulfate (SDS)

It is at this stage that the stringency of the hybridization is regulated.

The blotted membrane is removed from the hybridizing solution and placed into a clean container. It is then covered with 50 ml 5x SSC, 0.1% SDS and agitated at room temperature for 15 minutes. The solution is then poured off. The membrane is covered with 50 ml pre-warmed 0.5x SSC, 0.1% SDS and agitated at 42°C for 15 minutes. This stringency wash buffer is replaced with 50 ml pre-warmed 0.1x SSC, 0.1% SDS and the membrane is further agitated at 42°C for another 15 minutes.



2.3.10 Membrane Blocking, Antibody Incubations and Washes

The following reagents are required:

a. Buffer 1: NaCl 0.15 M
Tris base 0.1 M
Adjusted to pH 7.5 and made up to 1 litre.

b. Buffer 2: NaCl 0.4 M
Tris base 0.1 M
Adjusted to pH 7.5 and made up to 1 litre.

c. Bovine serum albumin (BSA) fraction V

d. Liquid Block (supplied)

e. Anti-flourescein HRP conjugate (supplied)

All the procedures are performed at room temperature and all incubations require agitation.

The membrane is placed in a clean container and then rinsed with 360 ml buffer 1 for 1 minute (2 ml buffer for each cm^2 of membrane). The buffer is discarded and 40 ml block solution is added. A 20-fold dilution of liquid block in buffer 1 is made up. The amount of solution is equivalent to 0.25 ml/cm^2 of membrane. The membrane is then incubated for 30 minutes. The block solution is discarded and the membrane is then rinsed with 360 ml buffer 1 for 1 minute. The buffer is then discarded. A 40 ml solution containing 0.5% bovine serum albumin (fraction V) and a 1000-fold dilution of anti-fluorescein HRP conjugate made up in buffer 2 is poured on the membrane. (The quantity of diluted antibody conjugate solution is equivalent to 0.25 ml/cm^2 of membrane). The membrane is incubated for 30 minutes, after which the solution is poured off. The blots are then rinsed with 200 ml buffer for 5 minutes. The buffer is poured off and the rinse step is repeated another three times to ensuring the removal of non-specifically bound antibody.

2.3.11 Signal Generation and Detection

The following equipment and reagents are required for this section:

a. Blue-light sensitive autoradiography film, (Hyperfilm^{TM}-ECL, RPN 2103)
b. Timer
c. Saran wrap
d. X ray film cassette
e. ECL Detection reagents 1 and 2 (RPN 2105)

The following steps should be carried out in a dark room. Since there is no lag phase with the ECL reaction, it is advised that the work be carried out with reasonable speed once the blots have been exposed to the detection solution.

Equal quantities of detection solution 1 and detection solution 2 are mixed to provide enough reagent to cover the membrane (0.125 ml/cm^2). Before the detection solution is added to the membrane, the membrane is drained from the excess buffer and then placed on a sheet of Saran wrap, RNA side up. The detection solution is then added to the blots and incubated for precisely 1 minute at room temperature. The excess detection buffer is then drained and the membrane is wrapped in the Saran wrap. The blots are placed RNA side up in the film cassette. The lights are switched off and a sheet of autoradiography film is placed on top of the blots. After closing the cassette, the film is exposed for 15 minutes and developed in the Agfa, Curix 60 (Type 9462/105) developing machine.

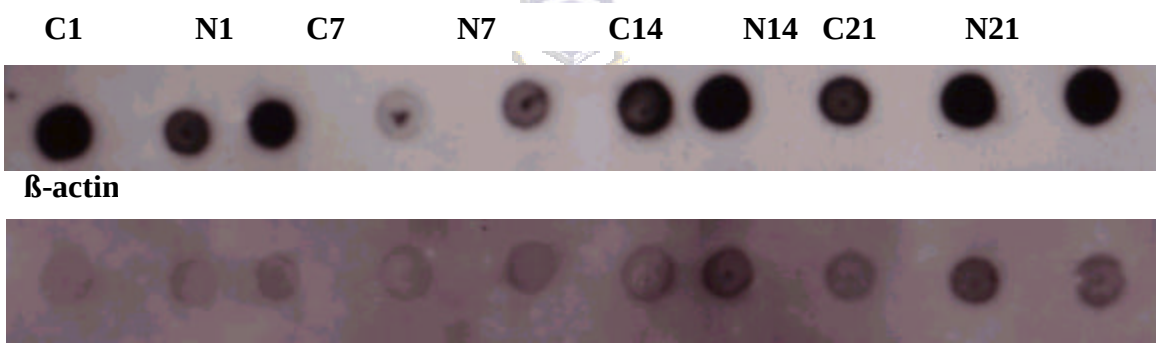


Fig. 5. Examples of dot blots obtained from 1, 7, 14, 21, and 49 day old control neonatal rat lungs and lungs of rats exposed to nicotine during gestation and lactation.

Since the mRNA levels are analyzed and expressed relative to the internal standard (ß-actin) and it is only possible to determine any differences using

densitometry and statistical analysis and not by directly viewing the blots, an example of the dot blots obtained is presented in this chapter (Fig.5).

2.3.12 Re-probing

Before re-probing, it is essential that the original probe is removed from the membrane. To ensure successful stripping of the probe, the membranes must never be allowed to dry out during hybridization and washing or after signal generation and detection.

The membrane is placed in 200 ml 0.1% SDS/DEPC treated water and incubated for 1 hour. The membrane is then rinsed in 100 ml DEPC treated water for 1 minute and poured off. 100 ml boiling 0.1% SDS/DEPC treated water is added to the membrane and incubated for 10 minutes. After pouring off the solution, the membrane is rinsed with 100 ml DEPC treated water for 1 minute and the solution is poured off. If the stripped membrane is not to be used immediately, it may be wrapped in Saran wrap and stored at 2-8°C to always ensuring that the membrane does not dry out.

2.3.13 Densitometry and Statistical Analysis

Densitometry is performed using the ImageJ freeware image analysis package [http://rsb.info.nih.gov/ij/index.html] written by W. Rasband, National Institute of Health, Bethesda, Maryland, USA. Before data is extracted from the programme the background is subtracted. The percentage data points for the individual subunits and ß-actin blots are recorded and the levels of mRNA for the individual sub-units analysed are expressed relative to ß-actin. Statistical outliers are determined by using the Box and Whisker plots and removed from the final data. The final data is analyzed statistically using the Wilcoxon unpaired T-test, in which $P<0.05$ is designated as significant.

CHAPTER 3

3. ANALYSIS OF HEXOKINASE ISOENZYME PATTERNS AND TRANSCRIPT LEVELS IN DEVELOPING NEONATAL LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

3.1 INTRODUCTION

The transport of glucose into cells and subsequently the phosphorylation of glucose to glucose-6-phosphate (G-6-P) are two vital steps for glucose utilization in mammalian tissue (Boileau *et al.*, 1998). Transportation of glucose across the cell membrane occurs via facilitated diffusion mediated by glucose transporters (GLUT) (Bell *et al.*, 1994), and it is subsequently phosphorylated to G-6-P by four Hexokinase (HK) isoenzymes. HKI, HKII, and HKIII are approximately 100-kDa proteins evolved by duplication and fusion involving an ancestral 50 kDa form (Sebastian *et al.*, 2001). HKI, II, and III all display a low K_m for glucose and a high susceptibility to G-6-P inhibition (Wilson, 1995). HKIV or glucokinase (GK) is a 50 kDa protein displaying a significantly higher K_m for glucose and is not inhibited by G-6-P (Wilson, 1995).

HKI is ubiquitously expressed and predominates in the highly oxidative tissues, brain and kidney (Grossbard and Schimke, 1966; Katzen *et al.*, 1970; Wilson, 1985). HKI is also found in significant quantities in cardiac tissue (Boileau, 1998; Reid and Masters, 1985). In many tissues this isoform can be found bound to mitochondria (Wilson, 1982). HKII is expressed in insulin-sensitive tissue, skeletal muscle, heart, and adipose tissue (Reid and Masters, 1985) and in tumor cells in which it has been implicated in playing a pivotal role in promoting cell growth and survival (Pedersen *et al.*, 2002; Shinohara *et al.*, 1998). HKII also binds to mitochondria in various mammalian tissues (Katzen *et al.*, 1970; Salotra and Singh, 1982b) and competes with HKI for binding (Kurokawa *et al.*, 1982). HKIII is expressed in most tissues, including liver and lung (Reid and Masters, 1985), but only in small amounts

(Furuta *et al.*, 1996). Furthermore, most studies indicate that it is not associated with the mitochondria (Katzen *et al.*, 1970; Salotra and Singh, 1982b; Radojkovic and Ureta, 1987). HKIV's (GK) presence is restricted to liver and pancreatic b-cells (Reid and Masters, 1985; Printz *et al.*, 1993) and is located in the cytosol (Lawrence *et al.*, 1984).

In lung, metabolism of glucose is regulated mainly at the hexokinase-catalyzed phosphorylation step (Salotra and Singh, 1982a). Salotra and Singh (1982b) report that HKI is the dominant HK isoenzyme in the adult rat lung. Furthermore, its mRNA expression in lung tissue has also been documented (Griffin, *et al.*, 1992). In a tissue that is considered predominantly glycolytic, in which HKI activity has been associated with glucose-derived ATP synthesis, equivalent amounts of HKII have also been found in adult lung tissue (Salotra and Singh, 1982b).
Determining the isoenzyme patterns in postnatal developing rat lung will therefore, provide insight into the metabolic development and need of the lung tissue. Furthermore, ascertaining whether the neonatal lung expresses the individual isoforms at the mRNA level will, provide information with respect to transcriptional and/or post-transcriptional modification of these isoforms.

Studies investigating the effects of maternal nicotine exposure on the developing rat lung demonstrates that, despite an increase in the total glucose turnover, an irreversible inhibition in the glycolytic pathway occurred which is reflected by a reduced rate in lactate production (Maritz, 1986). Furthermore, a significant increase in the adenine nucleotide pool is also observed in these neonatal lungs. It was suggested that the increase in the ATP levels in the lung tissue of the offspring may result in the observed inhibition of glycolysis (Maritz and Burger, 1992).

In follow up studies, Maritz (1997) shows that maternal nicotine exposure has no influence on HK activity in developing rat lung. However, the impact of maternal nicotine exposure on HK isoenzyme patterns and mRNA expression in this tissue has not been investigated. It is probable, that although the activity of this enzyme is

unaffected, the individual isoforms may be regulated at transcriptional and/or post-transcriptional levels.

Therefore, the objectives of this division of the investigation are (a) to determine the HK isoenzyme patterns and the mRNA expression of these isoforms in control developing neonatal rat lung as a function of age and, (b) to determine the impact of maternal nicotine exposure during pregnancy and lactation on the HK isozyme patterns and mRNA expression as the lung matures and ages.

3.2 RESULTS

3.2.1. Hexokinase (HK) Isoenzyme Pattern Analysis

3.2.1.1 HKI

Data presented in Table 3 and Fig.6 shows the isoenzyme pattern of HKI in control developing rat lung as well as in neonatal lung exposed to nicotine during pregnancy and lactation. HKI % Density/mg protein in control rat lung significantly increases ($P<0.01$) by 1.94 fold from postnatal day 1 to day 49. No difference is detected between day 1 and day 7, however, HKI % density /mg protein increased by 1.35-fold from 5.30 ± 0.31 on postnatal day 7 to 7.14 ± 0.17 at postnatal day 14 ($P<0.01$). On postnatal day 21 the % Density of HK 1 was at 12.44 ± 0.52, 1.79-fold ($P<0.01$) higher than at postnatal day 14. Conversely, between postnatal days 21 and 49 the % Density/mg protein decreased by 1.26-fold to 9.91 ± 0.54.

HKI % Density/mg protein in lung tissue exposed to nicotine during pregnancy and lactation also increases significantly from 3.92 ± 0.35 at postnatal day 1 to 8.95 ± 0.59 at postnatal day 49, an increase of 2.28-fold ($P<0.01$). % Density/mg protein at postnatal day 7 is 1.34-fold higher ($P<0.02$) than at postnatal day 1, however there is no difference between day 7 and day 14 ($P>0.10$). At postnatal day 21, HKI %

Density/mg protein was 1.75-fold higher (P<0.01) than that detected at day 14. At postnatal day 49 there is no difference when compared to day 21 (P>0.10).

Comparing the % Density/mg protein of HKI in control (5.09 ± 0.24) and experimental tissue (3.92 ± 0.35) at postnatal day 1 shows a significantly higher reading in the former (P<0.01). At postnatal day 7 however, control % Density/mg protein was 5.30 ± 0.31 and the experimental % Density/mg protein 5.26 ± 0.30 (P>0.10). This is due to a 1.34-fold increase in % Density/mg protein in the experimental animals, while no increase is observed in the control animals. Interestingly, at day 14, HKI in control tissue increased significantly to 7.14 ± 0.17%, whereas no difference is found in the experimental group. Thus the % Density/mg protein at postnatal day 14 in control tissue is 1.21-fold higher (P<0.01) than in the nicotine-exposed tissue. At postnatal day 21 the HKI % Density in control tissue is still significantly higher (P<0.01) than that found in experimental tissue (1.21-fold greater), even though the % Density/mg protein increased in both groups from day 14 to 21. At day 49 there was no difference in % Density/mg protein between the groups (P<0.05), owing to the significant decrease observed from day 21 to 49 in the control lung tissue.

Table 3: Influence of maternal nicotine exposure on HKI (% Density/mg Protein)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs Nic**
	(n=6)	(n=6)	
1	5.09±0.24	3.92±0.35	P<0.01
	(n=6)	(n=6)	
7	5.30±0.31	5.26±0.30	P>0.10
1 vs 7	P > 0.10	P > 0.10	
	(n=6)	(n=6)	
14	7.14±0.17	5.61±0.28	P<0.01
7 vs 14	P < 0.01	P < 0.01	
	(n=6)	(n=6)	
21	12.44±0.52	9.80±0.41	P<0.01
14 vs 21	P < 0.01	P > 0.10	
	(n=6)	(n=6)	
49	9.91±0.54	8.95±0.59	P>0.10
21 vs 49	P < 0.01	P < 0.02	

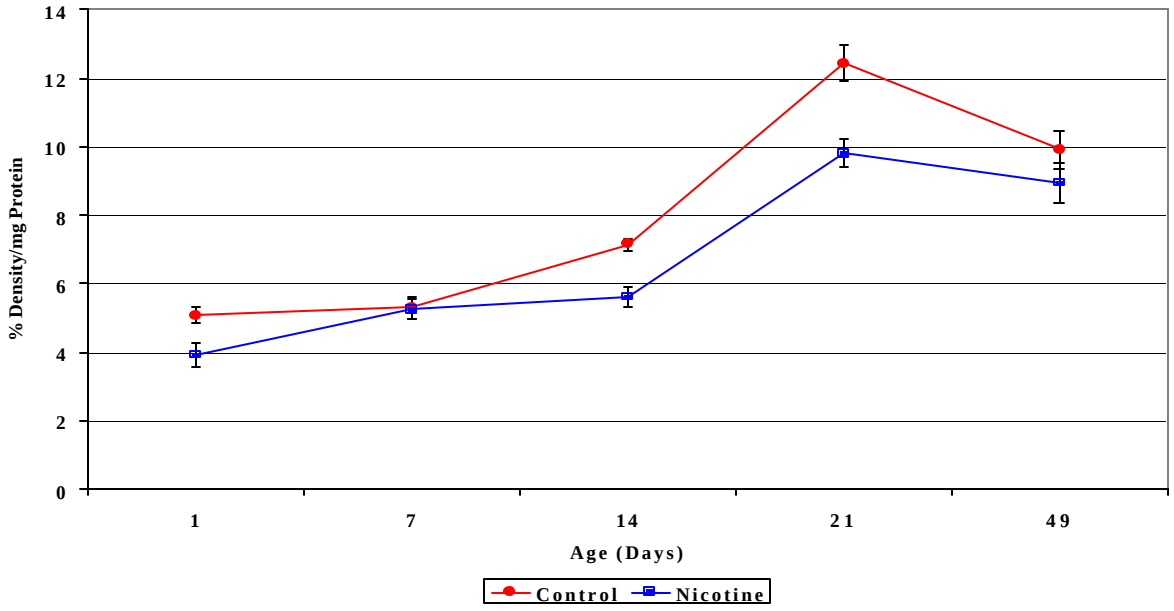


Fig.6. HKI isoenzyme pattern separated on PAGE gels in control neonatal rat lung and in lung tissue exposed to nicotine during pregnancy and lactation.

3.2.1.2 HKII

The results displayed in Table 4 and Fig.7 shows the HKII isoenzyme pattern in control rat lung and in lung exposed to nicotine during gestation and lactation. The developmental pattern and % Density/mg protein of HKII isoenzyme in control lung tissue is similar to that of HKI (Table 3). At postnatal day 1 the % Density/mg protein was at 5.33 ± 0.23 not different from the 5.49 ± 0.34 at postnatal day 7 ($P>0.10$). However, HKII % density increased from postnatal day 7 to postnatal day 14 by 1.16-fold ($P<0.05$) to 6.37 ± 0.0.31%. This elevation continues to postnatal day 21, in which the % Density/mg protein increased by 1.7-fold to 10.81 ± 0.47% ($P<0.01$). HKII % Density/mg protein decreased by 1.19-fold between postnatal days 21 and 49 ($P<0.01$) to 8.71 ± 0.32. HKII isozyme % Density/mg protein at postnatal day 49 was 1.63-fold higher ($P<0.01$) than at postnatal day 1.

The results obtained from the influence of maternal nicotine exposure on HKII isozyme pattern (Fig.6), is similar to the pattern displayed by HKI in the experimental tissue. HKII % Density/mg protein increased by 1.25-fold ($P<0.01$) from postnatal day 1 to postnatal day 7 (4.11 ± 0.37 to 5.46 ± 0.28). At day 14 the %Density/mg protein was at 5.33 ± 0.29 not different from that at postnatal day 7 ($P>0.10$). However, at day 21 the % Density/mg protein increased by 1.92-fold ($P<0.01$) to 10.23 ± 0.71. No difference is observed between postnatal day 21 and day 49 ($P>0.10$). HKII % Density/mg protein increased by 2.36-fold ($P<0.01$) from postnatal day 1 to day 49.

A comparison between control and experimental tissue shows that HKII % Density/mg protein is higher ($P<0.01$) in the former at postnatal day 1. No difference is detected at postnatal day 7 owing to the significant increase noted in the experimental group from day 1 to day 7 ($P>0.01$). At day 14 HKII % Density/mg protein in control tissue is 1.20-fold higher ($P<0.05$) than that found in lung tissue of the rat pups exposed to nicotine via the placenta and mother's milk. From postnatal days 14 to 21 both groups' % Density/mg protein increased so that no difference ($P>0.10$) was detected between the two groups at postnatal day 21. At postnatal day 49 there was also no difference in HKII % Density/mg protein between the control and nicotine-exposed lung tissue ($P>0.10$). The increase in % Density/mg protein from day 1 to day 49 is substantially higher in the nicotine-exposed animals (2.34-fold) than in control tissue (1.64-fold), owing to the lower density observed in the former at postnatal day 1.

3.2.1.3. HKIII

In this study, HKIII isozyme data is not obtainable in lung tissue when utilizing the PAGE techniques and crude lung extract. These findings are also documented by Allen *et al*. (1998).

Table 4: Effect of maternal nicotine exposure on HKII (% Density/mg Protein)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs Nic**
	(n=6)	(n=6)	
1	5.33±0.23	4.11±0.37	P<0.01
	(n=6)	(n=6)	
7	5.49±0.34	5.46±0.28	P>0.10
1 vs 7	P > 0.10	P < 0.01	
	(n=6)	(n=6)	
14	6.37±0.31	5.33±0.29	P<0.05
7 vs 14	P < 0.05	P > 0.10	
	(n=6)	(n=6)	
21	10.81±0.47	10.23±0.71	P>0.10
14 vs 21	P < 0.01	P < 0.01	
	(n=6)	(n=6)	
49	8.71±0.32	9.70±0.77	P>0.10
21 vs 49	P < 0.01	P > 0.10	

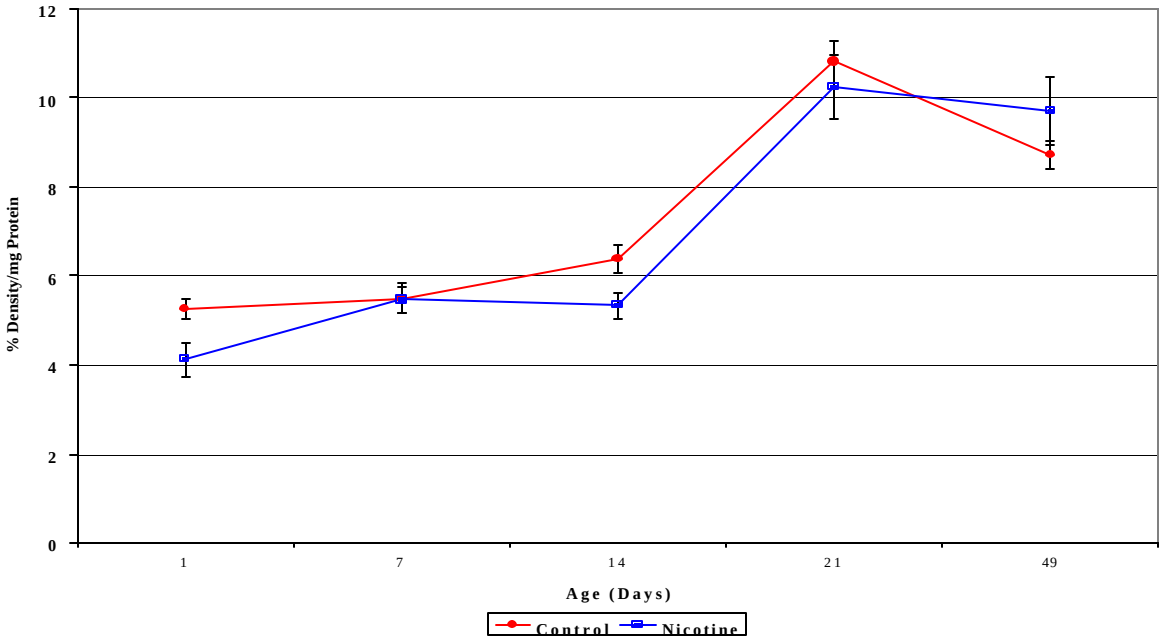


Fig.7. HKII isozyme patterns in control rat lung and the influence of maternal nicotine exposure during gestation and lactation.

3.2.1.4. Comparison of HKI and HKII in Control Developing Lung

HKI and HKII are both present in control developing neonatal lung tissue (Fig. 8) and increases significantly from postnatal day 1 to postnatal day 49 (1.95-fold and 1.64-fold, respectively). Equal quantities are detected at day 1 and day 7. At days 14 and 21, HKI is 1.12-fold and 1.15-fold respectively higher, ($P<0.05$) for both days than HKII. No difference is found between control and experimental tissue at postnatal day 49 ($P>0.10$). Both isoforms increase significantly from postnatal day 1 to postnatal day 21 (2.45-fold and 2.03-fold, respectively) after which a decrease in % Density/mg protein was noted in both isozymes. From postnatal day 21 to postnatal day 49, HKI decreased by 1.25-fold and HKII by 1.24-fold ($P<0.01$) for both. Thus from the results (Fig.8) HKI and HKII follow the same developmental patterns from postnatal day 1 to postnatal day 49, however, HKI is present at higher levels at day 14 and day 21.

3.1.2.5. Influence of Maternal Nicotine Exposure on HKI and HKII in Developing Lung

The data displayed in Fig. 9 shows HKI and HKII isozyme patterns in lung tissue of rats exposed to nicotine during gestation and lactation. At postnatal days 1 and 7 the relationship between HKI and II is comparable to that found in the control lung, in that no statistical differences were detected. However, at postnatal days 14 and 21, unlike the difference found between the two isoforms in control tissue, no difference is seen in the levels of HK I and II mRNA in lung tissue of rat pups exposed to nicotine during gestation and lactation. At postnatal day 49 no difference is found between the isoforms, which is comparable to the findings in the control lung tissue. There is a 2.28-fold ($P<0.01$) and 2.36-fold increase ($P<0.01$) in HKI and HKII % Density/mg protein, respectively, between postnatal days 1 and 49 in the nicotine-exposed tissue. Although HKI and HKII isoforms tend to display the same patterns in control and experimental tissue, the increases observed in the former is less (1.95-fold and 1.64-fold, respectively).

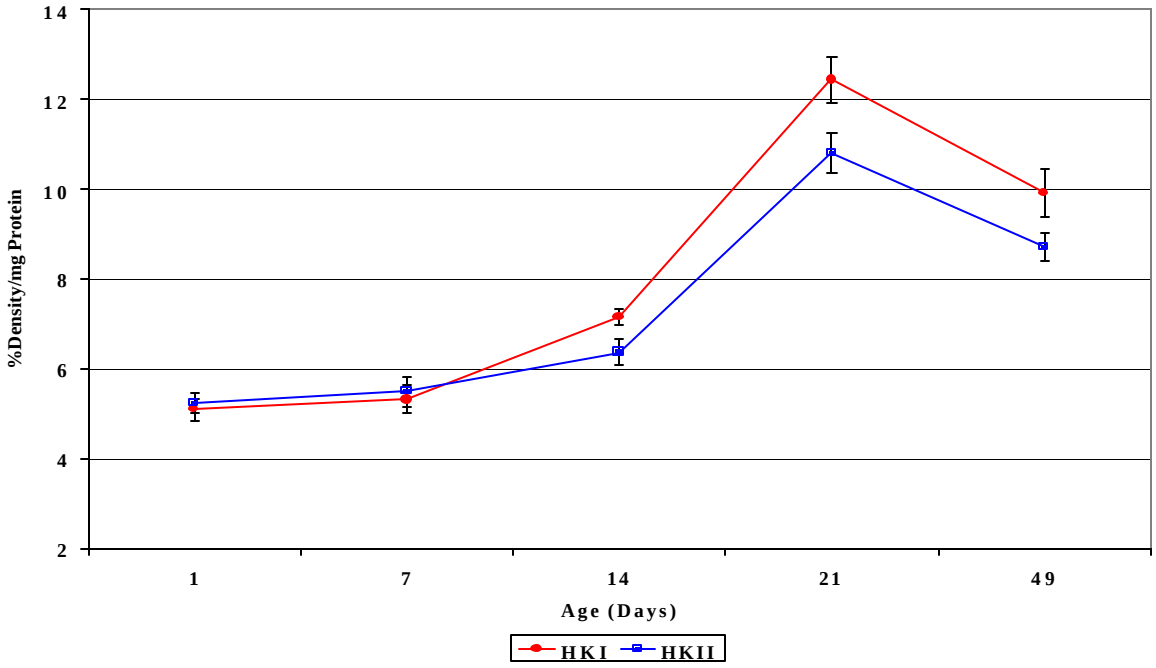


Fig.8. HKI and II isoenzyme patterns in control rat lung (%Density/mg Protein) as the animals aged.

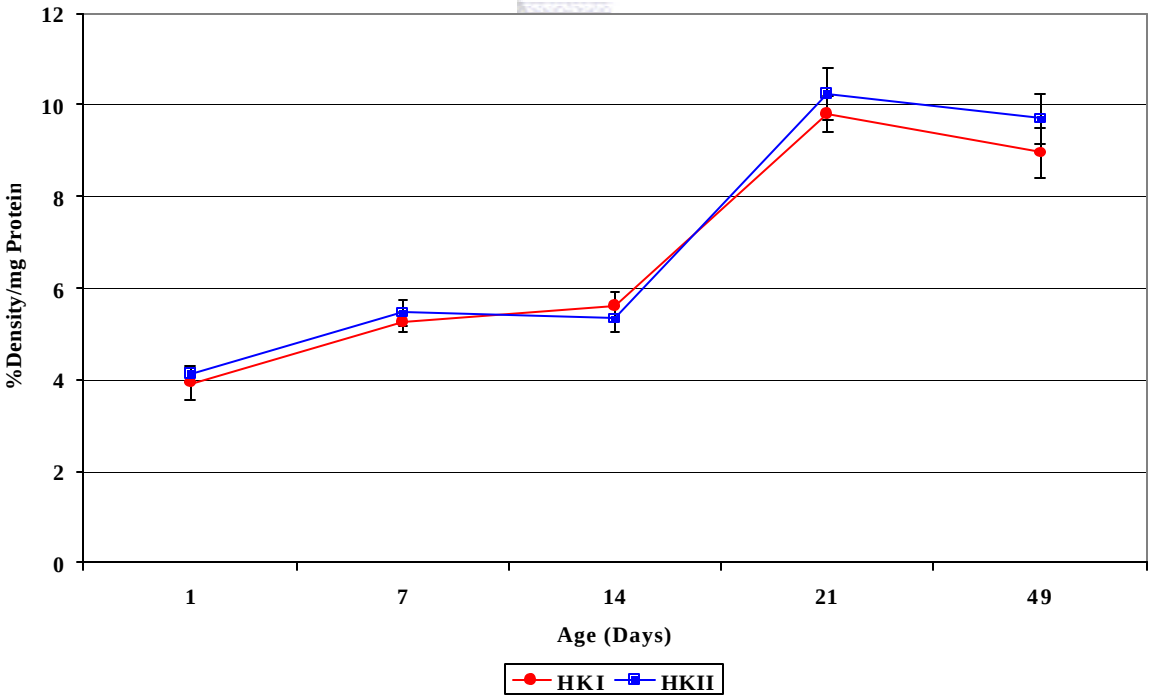


Fig.9. HKI and II isoenzyme patterns in rat lung exposed to nicotine during pregnancy and lactation (%Density/mg Protein) as the animals aged.

3.2.2 Analysis of mRNA Expression of HK Isoenzymes

3.2.2.1 mRNA Expression of HKI

HKI mRNA is detected in the lungs of rats just after birth (Table 5). The HKI mRNA/ß-actin ratio increased 1.5-fold between postnatal days 1 and 49 ($P<0.01$). No difference in the ratio was found between any of the consecutive age groups investigated. HKI mRNA levels increases gradually and at postnatal day 21, the levels were 2.1-fold higher ($P<0.01$) than at postnatal day 1. Between postnatal days 21 and 49 the HKI mRNA/ ß-actin ratio decreased from 0.54 ± 0.045 to 0.39 ± 0.035 ($P>0.10$), which is still higher than the levels detected at day 1 ($P<0.01$).

Expression of HKI at the mRNA level (Table 5) in the lungs of rat pups exposed to nicotine during pregnancy and lactation increased by 2.0-fold from postnatal day 1 to postnatal day 49 ($P<0.001$). As in the control lung, no difference is found between postnatal days 1 and 7, or between postnatal days 7 and 14. Unlike the levels seen in the control tissue, the mRNA levels increased 1.4-fold ($P<0.01$) from 0.42 ± 0.01 to 0.60 ± 0.03 between postnatal days 14 and 21. Thereafter HKI:ß-actin levels decreased 1.2-fold between postnatal days 21 and 49 ($P<0.02$). The expression however, remains significantly higher ($P<0.001$) than the mRNA levels detected at postnatal day 1.

A comparison of the control lung tissue and the tissue exposed to maternal nicotine shows no significant difference ($P>0.10$) at postnatal day 1 (control vs nicotine: 0.26 ± 0.038 and 0.25 ± 0.04). No difference is also detected at postnatal day 7, 14 and 21. At day 49 HKI mRNA levels in control tissue is at 0.39 ± 0.035, 1.3-fold lower ($P<0.05$) than the 0.51 ± 0.015 of the nicotine exposed rat pups.

The graph (Fig.10) clearly shows that HKI mRNA expression was the same for both control and nicotine-exposed tissue at postnatal day 1. Due to large standard error of means (SEMs) no statistical differences are found at days 7, 14 and 21 between the

two groups. However, on postnatal day 49 the HKI mRNA expression of the nicotine-exposed lung was 1.3-fold higher ($P<0.05$) than in lungs of control rats of the same age.

Table 5: HKI mRNA expression in control lung tissue and lung tissue of rat pups exposed to nicotine during pregnancy and lactation (HKI:ß-actin ratio)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs Nic**
	(n=7)	(n=8)	
1	0.26±0.038	0.25±0.04	P > 0.10
	(n=9)	(n=9)	
7	0.40±0.065	0.54±0.094	P > 0.10
1 vs 7	P > 0.10	P > 0.10	
	(n=8)	(n=8)	
14	0.51±0.047	0.42±0.005	P > 0.10
7 vs 14	P > 0.10	P > 0.10	
	(n=6)	(n=7)	
21	0.54±0.045	0.60±0.027	P > 0.10
14 vs 21	P > 0.10	P < 0.01	
	(n=7)	(n=6)	
49	0.39±0.035	0.51±0.015	P < 0.05
21 vs 49	P > 0.10	P < 0.02	

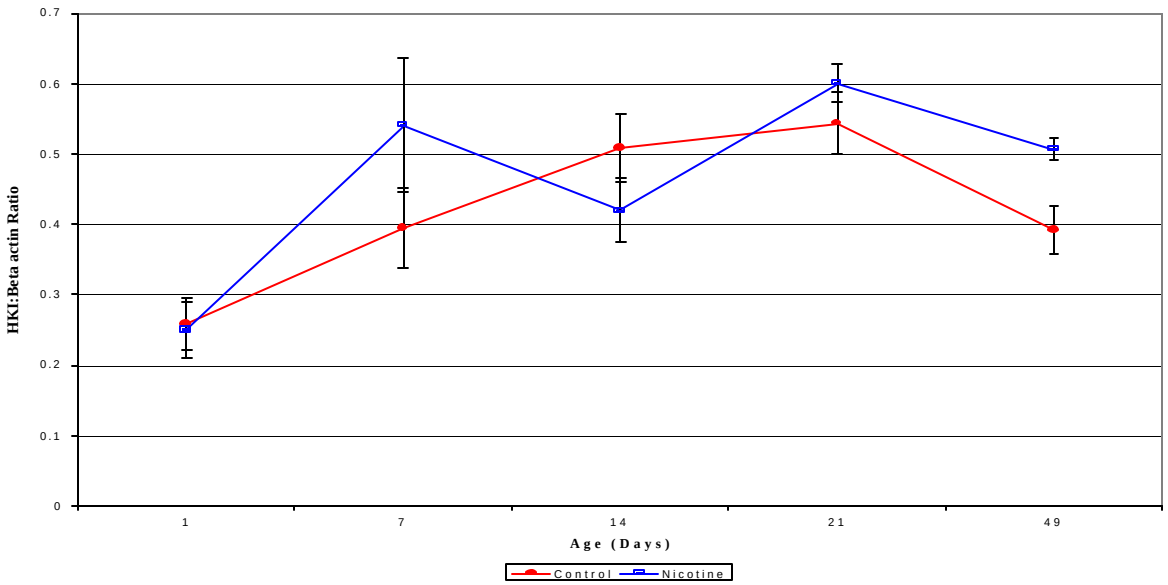


Fig.10. Influence of maternal nicotine on HKI mRNA levels (HKI/ß-actin ratio).

3.2.2.2 mRNA Expression of HKII

HKII mRNA levels, expressed as HKII mRNA: ß-actin ratio, was only detectable from postnatal day 14 in lungs of control rats as well as in the lungs of rats exposed to nicotine during pregnancy and lactation (Table 6). In control lung tissue, no difference is detected between postnatal days 14 (0.11 ± 0.01) and 21 (0.13 ± 0.01), or days 21 and 49 (0.12 ± 0.003) ($P>0.10$ for both) (table 6).

In the lung tissue exposed to nicotine during gestation and lactation, HKII mRNA expression increases markedly by 1.7-fold from postnatal day 14 (0.13 ± 0.01) to 21 (0.21 ± 0.02) ($P<0.001$). No differences occur between postnatal day 21 and 49 (0.19 ± 0.03). HKII mRNA levels increases by 1.5- fold from day 14 to day 49 ($P<0.01$).

At postnatal day 14 HKII is expressed at comparable levels in control and experimental tissue (0.11 ± 0.01 and 0.13 ± 0.01, ($P>0.10$) respectively). However, at day 21 HKII: ß-actin ratio is at 0.21 ± 0.02, 1.6-fold higher ($P<0.01$) in nicotine-exposed tissue than in control tissue. At day 49, mRNA expression remains markedly (1.6-fold) higher ($P<0.01$) in the experimental group than the control group. This implies that maternal nicotine exposure resulted in an increased expression of HKI mRNA in lungs of the control animals (fig. 11).

Table 6: HKII mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (HKII:ß-actin ratio)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs Nic**
	(n=9)	(n=8)	
14	0.11±0.011	0.13±0.01	P > 0.10
	(n=7)	(n=5)	
21	0.13±0.01	0.21±0.024	P < 0.0
14 vs 21	P > 0.10	P < 0.001	
	(n=8)	(n=7)	
49	0.12±0.003	0.192±0.025	P < 0.01
21 vs 49	P > 0.10	P > 0.10	

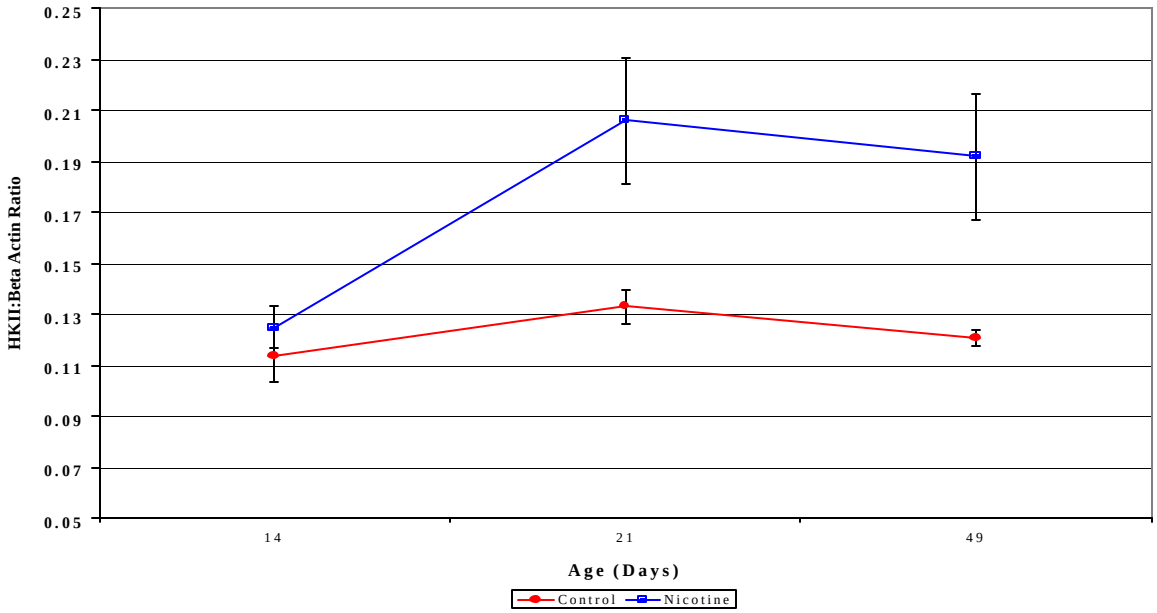


Fig.11. HKII mRNA expression in control lung tissue of neonatal rats and in the lungs of rat pups exposed to nicotine during gestation and lactation. The dot blot technique is not sensitive enough to detect mRNA levels at postnatal day 1 and 7.

3.2.2.3 mRNA Expression of HKIII

The data in Table 7 shows that the increases in HKIII mRNA expression in control lung tissue at postnatal days 1 and 7 and between postnatal days 7 and 14, 14 and 21 and, 21 and 49 are not significant. Thus, HKIII mRNA levels gradually increases with age after birth without marked increases in expression between specific age groups. However, due to the gradual increase over time the expression of HKIII mRNA increases 1.5-fold ($P<0.01$) from 0.15 ± 0.023 to 0.22 ± 0.023 from postnatal day 1 to day 49.

Similar results were obtained in the nicotine-exposed tissue and in the control tissue, except between days 14 and 21 in which HKIII mRNA levels increase from 0.19 ± 0.02 to 0.23 ± 0.03 ($P<0.01$) from postnatal day 14 to postnatal day 21. No difference in expression is thus found between postnatal day 1 and 7, 7 and 14 and,

21 and 49. Overall, HKIII mRNA expression increases from 0.12 ± 0.01 at day 1 to 0.26 ± 0.03 at day 49 ($P<0.01$), an increase of 2.2-fold.

HKIII mRNA expression (HKIII mRNA/ß-actin ratio) at postnatal day 1 is similar in control tissue when compared to nicotine-exposed tissue (control vs nicotine: 0.15 ± 0.02 and 0.12 ± 0.01). No difference is observed at day 7 (control vs nicotine: 0.15 ± 0.02 and 0.17 ± 0.02) and at day 14 (control vs nicotine: 0.19 ± 0.03 and 0.19 ± 0.02), either. However, at day 21, HKIII mRNA levels in the experimental group is 1.6-fold higher ($P<0.01$) than that detected in the control group. No difference is observed between the groups at day 49.

Fig.12 shows the steady increase in HKIII expression in control tissue. From postnatal day 1 to postnatal day 14, HKIII mRNA levels in the experimental tissue also display the steady increase observed in the control tissue. Between postnatal days 14 and 21, however, the expression sharply increases, unlike the results of the control group. Thereafter, the mRNA levels in the experimental group slightly decrease and no statistical difference is found at day 49 between control and nicotine-exposed lung tissue.

Table 7: HKIII mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (HKIII:ß-actin ratio)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs Nic**
	(n=5)	(n=5)	
1	0.15±0.023	0.12±0.010	P > 0.10
	(n=7)	(n=6)	
7	0.15±0.02	0.17±0.016	P > 0.10
1 vs 7	P > 0.10	P < 0.10	
	(n=9)	(n=9)	
14	0.19±0.025	0.19±0.020	P > 0.10
7 vs 14	P > 0.10	P > 0.10	
	(n=8)	(n=8)	
21	0.22±0.014	0.30±0.033	P < 0.10
14 vs 21	P > 0.10	P < 0.05	
	(n=8)	(n=9)	
49	0.22±0.023	0.26±0.030	P > 0.10
21 vs 49	P > 0.10	P > 0.10	

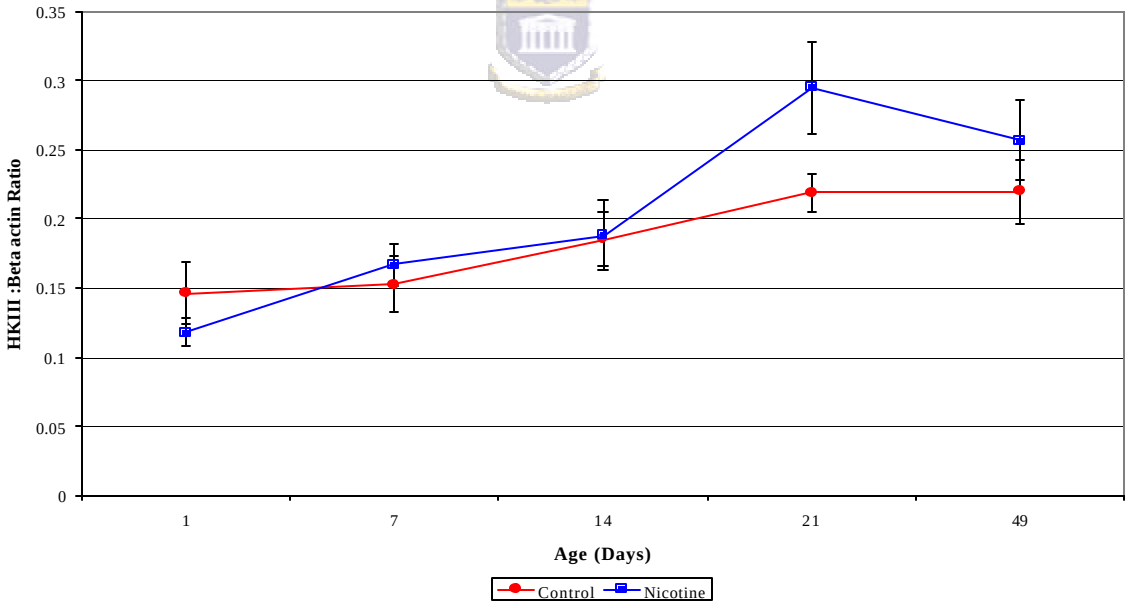


Fig.12. HKIII mRNA expression in normal developing rat lung and the influence of maternal nicotine exposure on the HKIII transcription.

3.2.2.4 A comparison of HKI, II and III mRNA Expression in Control lung Tissue.

Results in this division of the study (Fig.13) show that all three isoforms are expressed at the mRNA level in lungs of control rats. The dot blot technique employed is perhaps not sensitive enough to detect the mRNA levels of HKII just after birth and at postnatal day 7. A comparison of the data shows that HKI is the dominantly expressed isoform at all age groups analyzed. The data also indicates that HKII is expressed at lower levels than HKI and HKIII for all age groups analyzed. The HKI and HKIII mRNA levels increase ($P<0.01$) from postnatal day 1 to postnatal day 49. However, in contrast to HKI and III mRNA levels, the HKII mRNA levels at postnatal days 14, 21 and 49 did not change as the lungs of the control rats matured. At postnatal day 1 the mRNA levels of HKI is 1.8-fold higher than those found for HKIII. Comparisons at day 14 for all three isoforms show that HKI is dominantly transcribed in control lung; it is 4.5-fold higher than HKII and 2.7-fold higher than HKIII. At day 49, HKI is expressed 1.8-fold more that HKIII and 3.2-fold more that HKII.

3.2.2.5 A comparison of the Effect of Maternal Nicotine Exposure on HKI, II and III mRNA Expression in Developing Lung

Fig.14 shows that HKI in lungs of rats exposed to nicotine is dominantly expressed for all the age groups. HKII displays the lowest mRNA levels for all age groups. The most noticeable difference observed in mRNA expression is between HKII and HKIII when compared to findings in the control tissue. At postnatal days 21 and 49, HKIII mRNA levels is significantly higher than the levels detected for HKII in control pups, however in the nicotine-exposed pups no difference is detected between these isoforms for the same days. Thus the isoform's mRNA levels is most affected by maternal nicotine exposure, is HKII. Comparisons between expressions of the three isozymes show that at postnatal day 1, HKI is expressed 2.1-fold higher than HKIII. At day 14, transcription of HKI is 3.4-fold greater than HKII and 2.3-fold higher than HKIII. HKI mRNA levels 49 days after birth is 2.6-fold greater than

that of HK II and 2.0-fold greater than that of HKIII.

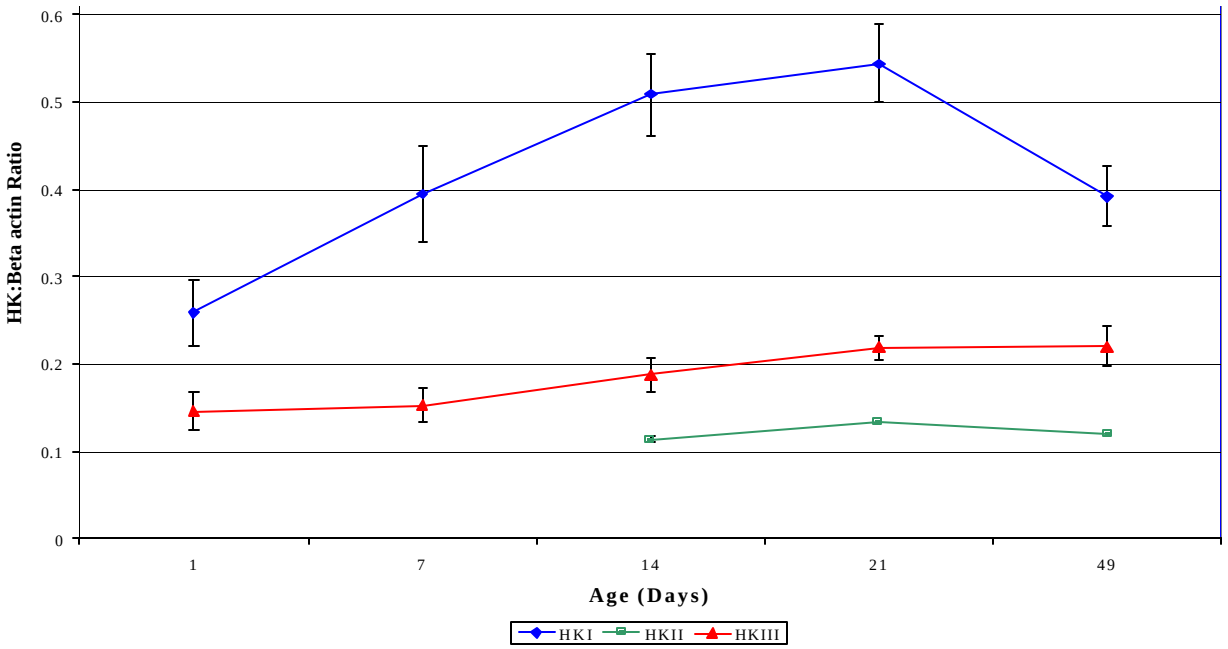


Fig.13. mRNA expression of HKI, II, and III in normal developing lung of neonatal rats (HK:ß-actin ratio).

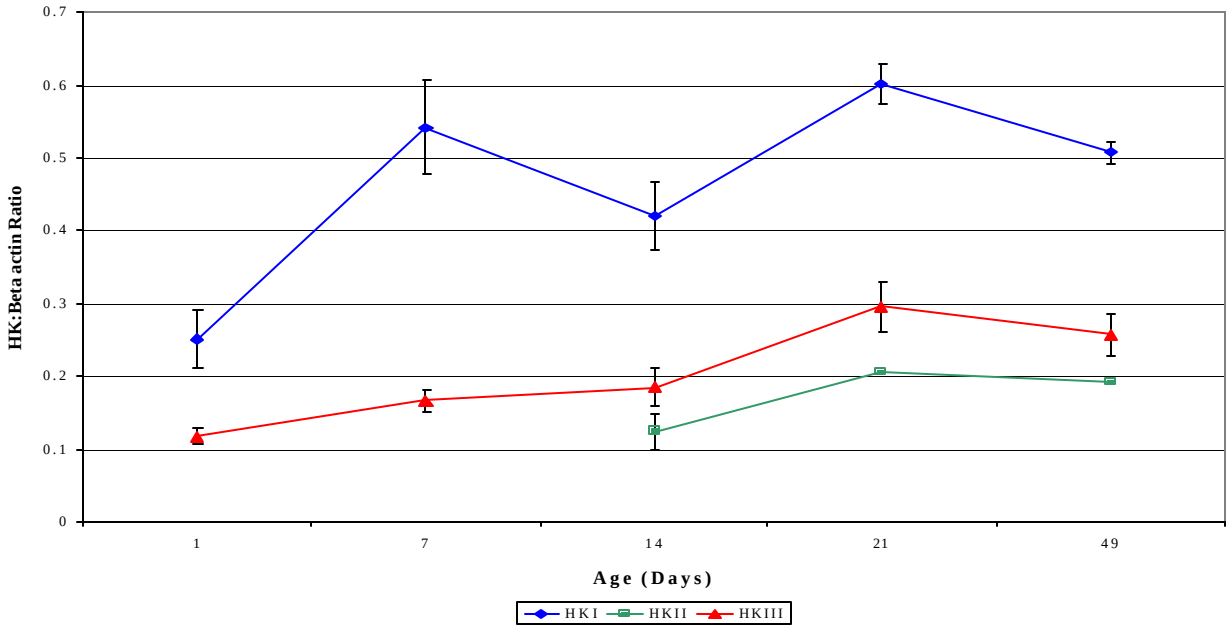


Fig.14. The influence of maternal nicotine exposure on HKI, II and III isozyme mRNA expression in developing rat lung (HK:ß-actin ratio).

3.3 DISCUSSION

Before the body tissues can utilize glucose it must first be phosphorylated to yield glucose-6-phosphate (Griffin *et al.*, 1992). This phosphorylation reaction is catalyzed by hexokinase (HK). An inhibition of this enzyme will thus result in a slower phosphorylation of glucose and thus a slower flux of this substrate via the glycolytic pathway and the hexose monophosphate pathway (HMP). Three hexokinase isoenzymes occur in lung tissue, namely HKI, II, and III (Wilson, 1984). HKI predominates in highly oxidative tissues such as brain and kidney (Grossbard and Schimke, 1966; Katzen *et al.*, 1970; Wilson, 1985), while HKII is thought to function anabolically, supporting glycogen and lipid synthesis (Wilson, 1984).

Results of this study show that all three isozymes are detectable at the mRNA level however this method is only sensitive enough to detect HKII from postnatal day 14. However, utilizing PAGE gels it was possible to detect only two HK isoforms in developing neonatal lung, namely HKI and HKII. The results show that normal developing rat lung have similar quantities of HKI and II at postnatal day 1 and day 7, but at day 14 and 21, HKI is present in markedly higher levels than II. This study show that HKI also predominates in developing and mature lung tissue such as in other highly oxidative tissues like the brain and kidney, (Grossbard and Schimke, 1966; Katzen *et al.*, 1970; Wilson, 1985). HKI is also the predominant isoenzyme in the lungs of the rats that were exposed to nicotine via the placenta and mother's milk. There is in this study little correlation between HKI isoenzyme patterns and the changes in mRNA levels from postnatal day 1 to day 49 in control and nicotine-exposed lung. It is reported (Griffin *et al.*, 1992) that in tissues such as brain and kidney where HKI is the predominant isozyme and where there seems to be poor correlation between activity and mRNA levels, regulation will be principally post-transcriptional. It is therefore plausible that control of HKI is post-transcriptional. Thus, further investigations have to be carried out to determine the influence of maternal nicotine exposure on the individual isoenzyme activity.

The change in the glycolytic status of the lung tissue as it matures is reflected in the significant increase in HKI % density from day 7 to day 14, which coincides with the phase of rapid alveolarization that occurs in developing rat lungs from postnatal day 4 to 13 (Brody and Vaccaro, 1979). During the phase of rapid alveolarisation, the rate of cell proliferation and synthesis of connective tissue, especially elastin, is increased (Kauffman, 1980). This implies that the demand for energy and materials for synthetic processes, such as NADPH, will increase. It is therefore plausible that the increase in HKI observed from postnatal day 7 to postnatal day 21 is supporting an increased flux of glucose through the glycolytic and hexose monophosphate pathways (HMP), whereas the latter may supply NADPH for synthetic processes. The lung has a limited ability to store glycogen and glucose, and the presence of HKI provides the lung with a mechanism for a high rate of aerobic glycolysis (Salotra and Singh, 1982b). After the phase of rapid alveolarisation, the rate of alveolar formation and thus cell proliferation decreases which implies that the demand for energy and NADPH is lower (Nardell and Brody, 1982). Since the demand for energy and NADPH will be lower, the rate of glucose phosphorylation and thus for utilization via the glycolytic and HMP pathways will conceivably also be lower and this may explain the decrease in HKI and II mRNA expression and HKI and II levels after postnatal day 21.

In a previous study it was shown that maternal nicotine exposure during gestation and lactation results in an irreversible inhibition of glycolysis despite an increase in the total glucose turnover in the lungs of these rats (Maritz, 1986). The increased glucose turnover is due to an increase in the flux of glucose via the HMP pathway for the production of NADPH (Maritz, 1983). Maternal nicotine exposure during gestation and lactation also suppress HKI between postnatal days 14 and 21. On the other hand, on postnatal day 49, the difference in HKI between control and nicotine-exposed rats was not significant. This implies that the lower HKI in lungs of the rats exposed to nicotine via the placenta and mother's milk (HKII is not affected by maternal nicotine exposure) had no adverse effect on the flux of glucose through the glycolytic pathway. Since the HMP pathway can only function after phosphorylation

of glucose, it implies that HK was not suppressed to a level that it will actually result in inhibition of glucose phosphorylation. This is supported by the fact that maternal nicotine exposure had no effect on the total activity of HK in the lungs of the nicotine exposed rat pups. This furthermore implies that the inhibition of glycolysis in the lungs of rat pups that were exposed to nicotine via the placenta and mother's milk cannot be attributed to changes in the isoenzyme levels of HK or expression of HK isoenzymes.

Since it is possible to detect HKIII mRNA in the lungs of the neonates, it is conceivable that the isoenzyme should also be present in the tissue of these animals. However, the method utilized did not make the analysis of HKIII isoenzyme possible. It is reported however, that HKIII is present in quite low amounts in most mammalian tissues, including lung tissue and this may account for the limitation in this study (Wilson, 1995). Moreover, studies attempting to analyze HKIII isoenzymes report that purifying this isozyme in substantial amounts is not easy. Since crude extract is used in this study, it may explain why HKIII could not be detected. Literature reveals scant information with regard to isoenzyme expression in lung and even less with regards to pre- or postnatal lung development. However, the data implies that HK plays a minor role in anabolic processes like glycogenesis and lipogenesis in developing lung. Based on the findings of this study, it can be conclude that HKI is the dominant isoenzyme in the lung of control as well as rats exposed to nicotine at both the transcriptional and post-translational levels during gestation and lactation. The developmental pattern of the isoenzymes as the lung matures follows the same trend in lung tissue of control and nicotine exposed offspring. It is also clear that the inhibition of glycolysis in lungs of rats exposed to nicotine during gestation and lactation is not due to changes in the HK isoenzyme levels in the lungs of the offspring.

CHAPTER 4

4. ANALYSIS OF PHOSPHOFRUCTOKINASE ISOENZYME PATTERNS AND TRANSCRIPT LEVELS IN DEVELOPING NEONATAL LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

4.1 INTRODUCTION

Regulation of glucose entry into pathways of energy production is the primary physiological role of 6-phosphofructo-1-kinase (PFK) (Mhasker and Dunaway, 1995; 2000). This oligomeric protein's smallest active species are tetramers formed by random association of available subunits. Three distinct subunit types have been demonstrated in rabbits (Foe and Kemp, 1984), rats (Dunaway and Kasten, 1985a; 1985b; 1987), and humans (Vora *et al*., 1980; Khan *et al*., 1979; Meienhofer *et al.*, 1979). PFK isozymes are, the M-type, the only subunit type in adult muscle, the L-type which is the major subunit type found in liver, and the C-type found at high levels in the brain and testes (Mhasker and Dunaway, 1995; 2000). The M-type subunit conveys a rapid and high affinity for fructose-6-phosphate and exhibits the greatest resistance to ATP inhibition (Kasten and Dunaway, 1993). The L-type and even more so, the C-type subunit has considerably lower affinity for fructose-6-phophate and increased susceptibility to inhibition by ATP (Kasten and Dunaway, 1993). The M-type subunit predominates in organs depending mostly on glycolysis, ie. skeletal muscle, heart and brain, whereas the L-type predominates in organs with active gluconeogenesis, ie. liver and kidney cortex. The C-type predominates in rapidly replicating cultured cells largely using aerobic metabolism (Vora, 1982; 1983).

It is shown that maternal nicotine exposure during gestation and lactation has no impact on PFK activity (Maritz, 1997). However, in the presence of ATP and ADP at levels that correspond with that of lung tissue, maternal nicotine exposure results

in PFK inhibition in lung tissue of offspring (Kordom *et al.*, 2003). This might be due to changes in isoenzyme activity since the isoenzymes display different sensitivities to ATP (Kasten and Dunaway, 1993). Thus determining the changes in mRNA expression may provide information with regards to the tissue's response to metabolic alterations observed by Maritz, (1983, 1986), Maritz and Burger, (1992) at the transcriptional level.

The objective of the study are a) to investigate the developmental changes in mRNA expression of the three PFK isozymes, PFK-M, PFK-L, and PFK-C in control rat lung as the animal ages, as well as the effect of maternal nicotine exposure during gestation and lactation on the mRNA expression of these isoenzymes and b) to establish whether changes in PFK isoenzymes might contribute to a lower flux of glucose via the glycolytic pathway.

4.2 RESULTS

A division of this study's objectives is to separate the PFK isoforms in crude lung tissue extract using polyacrylamide gel electrophoresis (PAGE). The separation of the PFK isoforms proved difficult since working with crude extract makes separating the PFK bands from other proteins in the crude tissue nearly impossible. This is also documented by Zeitschel *et al.* (1996) when they reported that it was impossible for them to separate the isoenzyme bands in crude tissue, because they could not separate the bands efficiently enough from other proteins even when using silver stained PAGE gels. This objective is unfortunately not accomplished in this study either.

4.2.1 Expression of PFK mRNA

4.2.1.1 PFK-M mRNA Analysis

Data in Table 8 represents changes in the mRNA expression of PFK-M in the developing lung of both control rats and rats exposed to nicotine during gestation and lactation. PFK-M mRNA expression in control neonatal lung displays an increase from postnatal day 1 to day 49. Overall, mRNA expression gradually increased by 1.85-fold ($P<0.05$) from day 1 to day 49. The most profound increase is between day 14 and day 21, in which expression increases by 1.10-fold ($P<0.05$). No differences are observed in the expression of the isoform between postnatal days 1 and 7, 7 and 14 and, 21 and 49 ($P>0.05$).

Expression of PFK-M mRNA in the lungs of nicotine exposed neonatal rats also displays an increasing trend from postnatal day 1 to day 49. No significant differences are observed between day 1 and 7, day 7 and 14, and day 21 and 49 ($P>0.05$). The increase in mRNA expression between day 14 and 21 is a significant elevation in which PFK-M mRNA increases by 1.15-fold ($P<0.01$). The mRNA levels gradually increase by 1.34-fold ($P<0.01$) from day 1 to day 49 in the lungs of the experimental animals.

Although both groups analyzed display the same pattern of mRNA expression, the difference in the levels of expression between the groups at any given postnatal day is significant ($P<0.05$). At postnatal day 1 PFK-M mRNA was 1.80-fold ($P<0.02$) higher in the experimental (0.37 ± 0.02) group than the control group (0.21 ± 0.03). At postnatal day 7 expression is 1.93-fold higher ($P<0.05$) in the nicotine exposed lung (0.33 ± 0.043) when compared to control tissue (and 0.17 ± 0.029). At day 14 (Control vs.. nicotine: 0.31 ± 0.02 vs.. 0.44 ± 0.02), 21 (0.34 ± 0.05 vs.. 0.5 ± 0.01) and 49 (0.38 ± 0.04 vs. 0.60 ± 0.03) the PFK: ß-actin mRNA ratio was 1.4-fold ($P<0.01$), 1.51-fold ($P<0.01$), and 1.56-fold ($P<0.01$) higher in the experimental groups in comparison to the control groups, respectively. From these experimental

findings it is apparent that maternal nicotine exposure during pregnancy and lactation alters the expression of PFK-M at the transcriptional level. From the data it is clear that on postnatal day 1 the expression of PFK-M is already 1.80-fold higher ($P<0.02$) than the 0.207 ± 0.031 of the control lung.

Figure 15 shows that the gradual increase of the PFK-M mRNA: ß-actin ratio of the lung tissue of the nicotine and nicotine-exposed rats followed the same developmental trend as the animals aged. It is also interesting to note that the difference in PFK-M mRNA expression in the lungs actually becomes bigger between the control and nicotine-exposed lungs between postnatal days 14 (1.42-fold) and 49 (1.58-fold). This implies that the effect of maternal nicotine exposure on the PFK-M isoenzyme of the lungs of the offspring is irreversible.

Table 8: PFK-M mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (PFK-M:ß-actin ratio)

Age (Days)	Control	Nicotine	Ctrl vs. Nic
	(n=7)	(n=6)	
1	0.21±0.03	0.37±0.02	P < 0.02
	(n=7)	(n=7)	
7	0.17±0.03	0.33±0.04	P < 0.05
1 vs. 7	P > 0.10	P > 0.10	
	(n=9)	(n=8)	
14	0.31±0.02	0.44±0.02	P < 0.01
7 vs. 14	P > 0.10	P < 0.02	
	(n=6)	(n=7)	
21	0.34±0.05	0.51±0.01	P < 0.01
14 vs. 21	P < 0.05	P < 0.01	
	(n=7)	(n=6)	
49	0.38±0.04	0.60±0.03	P < 0.01
21 vs. 49	P > 0.10	P > 0.10	

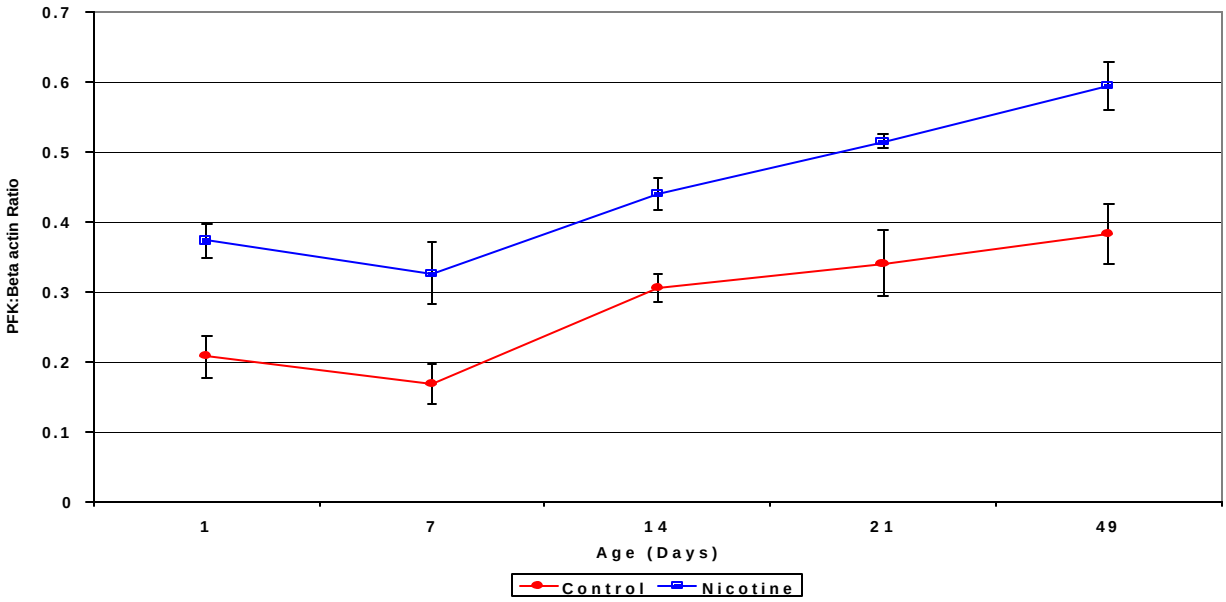


Fig.15. The influence of maternal nicotine exposure on the mRNA expression of PFK-M in developing rat lung.

4.2.1.2 PFK-L mRNA Analysis

The PFK-L mRNA expression (Table 9) in control developing rat lung shows an overall decline ($P<0.05$) from postnatal day 1 (0.18 ± 0.01) to postnatal day 49 (0.13 ± 0.01). After an initial increase of 1.31-fold ($P<0.05$) between postnatal days 1 and 7, the expression of PFK-L mRNA decreased by 1.52-fold ($P<0.01$) from 0.26 ± 0.04 on postnatal day 7 to 0.17 ± 0.02 at postnatal day 14. Between postnatal days 14 and 21 it decreased by 1.48-fold ($P<0.01$) to 0.11 ± 0.01. No difference is found between day 21 and 49.

PFK-L mRNA expression in the lungs of rats exposed to nicotine during pregnancy and lactation increase by 1.30-fold from postnatal day 1 (0.13 ± 0.03) to postnatal day 7 (0.19 ± 0.02) and by a further 1.14-fold between postnatal day 7 and 14 (0.22 ± 0.01). A decrease in expression of PFK-L is then observed from day 14 to day 49 (0.16 ± 0.02). However by day 21 mRNA expression is 1.35-fold greater at 0.20 ± 0.02 than the mRNA expression observed at day 1. At day 49 mRNA levels were not different to expression levels noted at day 1.

Comparisons between control and experimental mRNA levels of expression of PFK-L (Table 9) show no significant differences at day 1 and at day 7. However, at day 14, PFK-L mRNA levels in the experimental group are 1.44-fold higher ($P<0.05$) at 0.22 ± 0.01 than the 0.17 ± 0.02 observed in the control group. PFK-L mRNA levels remain significantly higher (1.51-fold) in the experimental group at day 21 after birth (control; nicotine: 0.11 0.17 ± 0.02; 0.20 ± 0.02) as well. At day 49 no difference is noted between the two groups.

Figure 16 shows that the patterns the mRNA levels of PFK-L follow between these age groups for the individual groups analyzed are distinctly unique. PFK-L mRNA expression increases in both control and nicotine-exposed lungs from day 1 to day 7. However, at day 7 the PFK-L mRNA levels of control lung start to decline up until day 21 where after it remains unchanged. It is also noted that mRNA levels in the experimental lungs steadily increases, plateaus at day 14 and then gradually decreases till day 49 to a level that resembles the level on postnatal day 1. It is therefore clear that the decrease in PFK-L mRNA expression of the control lung starts sooner (postnatal day 7), whereas that of the experimental animals begins at postnatal day 14.

Table 9: PFK-L mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (PFK-L:ß-actin ratio)

Age (Days)			
	Control	**Nicotine**	**Ctrl vs. Nic**
	(n=6)	(n=7)	
1	0.18±0.01	0.13±0.03	P > 0.10
	(n=7)	(n=9)	
7	0.26±0.04	0.19±0.02	P > 0.10
1 vs. 7	P < 0.05	P < 0.10	
	(n=9)	(n=6)	
14	0.17±0.02	0.22±0.01	P < 0.05
7 vs. 14	P < 0.01	P > 0.10	
	(n=9)	(n=9)	
21	0.11±0.01	0.20±0.02	P < 0.01
14 vs. 21	P < 0.01	P > 0.10	
	(n=6)	(n=7)	
49	0.13±0.01	0.16±0.02	P >0.10
21 vs. 49	P > 0.10	P > 0.10	

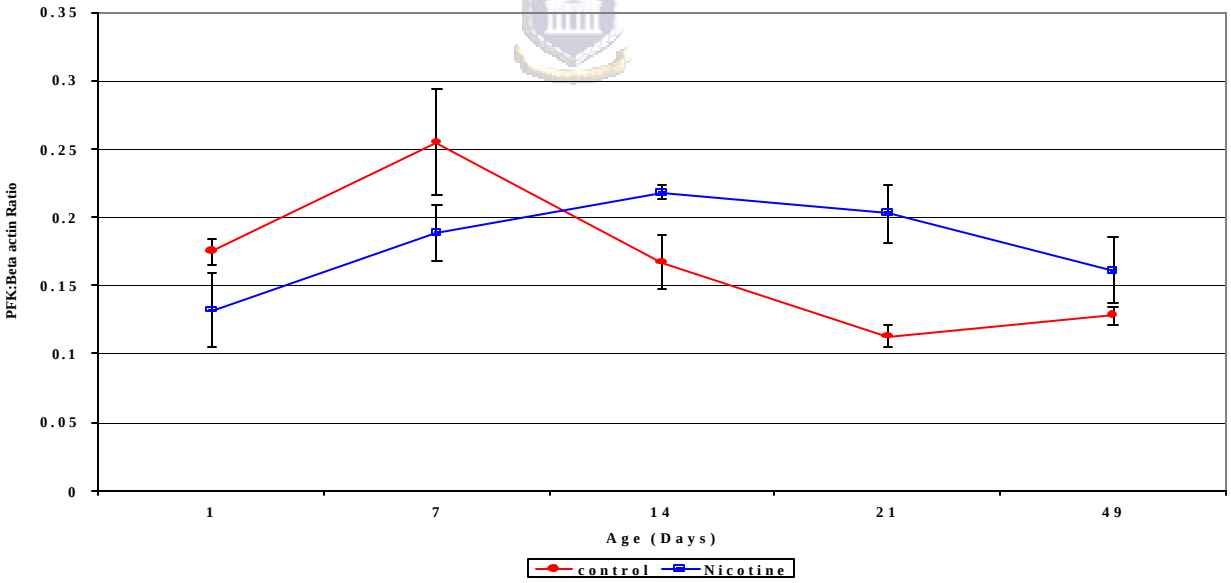


Fig.16 Influence of maternal nicotine exposure on PFK-L:ß-actin mRNA expression in developing neonatal rat lung during pregnancy and lactation.

4.2.1.3 PFK-C mRNA Analysis

The overall PFK-C mRNA expression (Table 10) in control developing rat lung increases significantly by 2.83-fold from postnatal day 1 to day 49 ($P<0.01$). PFK-C mRNA levels decrease by 1.15-fold from 0.16 ± 0.01 at postnatal day 1 to 0.13 ± 0.01 at postnatal day 7 ($P<0.05$). PFK-C expression then further increases to 0.17 ± 0.01 ($P<0.01$) at day 14. PFK-C mRNA expression continues to increase from day 14 to day 21 by 1.35-fold to 0.23 ± 0.02 ($P<0.02$) after which there is a further increase of 1.96-fold to 0.44 ± 0.05 ($P<0.01$) at day 49.

Results obtained in the lungs of rat pups exposed to nicotine during pregnancy and lactation show that PFK-C mRNA expression also increased by 3.18-fold from 0.16 ± 0.01 at postnatal day 1 to 0.45 ± 0.02 at day 49 by 3.18-fold ($P<0.01$). Unlike the decrease in mRNA levels observed from day 1 to day 7 in control lungs, no difference is observed in PFK-C mRNA expression in the experimental lungs at these ages. At day 14, mRNA levels are 0.20 ± 0.020 which is not significantly different from that on postnatal day 7. Expression increases significantly by 1.17-fold from day 14 to 0.24 ± 0.02 at postnatal day 21 ($P<0.05$) and continues to increase to 0.45 ± 0.02 at day 49. Between postnatal days 21 and 49 the PFK-C mRNA levels increase by 1.92-fold ($P<0.01$).

Figure 17 shows that the expression of PFK-C mRNA of control lungs and lungs exposed to nicotine during pregnancy and lactation at postnatal day 1, 7, 14, 21, and 49 follows the same pattern. This implies that maternal nicotine exposure during gestation and lactation does not alter the expression of PFK-C transcriptionally when compared to control lung. This is in contrast to the effect of maternal nicotine exposure on PFK-M and –L mRNA expression where the developmental pattern was significantly changed by maternal nicotine exposure during gestation and lactation.

Table 10: PFK-C mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (PFK-C:ß-actin ratio)

Age (Days)	Control	Nicotine	Ctrl vs. Nic
	(n=6)	(n=7)	
1	0.16±0.01	0.14±0.01	P > 0.10
	(n=6)	(n=6)	
7	0.13±0.01	0.16±0.02	P > 0.10
1 vs. 7	P < 0.05	P > 0.10	
	(n=9)	(n=9)	
14	0.17±0.01	0.20±0.02	P > 0.10
7 vs. 14	P < 0.01	P > 0.10	
	(n=9)	(n=8)	
21	0.23±0.03	0.25±0.01	P > 0.10
14 vs. 21	P < 0.02	P < 0.05	
	(n=6)	(n=6)	
49	0.44±0.05	0.45±0.02	P > 0.10
21 vs. 49	P < 0.10	P < 0.10	

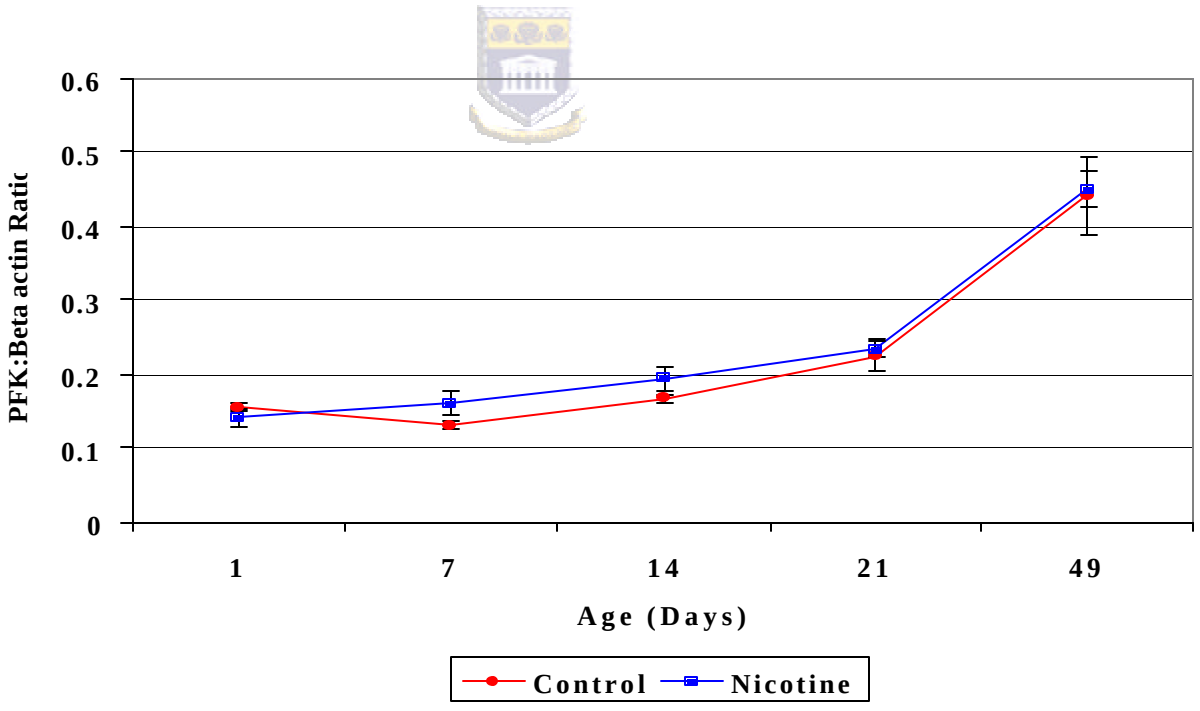


Fig.17 PFK-C mRNA expression in control neonatal rat lung and in lungs exposed to nicotine during pregnancy and lactation.

4.2.1.4 Comparison of the Three PFK Isoforms in Control Developing Lung

Results in this study show that all three isoforms are expressed at the mRNA levels in normal developing rat lung (Fig.18). As PFK-M and PFK-C mRNA levels increase with age, PFK-L mRNA levels significantly decreases from postnatal day 1 to day 49. No difference is found in mRNA levels between the three isoforms at day 1. At day 7, PFK-L mRNA levels is still higher than PFK-C mRNA levels ($P<0.05$). No difference is found between mRNA levels of the M-type and L-type or between the M-type and C-type mRNA levels. At day 14, however, PFK-M is clearly the dominant isoform being expressed at the mRNA level in normal lung. It is expressed 1.8-fold greater ($P<0.01$) than both C-type and L-type. Also apparent from Figure.17 is that there is no difference between the mRNA levels of PFK-L and PFK-C at postnatal day 14. At day 21 the neonatal lung is expressing the M-type isoform stronger than the other two. PFK-M expression is 3-fold higher ($P<0.01$) than PFK-L expression and 1.5-fold higher ($P<0.01$) than PFK-C expression. Furthermore, C-type mRNA levels is 2-fold greater ($P<0.001$) than the L-type mRNA levels at day 21. Analysis of PFK isoform mRNA levels at postnatal day 49 reveals that both PFK-M and PFK-C is dominantly expressed in normal lung and that the former is expressed 3.0-fold higher than PFK-L and the latter is expressed 3.5-fold greater ($P<0.01$) than PFK-L. No difference is found between PFK-M mRNA levels and PFK-C mRNA levels at day 49.

4.2.1.5 Influence of Maternal Nicotine Exposure on PFK Isoenzyme Transcript Levels in Developing Lung

The PFK isoenzyme mRNA levels in rat lungs exposed to nicotine during pregnancy and lactation presents a significantly different pattern when compared to its expression in the control lung (Fig.19). PFK-M is the dominantly expressed isoenzyme at the mRNA level from postnatal day 1 to day 49. Unlike the control lung tissue where no difference is found between the three isoforms mRNA levels at day 1, PFK-M is expressed at markedly higher levels than both PFK-L and PFK-C

(2.8-fold and 2.6-fold, respectively). At day 7, PFK-M levels are 1.7-fold higher ($P<0.02$) and 2-fold higher ($P<0.01$) than the L-type and C-type isoforms, respectively. Furthermore, no difference is found between the L- and C-type isoenzymes at day 7, 14 and 21. Whereas, PFK-M at day 14 is 2-fold higher ($P<0.001$) than the former and 2.3-fold higher ($P<0.01$) than the latter, mRNA levels for the M-type isoform at day 21 remains significantly higher than the L-type and C-type isoforms' mRNA levels (2.5-fold and 2.2-fold, respectively). At day 49 however, PFK-C mRNA levels increases by 1.9-fold, 2.8-fold higher than that of PFK-L expression ($P<0.01$). Comparison with the PFK-M levels however, reveals that this isoenzyme is expressed 1.3-fold higher ($P<0.01$) than the C-type and thus remains the principal isoform being expressed at the mRNA levels in lung tissue of neonatal rats exposed to nicotine during pregnancy and lactation.

Thus, from the illustrated data it is apparent that as the normal rat lung develops its metabolic needs change as is shown in Figure 17 by the significant changes in mRNA expression of the individual isoforms as the lung matures. However, when a developing lung is exposed to nicotine during pregnancy and lactation, the tissue predominantly expresses the M-type PFK isoform at the mRNA level (Fig.18). At postnatal day 49 the C-type is expressed at a lower level than the M-type, whereas in control lung it is not different from M-type.

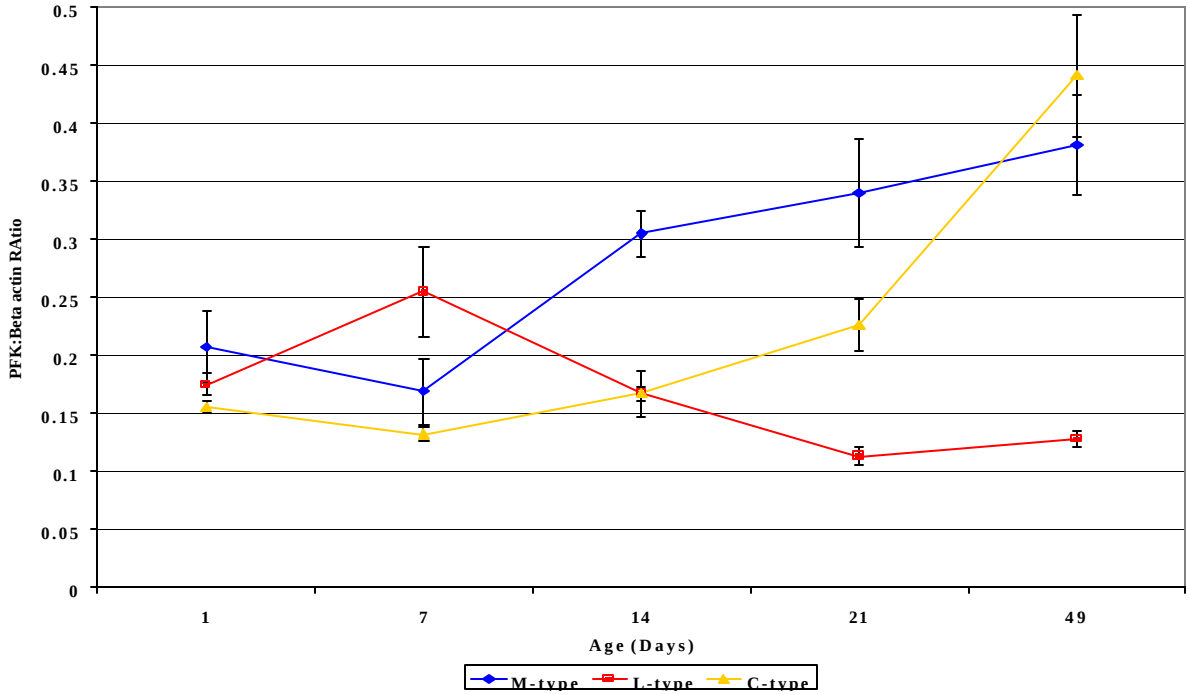


Fig.18. mRNA expression of PFK isoforms M-type, L-type, and C-type in control developing rat lung (PFK:ß-actin ratio).

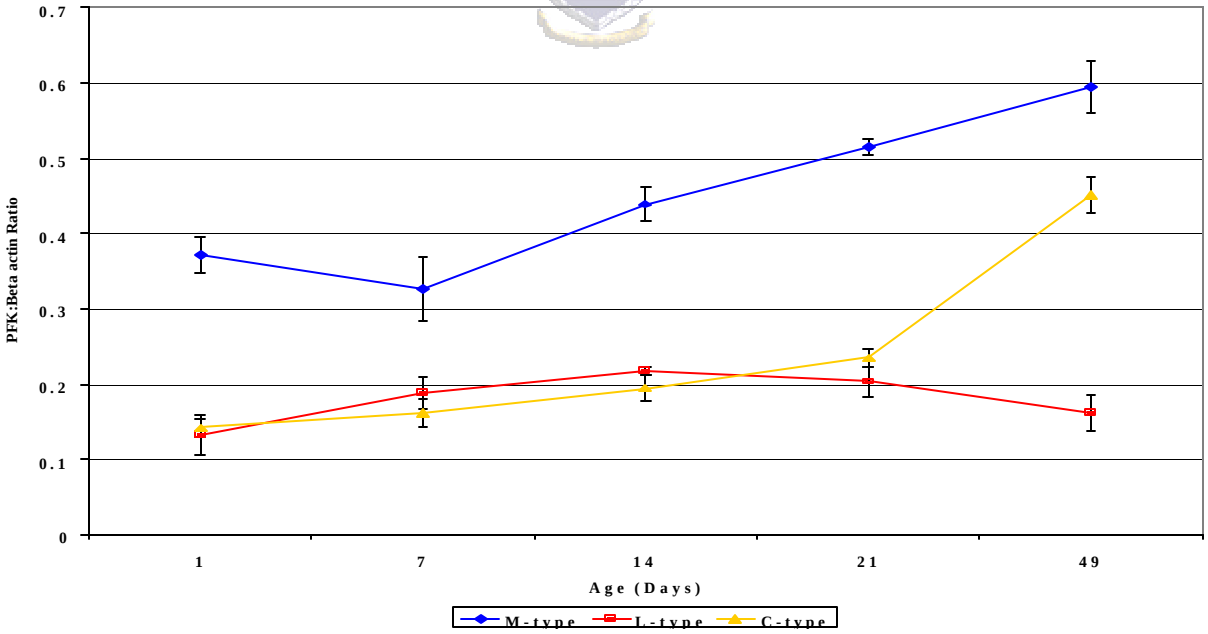


Fig.19. The effects of maternal nicotine exposure during pregnancy and lactation on PFK isoenzyme mRNA expression in developing rat lung. (PFK:ß-actin ratio)

4.3 DISCUSSION

Phosphofructokinase (PFK) plays an important role in the control of the glycolytic pathway and thus in the flux of glucose via this pathway. In most animal tissue PFK is a tetramer composed of 3 different subunits, which are assembled in various ways to produce isoenzymes that are characteristic of the tissue. The PFK isoenzymes have basically the same catalytic properties, but they differ quantitatively in their sensitivity to ATP inhibition and to other effectors, for example, muscle PFK (PFK-M) is less sensitive to fructose 2,6-biphosphate than is liver PFK (PFK-L) (Kasten and Dunaway, 1993). This implies that control of glycolysis in various tissues will depend largely on the isoforms that are dominant in that tissue. A change in the isoform during development reflects a change in the metabolic need of that tissue to meet the demand of the tissue to ensure proper development of the tissue. This implies that a change in the isoform composition or activity of PFK in developing lung, will also impact on the control of glycolysis in developing lung, and the role of the isoforms and glycolysis in the development of the lung. Any change in the isoform pattern might thus impact on the normal development and long term maintenance and health of the respiratory system.

4.3.1 Changes in PFK Isozyme Expression During Lung Development

The results obtained in this study show definitive developmental changes in mRNA expression of the three PFK isozymes in normal developing rat lung. It is clear from the data that all three sub-types are transcribed in control neonatal lung. Moreover, as the normal lung develops PFK-M and PFK-C mRNA expression increases from postnatal day 1 to day 49, whereas mRNA expression of PFK-L decreases markedly. This implies that the role of ATP in controlling glycolysis will decrease as the lungs mature since PFK-M is less sensitive to the inhibitory effect of ATP than PFK-L (Dunaway and Kasten, 1985a). This may ensure that the energy needs of the developing lung would be met to ensure proper development into an effective gas exchanger and for the maintenance of its integrity as a gas exchanger.

Initially PFK-L is expressed at levels comparable to that of PFK-M and –C (Fig.17). In fact, at postnatal day 7, it is expressed at significantly higher levels than PFK-C. The lung cannot store adequate amounts of glycogen (Griffin *et al.*, 1992) and is dependent on glucose as the main energy substrate. Very early post-partum tissue initially relies on gluconeogenic pathways for energy production, and since PFK-L activity is associated with gluconeogenic activity, its mRNA level of dominance reflects the metabolic need of lung tissue immediately after birth. After postnatal day 7, PFK-L mRNA expression decreases which probably reflects a reduced dependence on gluconeogenesis at this stage of lung development.

PFK-M activity is associated with organs which significantly utilizes the glycolytic pathways for energy production. In this study it is shown that PFK-M mRNA levels in normal developing rat lung increase by 1.9-fold from postnatal day 1 to day 49. The increase in M-type mRNA expression reflects the change in metabolic need of the developing rat lung. It is interesting to note that most of the increase (1.8-fold) occurred between postnatal days 7 and 14, which coincides with the phase of rapid alveolarization (Burri *et al*. 1978) and the increase in type I pneumocytes (Brody and Vaccaro, 1979). It is known that the type 1 pneumocytes depend on glycolysis for energy and the change in PFK-M activity may therefore reflect an increase in the overall glycolytic need of the tissue.

PFK-C mRNA expression in normal developing rat lung, and in all likelihood also the activity of the PFK-C isoenzyme, also increases with age. This isoform's activity, like that of HKI, is associated with aerobic metabolism (Vora, 1982; 1983). Interestingly, between postnatal day 1 and day 7, PFK-C mRNA levels decreased significantly, whereas the isoform associated with gluconeogenic activity (PFK-L) increases markedly. This decrease in PFK-C mRNA expression reflects a diminished ability of the tissue to extensively utilize aerobic metabolism since the tissue's glycogen stores are quickly depleted just after delivery. In a previous study it was shown that although the glycogen stores of the lung decreased rapidly from gestational day 21 to postnatal day 1, there is a pronounced increase in the glycogen

stores between postnatal days 1 and 7 (Maritz, 1988). This coincides with the increase in PFK-L mRNA expression observed in this study. After postnatal day 7 both the glycogen content of the developing lung and level of PFK-L mRNA decreased markedly and remained at a low level. HK isoenzyme % density and mRNA levels however, display an increase after postnatal day 7 (Chapter 3), this is perhaps an indication of increased glucose utilization.

On the other hand, PFK-C mRNA expression, and presumably PFK-C activity, like PFK-M mRNA expression, increased after postnatal day 7 and remained at a high level for the duration of the study. This increased expression indicating the tissue's change in metabolic needs to support growth and development of the lungs. The increased demand for energy is not only necessary to support the energy demands of the increased number of type I and II pneumocytes (Maritz, *et al.*, 1994), but also to supply energy for vascularization, septal formation, and elastin and collagen formation (Hislop, 1986). Thus, aerobic respiration in the lung tissue from postnatal day 7 increases significantly to meet the metabolic needs of the tissue.

In summary, normal developing neonatal lung thus expresses all three PFK isoforms, which, just after gestation are expressed at similar mRNA levels. However, just prior to and after the critical phase of alveolarization, PFK-M, the isoform associated with glycolytic activity and PFK-C, the isoform associated with aerobic glycolysis, are expressed at similar levels and are both expressed at markedly higher levels than PFK-L. In contrast, PFK-L, the isozyme associated with gluconeogenic activity is expressed at significantly lower levels to the other subunits.

4.3.2 Effect of Maternal Nicotine Exposure on the PFK Isozyme mRNA Expression in Developing Lung: Possible Consequences

PFK-C mRNA expression in the lungs of the offspring was not affected by maternal nicotine exposure. It is therefore plausible that the contribution of the PFK-C

isoenzyme to aerobic metabolism in the lungs of the offspring, and thus its contribution to lung development, was not affected by maternal nicotine exposure. The impact that maternal nicotine exposure has on the mRNA expression of PFK isoforms in developing neonatal rat lung is most obvious in the expression of PFK-M and PFK-L. At postnatal day 1 (Fig.18), PFK-M mRNA expression is significantly higher than both PFK-L and PFK-C. No difference is detected between the two latter isoforms, whereas in control tissue at postnatal day 1, the three isoforms are expressed at similar levels. Furthermore, the increased M-type expression in the experimental tissue is markedly higher than the levels found in the control tissue. Moreover, PFK-M remains at significantly higher levels up until postnatal day 49, when compared to control mRNA levels. It is also markedly higher expressed than PFK-L and PFK-C mRNA levels in the experimental tissue. The higher level of PFK-M mRNA expression suggests a higher PFK-M activity in the lungs of the nicotine exposed rat pups. If this is so, it is conceivable that the glucose flux through the glycolytic pathway of these lungs should be higher or equal to that in the lungs of the control rats. However, contrary to this statement, the flux of glucose through this pathway is irreversibly suppressed. This suggests that the PFK-M isoenzyme activity and in all probability the total PFK activity is not higher than in lungs of control animals despite the higher PFK-mRNA expression. In a study by Kordom *et al.* (2003) it was indeed found that the total PFK activity of rats exposed to nicotine via the placenta and mother's milk was lower than in lungs of control animals. Therefore, the need for the tissue to over-express this isoform probably stems from the inhibitory effect that maternal nicotine exposure has on the glycolytic pathway. Since glycolysis in lung tissue of nicotine exposed rats is irreversibly inhibited, it is plausible that the activity of PFK-M will be permanently lower in lung tissue of rat pups exposed to nicotine during gestation and lactation, especially since it was suggested that the lower PFK activity can be attributed to a conformational change in the structure of the enzyme (Kordom *et al*, 2003). Since expression of PFK-C and –L of nicotine-exposed lung tissue is the same as that of PFK-C and –L of control lung tissue, it is likely that only PFK-M was affected by maternal nicotine exposure during gestation and lactation. This also implies that the

long-term effect of maternal nicotine exposure on total PFK activity can be attributed to changes in the PFK-M isoenzyme activity.

Furthermore, Dewar *et al.* (2002) shows that nicotine in liver results in a significant increase in ATP synthesis. They attribute this to the surplus of ADP as a result of reduced production of pyruvate and lactate caused by the inhibition of glycolysis (Discussed in detail in Chapter 5). Maritz and Burger (1992) also demonstrated a significant increase in the ATP content of lung tissue of rat pups that were exposed to nicotine during gestation and lactation. This implies that maternal nicotine exposure will result in a reduction in the activities of PFK-L and –C. Despite a higher resistance to ATP inhibition compared to the other isoforms (Dunaway and Kasten, 1988), the higher ATP content that is maintained in the lungs of the nicotine-exposed pups will also reduce the activity of PFK-M. Although the level of reduction might be at a lower level than that for the other isoforms, the total PFK activity will be reduced resulting in a slower flux of glucose through glycolysis.

Since the half-life of nicotine is only 90-120 minutes (Zevin *et al.*, 1998), it is unlikely that there would be any nicotine in the lungs of the offspring at the alveoli after weaning on postnatal day 21. Therefore, the higher expression of PFK mRNA in nicotine-exposed lungs at postnatal day 49, suggest that exposure to nicotine during gestation and lactation causes irreversible over-expression of the PFK isoforms. It is therefore conceivable that the over-expression of PFK isozymes in lungs of nicotine-exposed pups has a short- as well as long-term effect. It is not clear whether the changes in the expression of the PFK mRNA and PFK activity contribute to the reported deterioration of the lung parenchyma in the long-term (Maritz and Windvogel, 2003).

Results in this study, demonstrate that all three PFK isoforms, namely PFK-M, PFK-L, and PFK-C are expressed in the normal developing neonatal rat lung. It is also shown that maternal nicotine exposure during pregnancy and lactation results in over-expression of PFK-M and PFK-L, but has no influence on PFK-C mRNA

levels. It is proposed, that the inhibition of glycolysis observed by Maritz (1983) is not as a result of inhibition of PFK mRNA expression, but might be due to an inhibition of PFK itself. Furthermore, it is evident that the maturing rat lung attempts to compensate for the glycolytic inhibition by over-expressing the isoforms associated with glycolytic activity and gluconeogenic activity. The influence of maternal nicotine exposure during pregnancy and lactation on PFK at the post-translational levels still needs investigating and will be discussed later in chapter 8 on Future Perspectives.

CHAPTER 5

5. ANALYSIS OF GLYCERALDEHYDE -3- PHOSPHATE DEHYDROGENASE TRANSCRIPT LEVELS IN DEVELOPING NEONATAL LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

5.1 INTRODUCTION

Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) is a key enzyme in glycolysis (Edwards *et al.*, 1985; Reid and Masters, 1986; Ryzlak and Pietruszko, 1988) and in the gluconeogenic pathway (Souza and Radi, 1998). As one of the most abundant cellular proteins it accounts for no less than 15% of all soluble proteins **(**Scopes and Stoter, 1982**).** GAPDH is encoded by a single functional gene (Reid and Masters, 1986) located on the short arm of chromosome 12 (Bruns and Gerald, 1976). Unlike other tetrameric components of the glycolytic pathway, this enzyme is not present in multiple forms. GAPDH may also exist in vivo as a dimer or a monomer, but its glycolytic function is restricted to its tetrameric form (Mazzola and Sirover, 2003) of approximately 150 KDa (Sirover, 1999).

Studies have also revealed that GAPDH is multidimensional in function in which it displays a variety of discrete activities apart from its glycolytic function. GAPDH has been shown to be involved in fusogenic activity **(**Glaser and Gross, 1995**)**, microtubule bundling **(**Kumagai and Sakai, 1983**),** autophosphorylation (Kawamoto and Caswell, 1986), and play a part in viral pathogenesis in human cells, (Duclos-Vallee *et al.*, 1998). Furthermore, GAPDH may be involved in nuclear RNA transport, (Singh and Green, 1993), play a role in apoptosis, (Sawa *et al.*, 1997), participate in DNA repair activity (Arenaz and Sirover, 1983; Vollberg *et al.*, 1987; Vollberg *et al.*, 1989), and there has also been studies reporting that GAPDH can specifically bind to macromolecules (Ryazanov, 1985). GAPDH also binds to sequences on mRNA essential for its modulation (Nagy and Rigby, 1995), its

oxidation enhances its binding to nucleic acids, (Arutyunova *et al.*, 2003). It also interacts with glutathione, thought to be a function of oxidative stress (Schuppe-Koistinen *et al.*, 1994).

Maritz (1997) showed that maternal nicotine exposure during gestation and lactation results in the inhibition of GAPDH activity in the lungs of the offspring. This investigation provided the exact location of interference in the glycolytic pathway. However, it is not known whether nicotine causes these changes at transcriptional levels as well as at post-transcriptional level or whether it is reversible. Therefore, this division of the study investigated GAPDH mRNA expression in normal developing neonatal rat lung (1) as a function of age, and (2) also determined the impact that maternal nicotine exposure during pregnancy and lactation may have on the mRNA expression of this key glycolytic protein.

5.2 RESULTS

5.3.1 GAPDH mRNA Expression

The results summarized in Table 11 show that the GAPDH mRNA:ß-actin ratio at postnatal day 1 of both the control and nicotine-exposed rat pups are not different from the ratio on postnatal day 7 ($P>0.10$). At postnatal day 1 however, GAPDH:ß-actin mRNA expression in nicotine exposed lungs was at 0.42 ± 0.03, 1.6-fold higher than its expression in control lungs (0.26 ± 0.02). At postnatal day 7 no significant differences were detected between the control and experimental groups ($P>0.10$). While the GAPDH mRNA expression stays the same ($P>0.10$) between postnatal days 7 and 14 in control rat lung, it increased in the nicotine exposed lung from 0.31 ± 0.04 at postnatal day 7 to 1.91 ± 0.19 ($P<0.01$) at postnatal day 14, an increase of 6.2-fold. At postnatal day 14 the GAPDH:ß-actin mRNA ratio was 4.1-fold higher in the lungs of rats exposed to maternal nicotine via the placenta and mother's milk than the control lungs. Between postnatal days 14 and 21 the GAPDH:ß-actin mRNA ratio in control tissue increased from 0.47 ± 0.05 to 1.05 ±

0.12 ($P<0.01$). On the other hand, no changes are observed in the experimental group between days 14 and 21 ($P>0.10$). However, mRNA expression in this group at postnatal day 21 is still significantly different when compared to the GAPDH:ß-actin mRNA in control tissue ($P<0.05$). The mRNA expression between postnatal days 21 and 49 increased significantly in lung tissue of both the control and experimental groups. In control lungs it increased by 2.2-fold from 1.05 ± 0.12 to 2.33 ± 0.23. In lungs of nicotine exposed rats it increased by 1.6-fold from 1.59 ± 0.22 on postnatal day 21 to 2.49 ± 0.29 on postnatal day 49 ($P<0.05$). At postnatal day 49, no difference ($P>0.10$) is found between the control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation.

Fig.20 displays a comparison of the expression of GAPDH:ß-actin mRNA between postnatal day 1 and 49 in control developing rat lung and lungs exposed to nicotine during gestation and lactation. In both groups the mRNA expression significantly increases between postnatal days 1 and 21 ($P<0.001$). During this period control lung GAPDH mRNA:ß-actin ratio increases by 4.0-fold and in the experimental group it increases by 3.8-fold. From the graph it is clear that the increase in GAPDH mRNA:ß-actin ratio is gradual as the lungs of the control rats mature. However, GAPDH:ß-actin ratio in nicotine-exposed pups show a marked increase after postnatal day 7 to reach a maximum at postnatal day 14. After postnatal day 14 the rate of increase decreases so that no difference is observed at postnatal day 49 between control and nicotine-exposed rat pups.

However, it is interesting to note that levels of expression are lowest during the phase of lung development which is associated with rapid alveolarization between postnatal day 4 and 13 (Brody and Vaccaro, 1979). Expression is highest after postnatal day 14. Since many of the non-glycolytic functions of GAPDH are associated with cell division it can be expected to be higher during this phase of lung development. The findings in this division of the study are thus unexpected. No data is available to explain the above-mentioned observation, but it is possible that the increased GAPDH mRNA expression after the phase of rapid alveolarization may be

due to (1) changes in dietary needs for example surfactant synthesis and (2) increase need for GAPDH to support glucose flux through the glycolytic pathway.

Table 11: GAPDH mRNA expression in control lung tissue and lung tissue exposed to nicotine during pregnancy and lactation (GAPDH:ß-actin ratio)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=6)	(n=5)	
1	0.26±0.02	0.42±0.03	P < 0.01
	(n=6)	(n=5)	
7	0.36±0.07	0.31±0.04	P > 0.10
1 vs 7	P > 0.10	P < 0.10	
	(n=9)	(n=6)	
14	0.47±0.05	1.91±0.19	P < 0.01
7 vs 14	P > 0.10	P < 0.01	
	(n=8)	(n=7)	
21	1.05±0.12	1.59±0.22	P < 0.05
14 vs 21	P < 0.01	P > 0.10	
	(n=9)	(n=7)	
49	2.33±0.23	2.49±0.29	P > 0.10
21 vs 49	P < 0.001	P < 0.05	

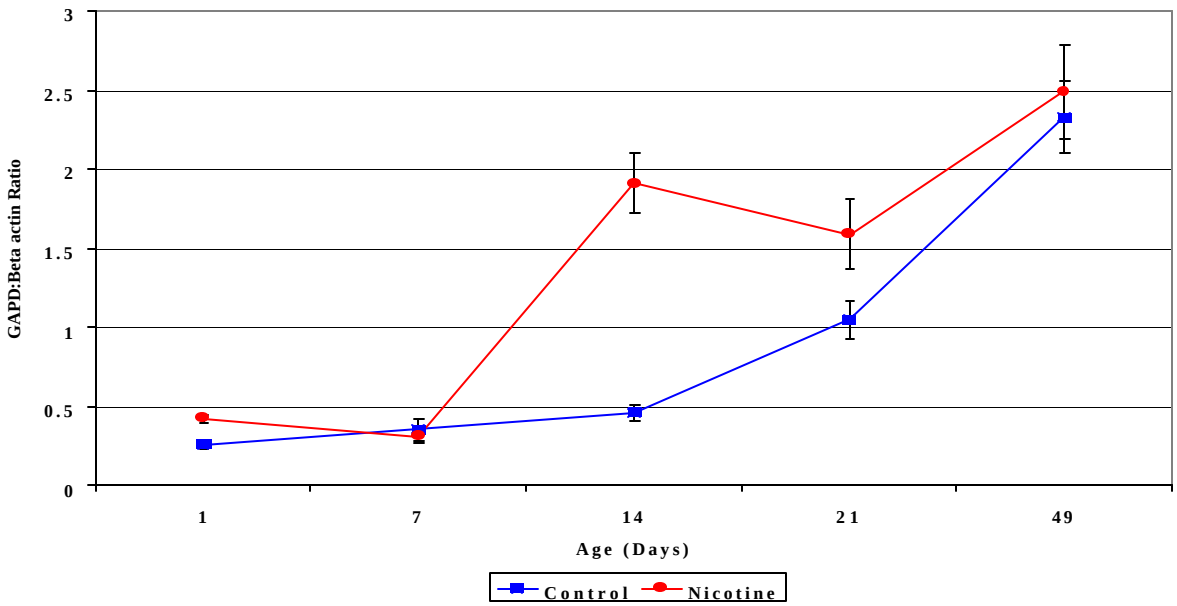


Fig.20. The influence of maternal nicotine exposure on GAPDH:ß-actin mRNA expression in developing neonatal rat lung.

5.3 DISCUSSION

GAPDH has long been recognized as a classical glycolytic protein and has been used as a "housekeeping" gene in studies of genetic expression and regulation. Recent studies have shown that, apart from its role in glycolysis and gluconeogenesis (Souza and Radi, 1998), it displays diverse non-glycolytic functions depending on its subcellular location, for example apoptosis (Mazolla and Sirover, 2003) and apoptosis is known to play an important role in lung growth and development. It is therefore plausible that the expression of GAPDH mRNA and activity of GAPDH will change as the lung matures to meet the demand for energy and for growth and development of the maturing lung. It is therefore also conceivable that an interference with changes in expression and activity of GAPDH that supports growth and development might have an adverse impact on lung development. If this interference causes permanent changes in the activity of this enzyme it might have an adverse effect on the maintenance of respiratory health in the long term.

The results in this study illustrate an increase in the GAPDH mRNA expression of 9.0-fold from postnatal day 1 to postnatal day 49 in control rat lung. The expression of GAPDH in developing lungs exposed to nicotine during pregnancy and lactation increased by 5.9-fold during the same period of time. However, this is considerably different when compared to control lung. While it initially was higher at postnatal day 1 than in control lung, the GAPDH mRNA expression in nicotine exposed lung was the same as that of the control lungs at postnatal day 7. When comparing the change in expression over time it is clear that the expression of GAPDH mRNA of control lung increased gradually between postnatal days 1 and 49. On the other hand, GAPDH mRNA expression in lungs of nicotine exposed rat pups showed a pronounced increase in expression after postnatal day 7 and reached a maximum at postnatal day 14 (a 6.1-fold increase versus the 1.3-fold increase of the control animals), where after it showed no further increase so that it was the same on postnatal day 49 as that of the control animals.

It is interesting to note that this rapid increase in GAPDH mRNA expression in the lungs of the nicotine exposed rat pups occurred during the phase of rapid alveolarisation between postnatal days 4 and 13 (Brody and Vaccaro, 1979), while no such pronounced increase occurred in the lungs of the control rat pups of the same age. This pronounced increase in GAPDH mRNA expression is most likely due to the inhibition of GAPDH in the lungs of rat pups that were exposed to nicotine via the placenta and mother's milk (Maritz, 1997).

Studies show (Maritz, 2002) that maternal nicotine exposure results in a gradual deterioration of the lung parenchyma of the offspring even after nicotine withdrawal. It was suggested that maternal nicotine exposure during gestation and lactation induces changes in the "programming' for the long term maintenance of lung integrity resulting in a quicker aging of the lungs of the nicotine exposed offspring. In this study, it was clearly shown that GAPDH mRNA expression returned to normal after nicotine withdrawal on postnatal day 21. This implies that over expression of GAPDH mRNA only occurred while the rat pups were exposed to nicotine. Maternal nicotine exposure, therefore, had no apparent impact on the "programming" of GAPDH mRNA expression, and thus on the long-term glycolytic and non-glycolytic functions of GAPDH and its role in lung maintenance as the animal ages. It is therefore unlikely that changes in the glycolytic and non-glycolytic functions of this enzyme will have any effect on the gradual deterioration of the lung parenchyma of nicotine-exposed rats.

The mechanism involved in GAPDH inhibition still remains unclear. However, GAPDH is especially vulnerable to the effects of oxidants (Knight *et al*, 1996). Studies show that superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) produced during oxidative stress conditions lead to the inactivation of GAPDH and thus this enzyme is recognized as an oxidant sensitive enzyme of the glycolytic pathway (Molina *et al.*, 1992; Stamler, 1994; McDonald and Moss, 1993; Mohr *et al.*, 1996). Nicotine has been associated with the production of oxygen reactive species. This alkaloid is chemotactic and leads to the increased responsiveness of polymorphonuclear

leukocytes (PMNs) to activated complement C_{5a} and thus producing oxygen free radicals (Totti *et la.*, 1984). Nicotine can also disrupt the mitochondrial respiratory chain leading to leakage from the electron transport chain (Gvozdjakova, *et al.*, 1992) and thus the formation of superoxide anions and hydrogen peroxides (H_2O_2). Studies performed on pancreatic tissue slices in rat, show that nicotine in a concentration comparable to that found in the saliva of smokers causes free radical production and consequently membrane damage in the pancreatic tissue (Wetscher *et al*., 1995). Nicotine is also capable of evoking long lasting nitric oxide (NO) release in the rat hippocampus (Smith *et al*., 1998). Thus, there is direct evidence implicating nicotine with the formation of reactive oxygen species and thus possibly the inhibition of GAPDH.

It is therefore plausible that exposure of pre- and post-gestational lung to nicotine can result in the formation of reactive oxygen species, which can then inactivate GAPDH. Consequently, inhibition of GAPDH results in the interference of carbohydrate metabolism, as observed by Maritz (1986). However, studies in the last decade have been investigating a toxic product, peroxynitrite (PN) anion, generated from the simultaneous production of NO and superoxides (Pryor and Sqauditro, 1996; Szabó, 1996). In *in vitro* systems, the ratio of superoxide and NO determines the reactivity of the PN, where excess NO reduces PN oxidative reactions (Rubbo *et al.*, 1994; Miles *et al.*, 1996). PN is capable of oxidizing thiols (Radi *et al.*, 1991), sulfides (Padmaji *et al.*, 1996), transition metal complexes (Goldstein and Czapski, 1995), halide ions (Goldstein and Czapski, 1995), ascorbate (Barlett *et al.*, 1995) and other aromatics. Even though PN can perform a variety of oxidation and nitration reactions among the most relevant, are the reactions with the thiol (-SH) groups (Souza and Radi, 1998).

PN has been classified as more cytotoxic than NO or superoxide in a variety of experimental systems. The presence of PN has been accompanied by marked changes in the levels of cellular energetics and DNA intergrity (Szabó, 1996). Zingarelli *et al.* (1996) has shown that endogenously produced PN in macrophages

from NO and superoxide results in DNA strands breakage and that the time course of the strand breakage parallels the time course of NO and PN production. Furthermore, studies performed in motor neurons axotomy and PN exposure results in a time dependent accumulation of DNA single strand breaks (Liu and Martin, 2001). Selected cytotoxic processes initiated by PN, have been documented by Szabó (1996). Thus the relationship between nicotine, PN and inhibition of GAPDH may well be the critical links to elucidating the mechanism behind glycolytic inhibition in developing rat lung.

Studies investigating myocardial ischemia-reperfusion may provide critical links to explaining the glycolytic inhibition observed. The significance of GAPDH in regulating myocardial glycolysis has long been recognized (Knight *et al.*, 1996). During anoxic (Williamson, 1996) and ischemic (Rovetto *et al.*, 1975) conditions GAPDH's contribution to glycolytic regulation increases. During reperfusion GAPDH's activity is inhibited and this inhibiton is thought to be attributed to covalent modification of the GAPDH enzyme (Knight *et al.*, 1996). Furthermore, Lineweaver-Burk plots show a noticeable reduction in V_{max} without significant change in K_m for glyceraldehyde-3-phosphate (GAP), consistent with a non-competitive form of inhibition or a decrease in the amount of enzyme (Knight *et al.*, 1996). A likely mechanism for the covalent modification is the action of the reactive oxygen species. During myocardial reperfusion superoxide anions, NO, H_2O_2, and hydroxyl radicals are produced (Schultz and Wambolt, 1995; Bolli, 1990). When GAPDH is exposed to H_2O_2 the critical reactive thiol group of cystein-149 is oxidized resulting in the loss in the activity of the enzyme (Brodie and Reed, 1990; Vaidyanathan *et al*, 1993; Janero *et al.*, 1994). GAPDH has been implicated as one of the primary targets of NO, and the loss of activity has been attributed to the linkage of NAD(H) to a cystein residue of the active site (Souza and Radi, 1998). It suggested that NO could mediate GAPDH inactivation independent of NAD(H)-dependent covalent modification. Under anaerobic conditions inactivation of GAPDH by authentic NO is slow, demonstrated by a slow NO-mediated oxidation of thiols (Souza and Radi, 1998) whereas under aerobic conditions NO-mediated

inactivation is rapid. Studies by Souza and Radi (1998) demonstrated that GAPDH is highly reactive to PN and that this reactivity is mostly dependent on the rather unique properties of the critical thiol group, Cys-149. The high sensitivity is due to a high second-order rate constant obtained for the GAPDH-PN reaction and the 4:1 (PN:GAPDH tetramer ratio) inactivation stoichiometry. Maximal inactivation is strongly pH-dependent and results at an alkaline pH, although significant inactivation is also observed at an acidic pH. Since GAPDH accounts for no less than 15% of all soluble proteins **(**Scopes and Stoter, 1982**),** it is possible that inactivation of GAPDH by PN may present a significant mechanism, which may alter glycolysis (during the rapid phase of alveolarization), gluconeogenesis as well as other non-carbohydrate functions associated with this enzyme.

Another significant set of reactions as a consequence of the presence of nicotine that may result in GAPDH inactivation also stems from the formation of reactive oxygen species. Superoxide over-production (Du *et al.*, 2003), and reactive NO species such as PN (Kim *et al.*, 2001), can directly damage DNA (Crow and Beckman, 1995; Beckman and Koppenol, 1996). When DNA is damaged, poly (ADP-ribose) polymerase (PARP), a nuclear DNA-repair enzyme is activated and consequently GAPDH is inhibited. The nuclear PARP isoform, $PARP_1$ is exclusively responsible for GAPDH poly (ADP-ribosyl)ation. Inactivation of GAPDH results when $PARP_1$ catalyzes the addition of ADP-ribose units from NAD^+ to, nuclear proteins (Du *et al.*, 2003). It is shown that during cell death, GAPDH translocates into the nucleus (Sawa *et al.*, 1997; Schmitz, 2001). Thus when GAPDH undergoes poly (ADP-ribosyl)ation it probably occurs with nuclear translocation. The extent of inhibition of GAPDH by PARP may have a significant impact on carbohydrate metabolism in conjuction with the direct inactivation of GAPDH by the reactive oxygen species (ROS) already discussed.

The structural and functional integrity of a developing and maturing fetal and neonatal lung is critically dependent on carbohydrate metabolism (Tierney and Levy, 1976; Gilden *et al.*, 1977; Maniscalco *et al.*, 1978; Bourbon and Jost, 1982).

Maternal nicotine exposure during pregnancy and lactation results in the inhibition of glycolysis in developing rat lung (Maritz, 1983; 1986). Clearly exposure of neonatal lung to nicotine compromises the structural and functional integrity of the developing organ. The studies discussed above provide sound theories that may aid in understanding the damage observed in developing rat lung by maternal nicotine exposure (Maritz and Woolward, 1992; Maritz *et al.*, 1994; Maritz and Najaar, 1995; Maritz and Windvogel, 2003).

It is therefore proposed that inhibition of GAPDH activity by maternal nicotine exposure in developing rat lung may be as a result of the following mechanism: Maternal nicotine exposure during pregnancy and lactation results in over-expression of ROS, namely superoxides, NO, and PN in the lungs of rat pups. The oxidants are produced by activated macrophages and PMNs in response to the presence of nicotine (a chemotactic product). These reactive oxygen species react with the critical thiol group, Cys-149 on GAPDH and inactivate the enzyme. GAPDH is a key regulatory enzyme in glycolysis and gluconeogenesis, and inhibition of the enzyme leads to interference with carbohydrate metabolism. Furthermore, the neonatal lung over-expresses the enzyme at the transcriptional level in an attempt to compensate for activity loss, which is not as a result of substrate inhibition. Interference with carbohydrate metabolism results in an increase in glucose turnover, accompanied by a decrease in lactate production. The high levels of oxidants cause direct damage to DNA and this activates the DNA-repair enzyme, PARP. More particularly the isoform $PARP_1$ that specifically causes poly (ADP-ribosyl)ation of GAPDH and results in its inactivation. Since GAPDH accounts for no less that 15% of all soluble protein, the combination of oxidant reactivity with GAPDH and PARP poly (ADP-ribosyl)ation of GAPDH presents with a significant mechanism that can result in glycolytic inhibition and therefore significant interference in carbohydrate metabolism.

In conclusion it is clear that although maternal nicotine exposure results in the inhibition of GAPDH activity, and over-expression of GAPDH mRNA takes place

only when nicotine is present, it has no long-term effects on GAPDH mRNA expression. Thus maternal nicotine exposure also has no effect on the "programmed" deterioration of lungs of rats exposed to nicotine via the placenta and mother's milk.

CHAPTER 6

6. ANALYSIS OF LACTATE DEHYDROGENASE ISOENZYME PATTERNS AND TRANSCRIPT LEVELS IN DEVELOPING NEONATAL LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

6.1 INTRODUCTION

The adult lung, like most other organs can oxidize to a certain degree glucose, fatty acids, amino acids, lactate and glycerol (Rhoades, 1974). Under normal physiological conditions the rate of glucose oxidation is highest in the lung in comparison to the oxidation of all of the other above-mentioned substrates (Shaw and Rhoades, 1977). This substrate, upon entering the lung is mainly metabolized via the glycolytic pathway after which 40 to 50% of it exits as lactate (Bassett and Fisher, 1976).

The rate at which lactate is produced remains an impressive feature of the lung. Despite the high partial pressure for oxygen, the rate of lactate production remains consistently high, accounting for almost 10% of the total body lactate production (George et al., 1978). Thus the rate at which lactate is produced is an important marker for the activity of the glycolytic pathway in the lung (Kerr et al., 1979).

The cytoplasmic enzyme lactate dehydrogenase (LDH) catalyses the interconversion between pyruvate and lactate. In vertebrates, LDH is a tetramer and may be found in five isoenzyme forms. These isoenzymes exist as a result of the random association of two sub-unit types, namely; H-type and M-type (Laplace-Marieze et al., 1994). The two peptide chains are under individual genetic control and the five isoforms differ in their molecular structure (Reid and Masters, 1985). A third sub-unit (LDH C) also exists in mammalian tissue, but is exclusively expressed in the testis following the onset of spermatogenesis (Skidmore and Beebee, 1990).

The H-type sub-unit is associated with oxidative metabolism and the M-type sub-unit is associated with anaerobic metabolism (Leberer and Pette, 1984). The isoenzymes are designated LD-1 (H_4), LD-2 (H_3M), LD-3 (H_2M_2), LD-4 (HM_3) and LD-5 (M_4) in order of their decreasing anodal mobility in an alkaline medium (Drent et al., 1996).

Assessing LDH isoenzyme patterns provides valuable information with regards to tissue oxygen utilization and the need of that tissue to utilize anaerobic glycolysis (Cahn et al., 1962; Dawson et al., 1964). It also reflects the balance between the rate of synthesis and degradation of each isoform (Kachmar and Moss, 1976).

To date, very little work has been performed on LDH isoenzyme developmental patterns in normal developing rat lung. Clinically, LDH isoform patterns are being recognized as powerful tools in the diagnosis of certain lung conditions. For example, an isomorphic isoenzyme pattern is observed in the serum of patients with pulmonary alveolar proteinosis (Hoffman and Rogers, 1991). When analyzed in the BALF of the same patients, LD-1 and LD-2 are lower in percentage and LD-3, -4 and –5 are higher. Rats injected with small doses of monocrotaline pyrrole (MCTP), a substance that causes injury only to the lung and no other organ, showed an increase in LD-4 and LD-5 (Schultze et al., 1994).

The lung in comparison to most tissues consists of a heterogeneous mixture of cell types (George et al., 1978). The alveolar surface of a postnatal lung is composed of up to 90% type I pneumocytes (Naimark, 1977). They have very few mitochondria compared to the type II pneumocytes and depend heavily on glycolysis for ATP production (George et al., 1978). The type II pneumocytes, macrophages, lymphocytes, to name a few, are predominantly oxidative, thus illustrating the diversity of the metabolic states of the cells of the lung.

As a neonatal lung matures and develops, so too does the physiological need of the tissue. Monitoring the developmental isoenzymatic patterns of LDH in the lung

tissue homogenate should provide a clear understanding with respect to the overall metabolic state of the lung as it grows and matures.

This section of research aims: a) To determine the effect of lung growth and maturation as the animal ages and, b) To determine the influence of maternal nicotine exposure during gestation and lactation on 1) the changes, if any, of the LD-isoenzymes 2) and, the expression of the H and M subunits at the mRNA levels of the offspring as the lung matures. This would give an indication of the changes in the metabolic status of the lung as it matures, and the impact of maternal nicotine exposure.

6.2 RESULTS

6.2.1 Lactate Dehydrogenase (LDH) Isoenzyme Pattern Analysis in rat lung tissue as a function of age and the effect of maternal nicotine exposure during gestation and lactation.

6.2.1.1. LD-1 (%Density/mg protein)

The data summarized in Table 12 show that the %Density/mg protein of LD-1 in control and nicotine exposed rat lung at postnatal days 1 and 7 were the same ($P>0.01$). Between postnatal days 7 and 14 LD-1 increased by 1.8-fold in the experimental group (66.49 ± 7.54 to 118 ± 20.38). In lungs of the control animals the values on postnatal day 7 is the same as on postnatal day 14 ($P>0.10$) (77.83 ± 6.80 to 61.78 ± 7.41).

LD-1 remains higher in the nicotine exposed lungs than in the lungs of the control animals from postnatal day 14 for the duration of the study. At postnatal day 21 a slight decrease is observed in experimental lungs, in which LD-1 (%Density/mg protein) in the nicotine-exposed animals is 1.4-fold higher than controls ($P<0.05$). At postnatal day 49 LD-1 in the experimental lung tissue is 1.8-fold higher ($P<0.001$)

than that in the control tissue (66.53 ± 6.61 vs 36.55 ± 3.75). The %Density decreased significantly by 43.77% from day 21 to day 49 (P<0.001) in control tissue in comparison to the 28.11% decline (P<0.10) observed in experimental lung tissue. Fig.21 illustrates a declining trend from day 1 to day 49 in both groups; in control animals the % density at day 1 is 2.4-fold higher than at day 49 (P<0.001), 85.71 ± 6.02 and 36.55 ± 3.75, respectively. In the nicotine exposed animals it decreased by the % density at day is 1.4–fold higher at day 1 than at day 49 (P<0.02), 95.23 ± 5.4 and 66.53 ± 6.61, respectively.

Table 12: Changes in LD-1 in developing lung and the influence of maternal nicotine exposure during gestation and lactation on LD-1 in lungs of the offspring (% Density/mg Protein)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=8)	
1	85.71±6.02	95.23±5.4	P > 0.10
	(n=6)	(n=7)	
7	77.83±6.80	66.49±7.54	P > 0.10
1 vs 7	P > 0.10	P < 0.05	
	(n=7)	(n=8)	P < 0.05
14	61.78±5.76	119.46±17.71	
7 vs 14	P > 0.10	P < 0.05	
	(n=7)	(n=8)	P < 0.05
21	65.00±3.91	92.54±8.33	
14 vs 21	P > 0.10	P > 0.10	
	(n=8)	(n=6)	P < 0.001
49	36.55±3.75	66.53±6.61	
21 vs 49	P < 0.001	P < 0.01	

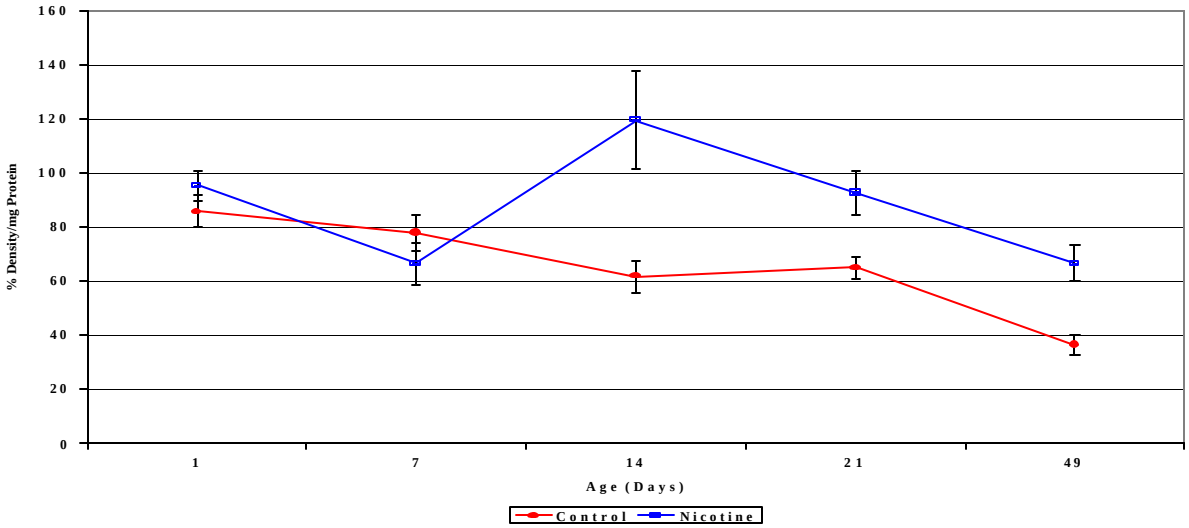


Fig.21. Effect of maternal nicotine exposure during gestation and lactation on the LD-1 pattern in developing rat lung.

6.2.1.2. LD-2 (%Density/mg protein)

The data summarized in Table 13 show there is no difference between the two groups at day 1 ($P>0.10$) and at day 7 ($P>0.10$) and LD-2 increases in both control ($P<0.001$) and experimental groups ($P<0.02$) between these age groups (control vs nicotine: 43.38 ± 3.18 and 35.31 ± 3.89). At day 14 however, LD-2 has decreased by 2.0-fold ($P<0.001$) in control rat lungs to 21.65 ± 2.83 in comparison to an increase of 2.0 fold (35.31 ± 3.89 to 71.9 ± 12.48) observed in the lungs of nicotine-exposed rats. This significant change is very similar to that of the LD-1 pattern in which it increased by 1.8 fold (Table 12) at this stage of development in the lung. At day 14 this isoform is significantly greater (3.3 fold) in nicotine exposed rat lung in comparison to that found in controls ($P<0.001$). At day 21, the % density in control lung (29.59 ± 1.75) is not different ($P<0.10$) from those found in 14-day old lung (21.65 ± 2.83). In nicotine-exposed lung LD-2 decreases by 3.3-fold ($P>0.10$). These findings are also similar to the trend of LD-1 observed in control and experimental rat lung tissue. By day 49 LD-2 decreased to 11.07 ± 1.03 in control lung tissue, a difference of 2.0-fold ($P<0.02$) from day 21. In the nicotine exposed animals there is no difference between day 21 and 49 ($P<0.10$). However at postnatal day 49 LD-2 is 3.3-fold higher in the experimental lungs when compared to the control tissue

(P<0.001). There is a significant decline of 2.3-fold (P<0.02) in the control rat lungs between day 1 and day 49 (25.93 ± 2.68 to 11.07 ± 1.03). In comparison, LD-2 increased markedly (P<0.01) by 1.6-fold in the experimental rat lungs (23.24 ± 2.35 to 36.45 ± 3.12).

Fig.22 clearly shows a decreasing trend in the lungs of control animals between postnatal days 7 and 49. In contrast, results from the lungs of nicotine-exposed rat pups shows an increase from postnatal day 7 to postnatal day 14.

Table 13: Changes in LD-2 in developing lung and the influence of maternal nicotine exposure during gestation and lactation on LD-2 in lungs of the offspring (% Density/mg Protein)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=8)	
1	25.93±2.68	23.24±2.35	P > 0.10
	(n=7)	(n=8)	
7	43.38±3.18	35.31±3.89	P > 0.10
1 vs 7	P < 0.001	P < 0.02	
	(n=8)	(n=7)	
14	21.65±2.83	71.9±12.48	P < 0.001
7 vs 14	P < 0.001	P < 0.10	
	(n=7)	(n=8)	
21	29.59±1.75	39.91±4.43	P < 0.10
14 vs 21	P < 0.10	P > 0.10	
	(n=8)	(n=7)	
49	11.07±1.03	36.45±3.12	P < 0.001
1 vs 49	P < 0.02	P > 0.10	

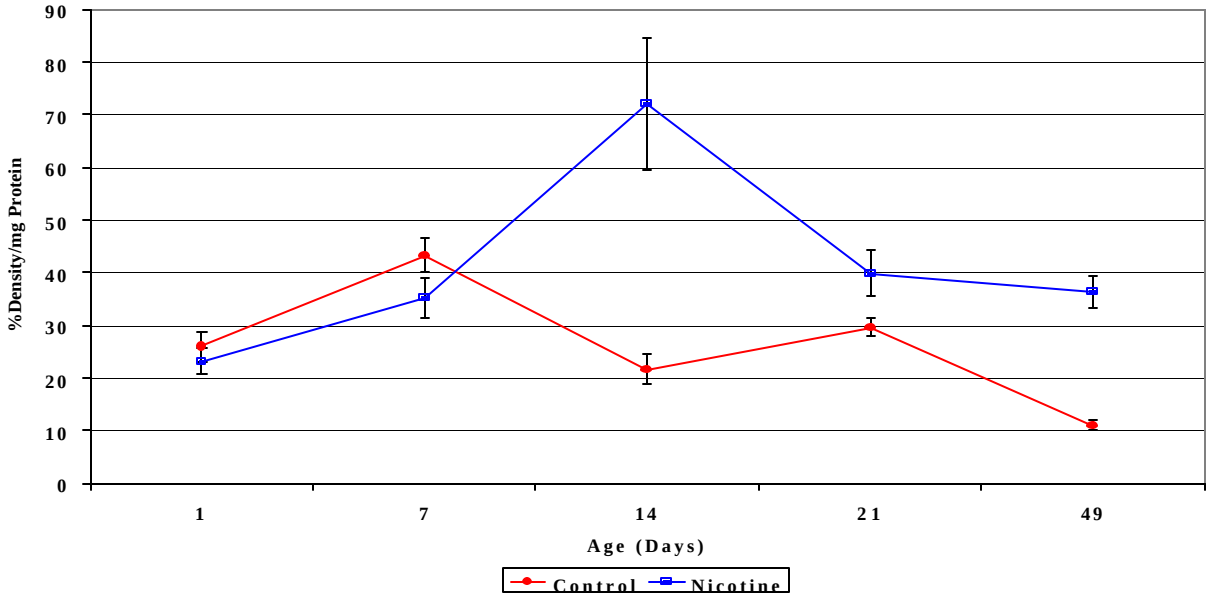


Fig. 22: Effect of maternal nicotine exposure during gestation and lactation on the LD-2 pattern in developing rat lung.

6.2.1.3. LD-3 (%Density/mg protein)

The data in Table 14 shows that at day 1 and at day 7 no differences occur between the two groups analyzed (P>0.10, for both groups). An increase in LD-3 of 1.6-fold (P<0.01) (28.28 ± 2.66 to 43.70 ± 3.19) and 1.5-fold (P<0.02) (23.25 ± 2.12 to 35.83 ± 3.97) is noted from day 1 to day 7 in control and experimental lung; respectively. There is a marked decrease of 2.3-fold (P<0.001) between day 7 and 14 in the control group (43.70 ± 3.19 to 19.06 ± 1.65). In contrast, LD-2 in the experimental group increases (P<0.05) by 1.9-fold (35.83 ± 3.97 to 68 ± 11.7). Thus at day 14 LD-3 in the nicotine exposed lung is 71.97% higher than in the control lungs (P<0.001). There is an increase of 1.5-fold (P<0.01) in the control lungs between day 14 and day 21, whereas LD-2 decreased by 1.8-fold in the lungs of nicotine exposed rat pups. At day 21 LD-3 in nicotine-exposed lung is 1.4-fold higher (P<0.01) than the control lungs. By day 49 LD-3 has decreased by 2.5-fold (P<0.001) in control lungs and by only 1.1-fold (P>0.10) in nicotine exposed lungs from day 21. At day 49 LD-3 is found to be significantly greater (P<0.001) in the

latter group than in the control group (36.99 ± 3.16 and 11.45 ± 1.13; respectively). The results obtained also show that LD-3 significantly decreased (P<0.001) from day 1 to day 49 in control lung (a drop of 2.5-fold). In the nicotine exposed lung the % Density increased by 1.6-fold (P<0.01). These findings are very similar to the effect maternal nicotine exposure has on LD-2 patterns, with the exception of the changes observed between day 14 and 21 in the control rat lung. This is clearly shown when comparing the LD-2 and LD-3 graphs (Fig.22 and Fig.23).

The graphic results of LD-3 (Fig.23) shows a decreasing trend over time in the control lung tissue. Although there were significant increases in LD-3 %Density/mg protein between postnatal days 1 and 7, and 14 and 21, the %Density/mg protein show a decreasing trend so that on postnatal day 49 the %Density/mg protein of LD-3 was lower (P<0.001)) than on postnatal day 1. Contrary to this the %Density/mg protein of LD-3 was higher (P<0.01) in lung tissue of the nicotine-exposed rats on postnatal day 49 than on postnatal day 1.

Table 14: Changes in LD-3 in developing lung and the influence of maternal nicotine exposure during gestation and lactation on LD-3 in lungs of the offspring (% Density/mg Protein)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=8)	
1	28.28±2.66	23.25±2.12	P > 0.10
	(n=7)	(n=8)	
7	43.70±3.19	35.83±3.97	P > 0.10
1 vs 7	P < 0.01	P < 0.02	
	(n=8)	(n=7)	
14	19.06±1.65	68±11.7	P < 0.001
7 vs 14	P < 0.001	P > 0.10	
	(n=7)	(n=8)	
21	28.76±1.83	40.67±5.04	P < 0.01
14 vs 21	P < 0.01	P > 0.10	
	(n=8)	(n=7)	
49	11.45±1.13	36.99±3.16	P < 0.001
21 vs 49	P < 0.001	P > 0.10	

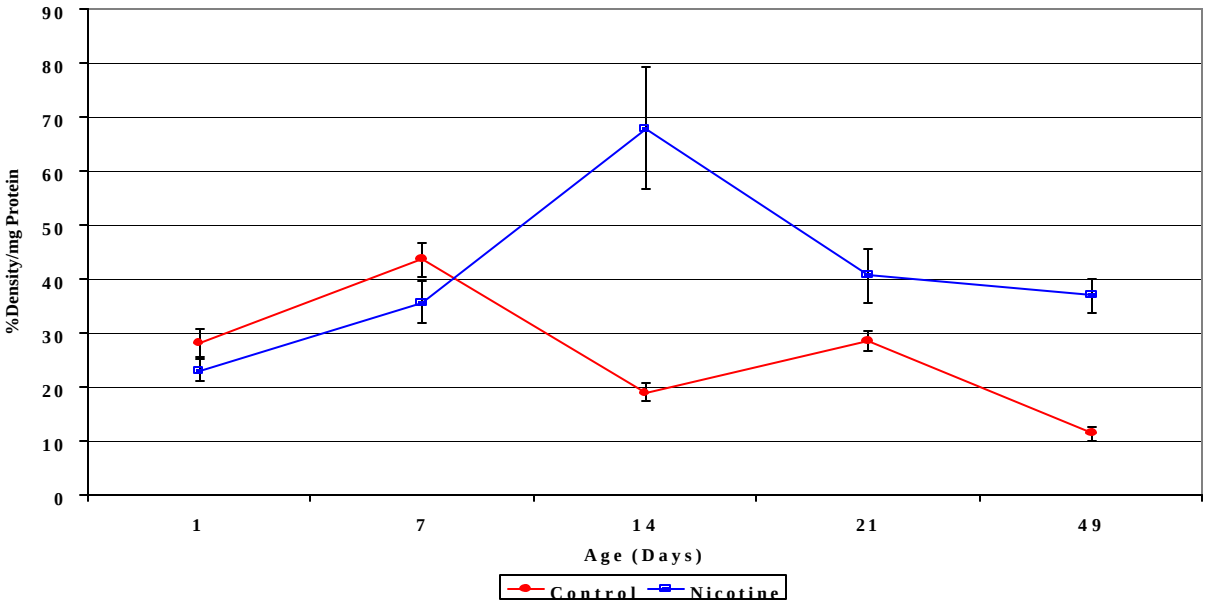


Fig.23: Effect of maternal nicotine exposure during gestation and lactation on the LD-3 pattern in developing rat lung.

6.2.1.4. LD-4 (%Density/mg protein)

Results in Table 15 show no changes between control and nicotine exposed animals at postnatal days 1 and 7 ($P>0.10$ for both). Between postnatal days 1 and 7 LD-4 increased by 1.8-fold ($P<0.01$) in the control group and by 2.1-fold ($P<0.01$) in the experimental group. At day 14 this isoform is markedly greater (4.1-fold) in the nicotine-exposed group ($P<0.001$) when compared to controls. Between postnatal days 7 and 14 LD-4 decreased significantly by 2.5-fold in the controls, whereas a marked increase from 30.77 ± 3.56 to 61.7 ± 9.73 ($P<0.01$) is observed in the experimental group. LD-4 remains significantly higher in the nicotine exposed lungs for postnatal days 21 and 49 when compared to the lungs of the control rats of the same age groups ($P<0.01$, $P<0.001$; respectively), despite the fact that it decreases by 1.9-fold ($P<0.05$) between postnatal days 14 and 21 in the nicotine exposed lungs, and increased by 1.4-fold ($P<0.05$) in the control lung. LD-4 remains relatively the same between postnatal days 21 and 49 in the nicotine exposed group,

but it decreases by 2.5-fold (P<0.001) in the control animals. Overall (Fig.24), between postnatal days 1 and 49, LD-4 decreased by 2.5-fold (P<0.001) in the control lungs (20.46 ± 2.97 to 8.25 ± 0.73), while it increased by 2.1-fold (P<0.001) in the nicotine exposed lungs (14.82 ± 1.5 to 31.25 ± 2.79).

Table 15: Changes in LD-4 in developing lung and the influence of maternal nicotine exposure during gestation and lactation on LD-4 in lungs of the offspring (% Density/mg Protein)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=8)	
1	20.46±2.97	14.82±1.5	P > 0.10
	(n=7)	(n=8)	
7	37.48±2.42	30.77±3.56	P > 0.10
1 vs 7	P < 0.01	P < 0.01	
	(n=8)	(n=7)	
14	15.11±1.89	61.7±9.73	P < 0.001
7 vs 14	P < 0.001	P < 0.10	
	(n=7)	(n=8)	
21	20.88±1.68	31.86±3.30	P < 0.01
14 vs 21	P < 0.05	P > 0.10	
	(n=7)	(n=7)	
49	8.25±0.73	31.25±2.79	P < 0.001
21 vs 49	P < 0.001	P > 0.10	

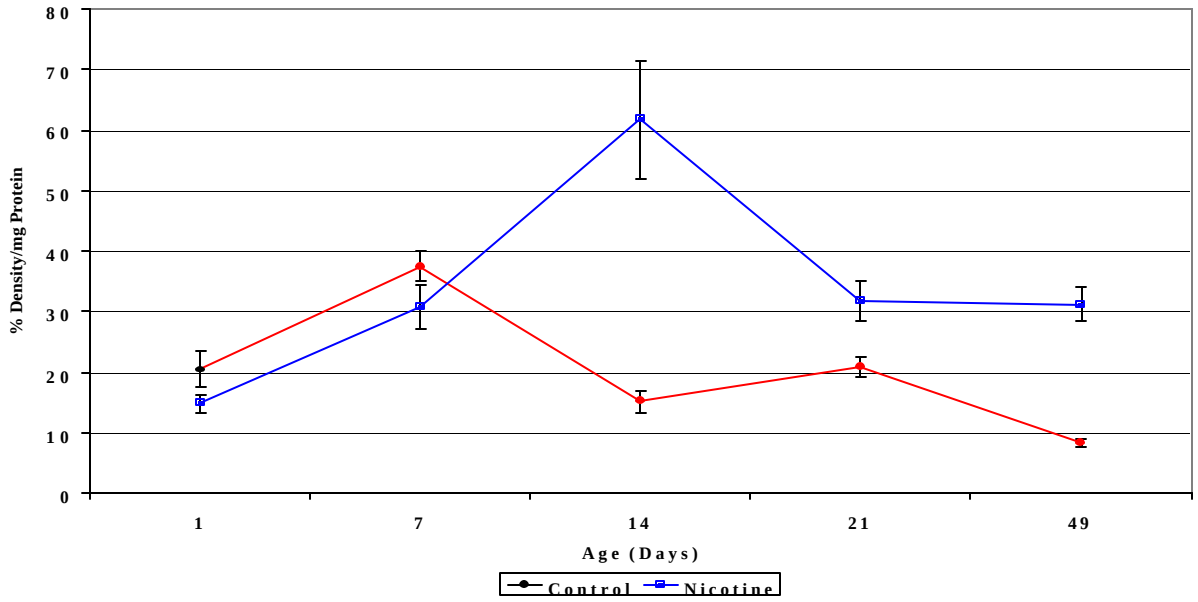


Fig.24: Effect of maternal nicotine exposure during gestation and lactation on the LD-4 pattern in developing rat lung.

6.2.1.5. LD-5 (%Density/mg protein)

LD-5 is a homotetramer of the glycolytic sub-unit of LDH. The data summarized in Table 16 and the developmental trend (Fig.25) shows definite changes between the individual age groups in the control as well as the experimental animals. Between postnatal days 1 and 7 LD-5 increases by 1.8-fold ($P<0.01$) from 20.29±2.89 to 37.27±3.01 in the control lung tissue, and by 2.0-fold ($P<0.01$) from 14.66±1.54 to 29.29±3.37 in the nicotine exposed lung. There are no differences between the groups at postnatal days 1 and 7 ($P> 0.10$). The isoform's % Density significantly decreased from 37.27 ± 3.01 to 15.38 ± 0.54 ($P<0.001$) in control lungs between postnatal days 7 and 14. In contrast, it markedly increased in the nicotine-exposed lungs from 29.29 ± 3.37 to 60.54 ± 10.18 ($P<0.05$). At this stage of development LD-5 is higher (3.9-fold) in the experimental tissue than in the control tissue ($P<0.001$) and it maintains this elevated level of significance up until day 49. Between postnatal days 14 and 21 this isoform increased by 1.6-fold ($P<0.01$) in the

control group and decreased by 1.3-fold in the nicotine exposed lung. At day 49 LD-5 reaches its lowest value of 11.9 ± 0.91 in the control tissue and in the experimental lung it seems to plateau at a value of 37.01 ± 3.2. Analysis performed between day 1 and day 49 in control rat lung shows a significant decrease of 1.7-fold ($P<0.05$) over this time period. In the experimental lungs a marked increase of 2.5-fold ($P<0.001$) is observed.

Table 16: **Changes in LD-5 in developing lung and the influence of maternal nicotine exposure during gestation and lactation on LD-5 in lungs of the offspring (% Density/mg Protein)**

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=8)	
1	20.29±2.89	14.66±1.54	P > 0.10
	(n=7)	(n=8)	
7	37.27±3.01	29.29±3.37	P > 0.10
1 vs 7	P < 0.01	P < 0.01	
	(n=7)	(n=8)	
14	15.38±0.54	54.71±10.6	P < 0.02
7 vs 14	P < 0.001	P < 0.05	
	(n=7)	(n=8)	
21	24.02±1.57	42.65±4.47	P < 0.001
14 vs 21	P < 0.01	P > 0.10	
	(n=8)	(n=7)	
49	11.90±0.91	37.01±3.2	P < 0.001
21 vs 49	P < 0.01	P > 0.10	

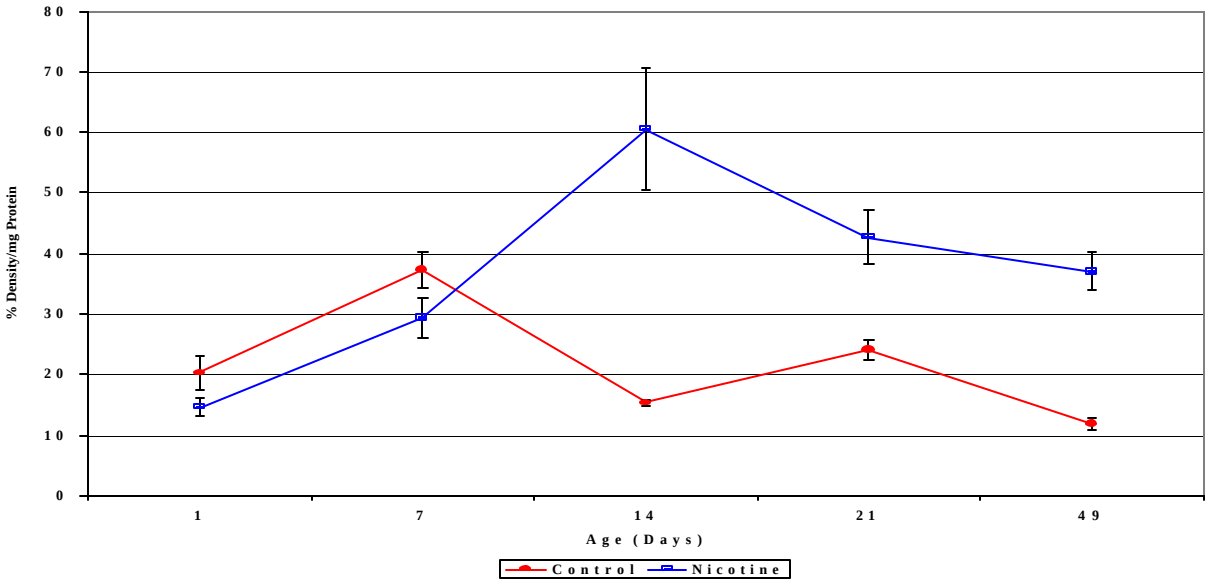


Fig.25. Effect of maternal nicotine exposure during gestation and lactation on the LD-5 pattern in developing rat lung.

6.2.1.6 Comparison of LDH Isoenzyme Patterns in Control Developing Lung and Lungs Exposed to Nicotine during gestation and lactation.

Overall, results attained in this study with the aid of electrophoretic and densitometry techniques, undoubtedly shows that LD-1 (Fig. 26 and Fig. 27) is the dominant LDH isoform in the developing rat lung. Comparisons between LD-1 and LD-5 reveal that LD-5 in the developing rat lung responds in a more pronounced way to the exposure of maternal nicotine than LD-1. The LD-5 decreases by 2.4-fold ($P<0.05$) between postnatal days 7 and 14 whereas the increase observed in the LD-1 was 1.8-fold ($P<0.05$). Furthermore, at postnatal day 49 in control animals, LD-1 is 3.1-fold higher than LD-5 ($P<0.001$). However, in the nicotine-exposed animals LD-1 is 1.8-fold higher than LD-5 ($P<0.01$). This clearly demonstrates that maternal nicotine has a more profound effect on LD-5, the glycolytic isoform than on LD-1, the oxidative form.

All the LDH isozymes in control lung show the same developmental pattern as the animal age. The lowest levels occurring in the mature lung. Apart from LD-1, which shows a gradual decrease in % Density, all the other LDH isozymes display an increase after postnatal day 1 which again decreases after postnatal day 7. Maternal nicotine exposure also changes the developmental patterns of the LDH isoenzymes resulting in significantly higher % Densities in all the isoforms after postnatal day 7. In contrast LD-1 shows a gradual decrease from postnatal day 1, it decreases between postnatal day 1 and 7, after which it again increases between day 7 and 14 to its highest level (119.46 ± 17.71).

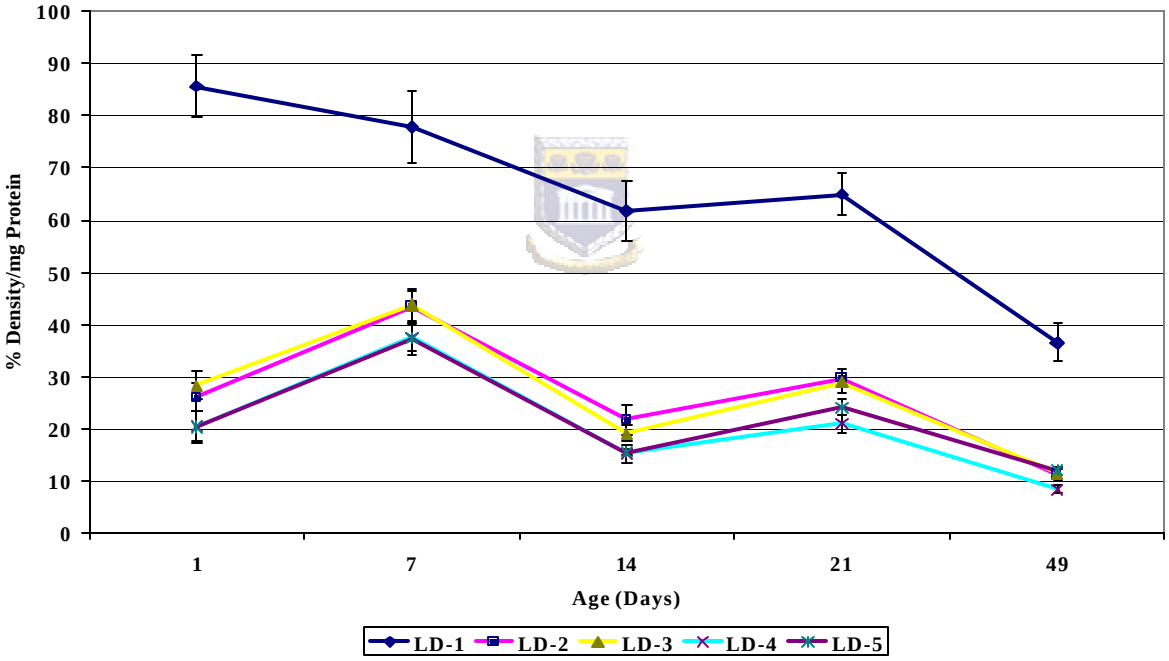


Fig.26. Isoenzyme patterns of the five LDH isoforms in control developing rat lungs.

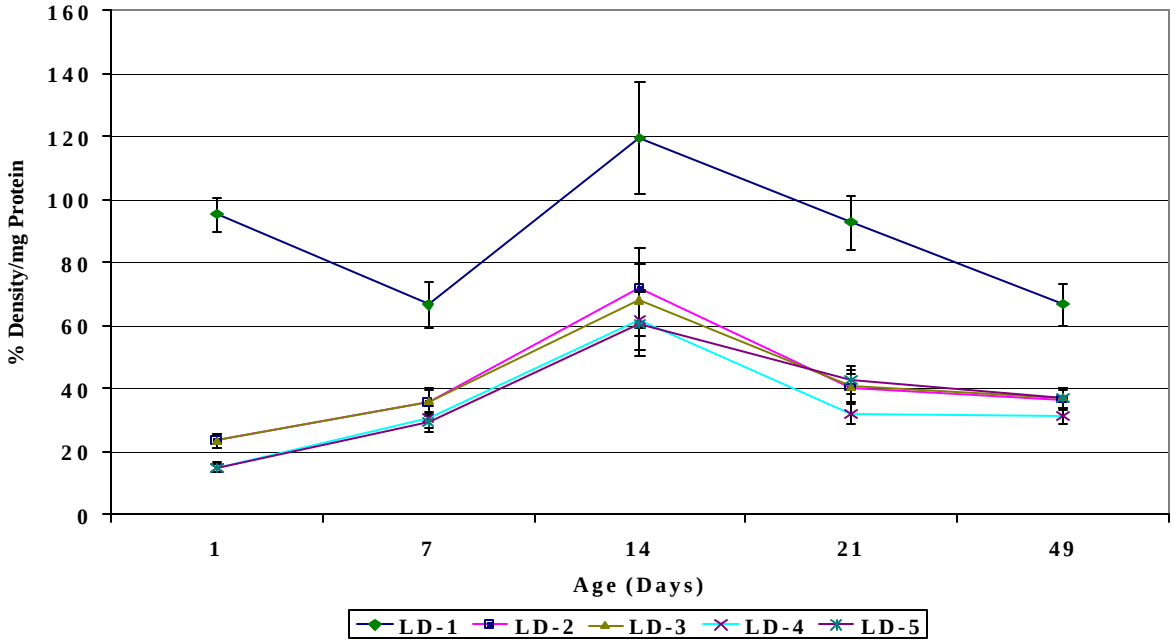


Fig.27. Influence of maternal nicotine exposure during pregnancy and lactation on LDH isoenzyme patterns in the lungs of developing rat pups.

6.2.1.7 Comparison of LD-1 and LD-5 Isoenzyme Patterns in Control Developing Lung and Lungs Exposed to Nicotine during gestation and lactation.

The LD-1/LD-5 ratio of control lung on postnatal day 1 (4.74 ± 0.2) was 0.7-fold lower ($P<0.001$) than that of the nicotine exposed animals (6.79 ± 0.44) (fig.28). At postnatal day 7 there was no difference (Control vs Nicotine: 2.13 ± 0.16 vs 2.21 ± 0.13). At postnatal days 14 (Control vs Nicotine: 2.00 ± 0.22 vs 3.94±0.10), 21 (Control vs Nicotine: 2.19 ± 0.059 vs 2.72 ± 0.05), and 49 (Control vs Nicotine: 1.67 ± 0.14 vs 2.84 ± 0.08) the ratio was 2.0-fold, 1.24-fold and 1.67-fold, respectively higher ($P<0.001$) in the lungs of the nicotine-exposed rats than in the control animals. This can be attributed to a large increase in the LD-5 levels after postnatal day 7 compared to that of the control lung tissue.

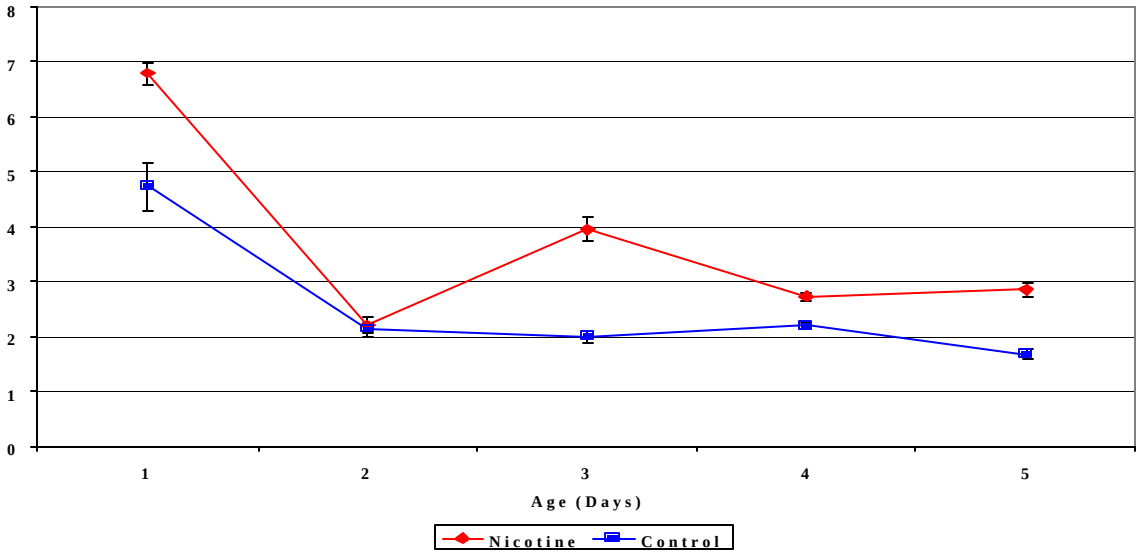


Fig.28. Influence of maternal nicotine exposure during pregnancy and lactation on LD-1/LD-5 ratio isoenzyme patterns in the lungs of developing rat pups.

6.2.2 Analysis of LDH Isoenzyme mRNA Expression in rat lung tissue as a function of age and the effect of maternal nicotine exposure during gestation and lactation

6.3.2.1 mRNA Expression of LD-M .

LD-M is the sub-unit associated with glycolytic metabolism in the cell. The data summarized in table 17 and illustrated in figure 29 shows no difference at postnatal days 1 and 7 between control and experimental tissue. Furthermore, no significant differences exist between days 1 and 7 for both groups either. Between postnatal days 7 and 14 LD-M in control lung increased by 1.5-fold ($P<0.05$) from 0.19 ± 0.025 to 0.28 ± 0.024 and in nicotine-exposed lung it increases by 1.9-fold ($P<0.01$) from 0.18 ± 0.005 to 0.35 ± 0.018. A significant difference is found between the groups at day 14 in which the experimental tissue is 1.3-fold ($P<0.01$) higher than the control. Between postnatal days 14 and 21 the sub-unit's expression stays the same in the control lung but increased ($P<0.05$) in the nicotine-exposed lung by 1.5-fold. Comparison between the groups at day 21 reveals that LD-M is 1.7-fold

(P<0.01) higher in the latter. Expression of LD-M from day 21 to 49 shows no differences for both control and experimental tissue, but the latter remains markedly higher (P<0.02) than the former. Between postnatal days 1 and 49 LD-M mRNA expression in control lung tissue increased by 2.1-fold (P<0.02) and in the nicotine-exposed lung tissue it increased by 2.6-fold (P<0.01).

Table 17: Changes in LD-M m RNA expression (LD-M: ß-actin ratio) in rat lung tissue as a function of age and the effect of Maternal Nicotine Exposure during gestation and lactation.

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=6)	(n=6)	
1	0.13±0.020	0.18±0.030	P > 0.10
	(n=6)	(n=5)	
7	0.19±0.025	0.18±0.005	P > 0.10
1 vs 7	P > 0.10	P > 0.10	
	(n=7)	(n=5)	
14	0.28±0.024	0.35±0.018	P < 0.01
7 vs 14	P < 0.05	P < 0.01	
	(n=9)	(n=9)	
21	0.31±0.027	0.51±0.064	P < 0.01
14 vs 21	P > 0.10	P < 0.05	
	(n=9)	(n=6)	
49	0.28±0.044	0.47±0.070	P < 0.02
21 vs 49	P > 0.10	P > 0.10	

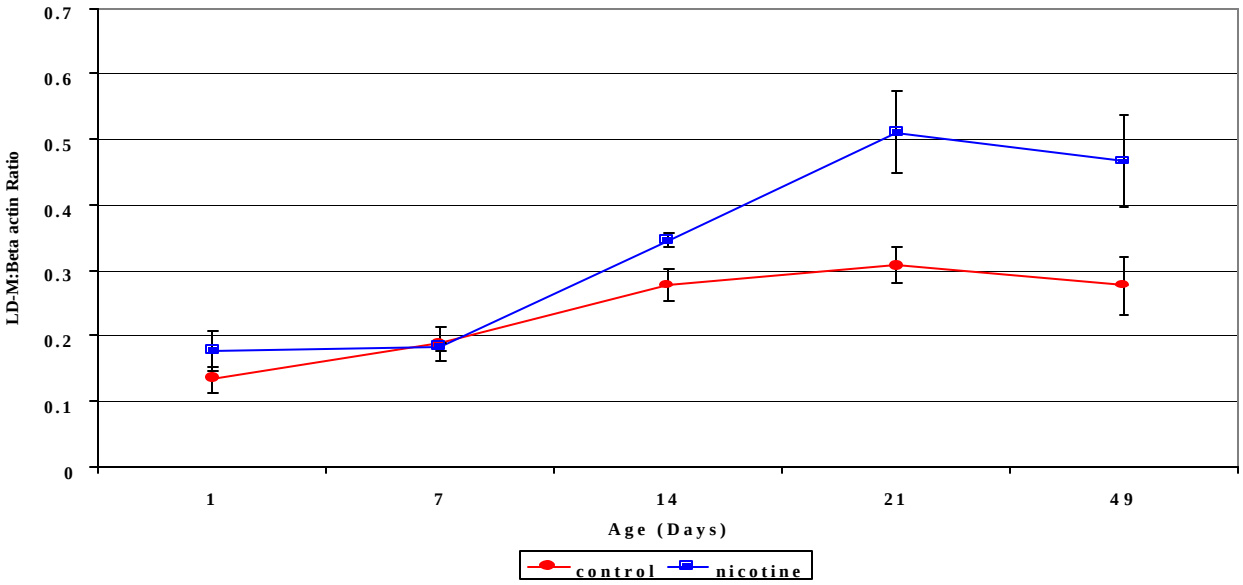


Fig.29. Illustration of the Effect of Maternal nicotine exposure during gestation and lactation on LD-M mRNA expression in developing rat lung.

6.3.2.2 mRNA Expression of LD-H

The LDH sub-unit closely linked to the oxidative aspect of cellular metabolism is LD-H. Interestingly, data in Table 18 shows that expression of LD-H in the control tissue at postnatal days 1 and 7 is higher ($P<0.05$) than in nicotine-exposed tissue. However, between postnatal days 7 and 14, LD-H in the nicotine-exposed group increased by 1.2-fold ($P<0.01$), whereas no change is observed in the control group ($P>0.10$). During this period of lung development, LD-H expression in both groups remains unchanged between day 14 and day 21. Comparisons made between the groups at day 21 show that LD-H expression in experimental lung tissue is 1.3-fold higher ($P<0.02$) than in controls. Between day 21 and day 49 this sub-unit's expression decreases from 0.56 ± 0.033 to 0.46 ± 0.021 ($P<0.05$) in the control animals and in the experimental animals a decline from 0.71 ± 0.041 to 0.55 ± 0.026 ($P<0.02$) is observed. Although a significant decline occurs at this stage, LD-H remains higher ($P<0.01$) in the nicotine-exposed lung than in the control lung. Comparing between days 1 and 49 reveals that LD-H in control lung tissue LD-H

mRNA expression decreased by 1.3-fold (P<0.02) and in experimental lung tissue it increased by 1.2-fold (P>0.10). This finding clearly demonstrates that maternal nicotine exposure alters LD-H expression at the mRNA level.

From Fig.30 it is clear that LD-H mRNA expression in control lung gradually decreased from postnatal day 1 to its lowest level on postnatal day 49. In nicotine-exposed lung, it is highest on postnatal day 21 where after it decreased to a level on postnatal day 49 that was not different from that on postnatal day 1 (P<0.01). From postnatal day 14 the expression of LD-H mRNA of lungs of nicotine-exposed rat pups remains higher (P<0.01) than that of the control animals.

Table 18: Changes in LD-H mRNA expression (LD-H: ß-actin ratio) in rat lung tissue as a function of age and the effect of Maternal Nicotine Exposure during gestation and lactation.

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=8)	(n=6)	
1	0.58±0.023	0.48±0.025	P < 0.05
	(n=7)	(n=8)	
7	0.64±0.033	0.54±0.028	P < 0.05
1 vs 7	P > 0.10	P > 0.10	
	(n=8)	(n=6)	
14	0.60±0.017	0.66±0.019	P < 0.05
7 vs 14	P > 0.10	P < 0.01	
	(n=8)	(n=9)	
21	0.56±0.033	0.71±0.041	P < 0.02
14 vs 21	P > 0.10	P > 0.10	
	(n=8)	(n=6)	
49	0.46±0.021	0.55±0.026	P < 0.01
21 vs 49	P < 0.05	P < 0.02	

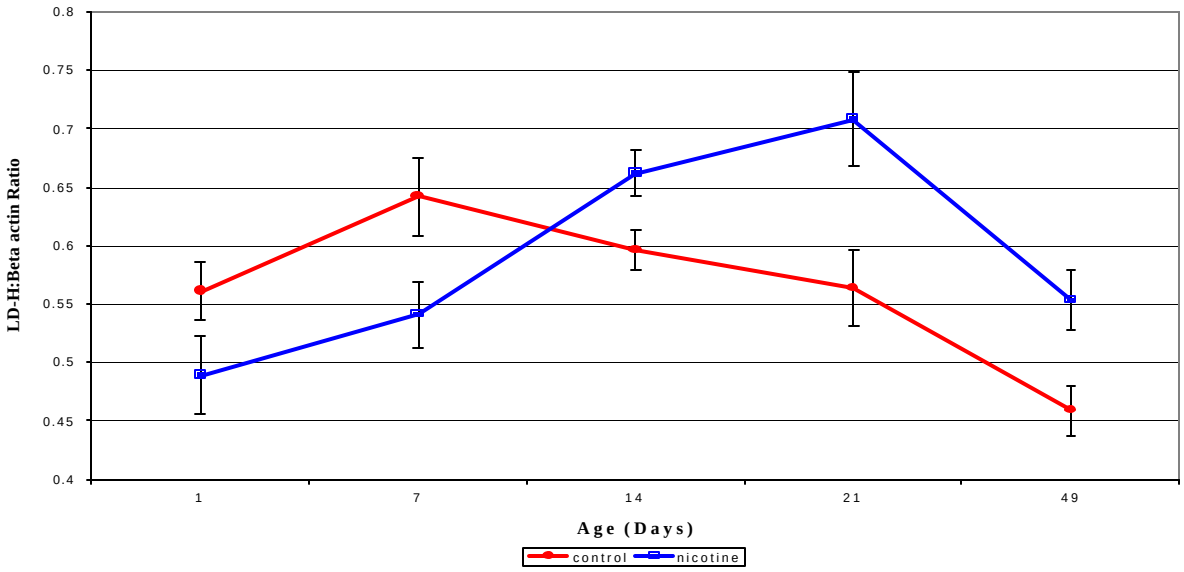


Fig.30. Illustration of the Effect of Maternal nicotine exposure during gestation and lactation on LD-H mRNA expression in developing rat lung.

6.2.2.3 Comparison of LD-H and LD-M at the mRNA level

This investigation shows that LD-H is the dominant LDH sub-unit in control developing rat lungs (Fig.31). A graphical representation of LD-H and LD-M in both control and experimental lung tissue clearly reflects the altering effects of maternal nicotine on the expression of these sub-units at the transcriptional level. From the graph, LD-H remains significantly higher than LD-M in the control lung tissue in all the age groups analyzed. In the nicotine-exposed lung its dominance is observed from day 1 to day 21. At day 49 no statistical difference is found between the two sub-units. Thus, between postnatal days 1 and 21 (in control and experimental lung) the LDH sub-unit mainly expressed is the oxidative associated sub-unit. However, by day 49 the lungs of nicotine exposed pups are almost equally expressing both glycolytic and oxidative sub-units.

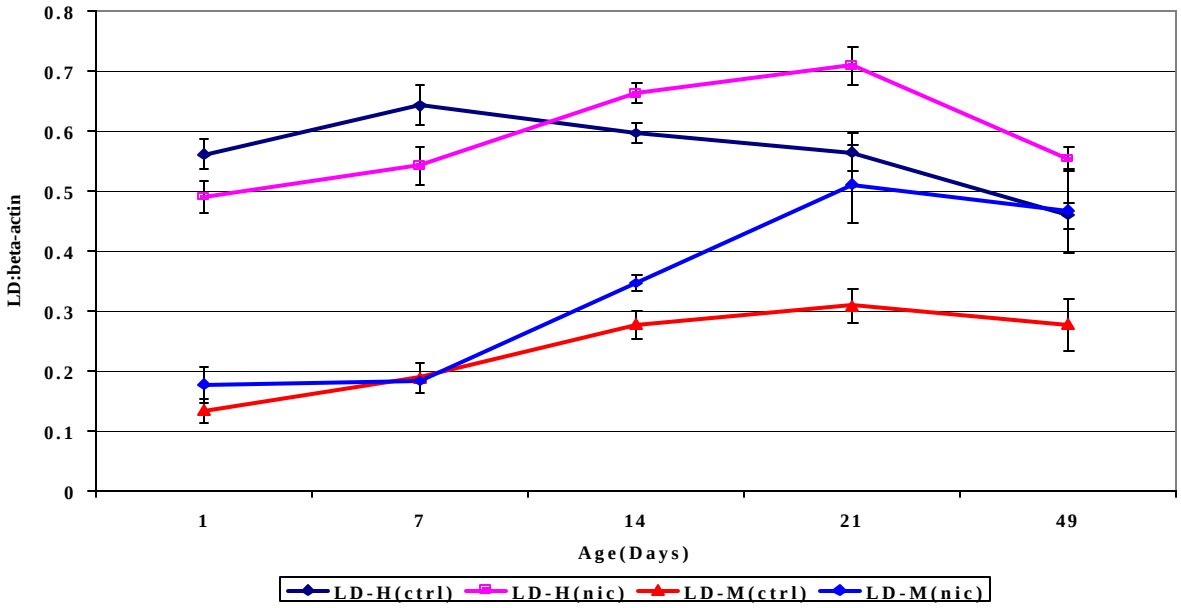


Fig.31. Expression of LD-H and LD-M at the mRNA level in control neonatal rat lung and lung exposed to nicotine during pregnancy and lactation.

6.3 DISCUSSION

6.3.3 Isoenzyme patterns of LDH during normal lung development and the influence of maternal nicotine exposure during gestation and lactation

Previous work by Maritz (1986) shows that exposure of neonatal rat lung to nicotine during pregnancy and lactation results in the significant decrease in lactate production despite a marked increase in glucose turnover. These findings lead to the proposal that maternal nicotine exposure causes the inhibition of glycolysis in developing rat lung. Furthermore, this inhibition is still observed four weeks after nicotine had been withdrawn (Maritz, 1988).

It is interesting to note that, although lactate production *in vitro* in lungs of rats exposed to nicotine during pregnancy and lactation is lower than in control lungs,

the LD-M and LD-H isozyme levels are higher in lung tissue of rats that were exposed to nicotine via the placenta and mother's milk.

To date, data on isoenzyme patterns and mRNA expression in rat lung during postnatal development has not been documented. Assessing LDH isoenzyme patterns may provide valuable information with regards to tissue oxygen utilization and the need of that tissue to utilize anaerobic glycolysis (Cahn et al., 1962; Dawson et al., 1964) to supply its energy demands. Furthermore, LDH isoenzyme patterns should provide critical information with regards to overall metabolic state as a normal rat lung matures and indicate exactly how maternal nicotine exposure during gestation and lactation affects this cytoplasmic enzyme. This will also give an indication whether maternal nicotine exposure changes the flux of glucose via glycolysis at the LD level.

This study clearly shows that LD-1 (the homozygous LD-H isoenzyme) is the dominant isoform in the developing control rat lung. This finding implies that between postnatal days 1 and 49 the rat lung is predominantly in an oxidative state. With the diversity of cells found in the lung (alveolar macrophages, fibroblasts, alveolar pneumocytes type I and II), and the fact that the age groups analyzed depict the period in which the neonatal lung undergoes significant structural, biochemical and molecular changes (Burri et al.,1974; Burri, 1974; Powell and Whitney, 1980), this result is not surprising since changes in LD-1 reflects the high oxidative need of the tissue as it develops. Generally, the % Densities for all the isoforms decreased significantly between postnatal days 1 and 49. Closer evaluation shows that between day 7 and day 14, a period associated with the phase of rapid alveolarization in rat (Burri et al., 1974) all the isoenzymes, except LD-1, decreased. LD-4 and LD-5 (the homozygous LD-M isoenzyme), the glycolytic associated isoenzymes, displayed the greatest decrease. LD-1 only markedly decreases after postnatal day 21.Thus it is likely due to the fact that at this stage the developing lung depends on a high rate of glucose oxidation via the Krebs cycle and ATP production via the respiratory chain to meet the increased energy demands rather than the lower energy output due to the

conversion of large quantities of glucose to lactic acid. On the other hand, after postnatal day 21 the energy requirements are less due to slower cell proliferation and hence it is plausible that the LD1 activity will decrease accordingly.

In this study, we showed that maternal nicotine exposure during gestation and lactation increased both the levels of both the oxidative (LD-1) and glycolytic (LD-5) isoforms of LD; the increase in the glycolytic form was, however, more prominent than that of the oxidative form. This implies that lactate production should increase. Since the levels of all the LD isoforms were higher in the lungs of the nicotine exposed rats, it is conceivable that the rate of lactate production in these lungs should be higher than in that of the control rats. If that is indeed so a great flux of glucose through the glycolytic pathway could be expected. Previous studies indeed show that glucose utilization by lung tissue of nicotine exposed rat pups are higher than in the lungs of control rats. However, despite this the lactate production is lower despite the higher levels of glycolytic LD isoforms. This implies that the decreased flux of glucose via the glycolytic pathway was not due to a lower LD activity or LD mRNA expression. This furthermore implies that the site where the flux of glucose through the glycolytic pathway is inhibited is above the LD catalyzed reaction.

Clearly maternal nicotine exposure during pregnancy and lactation results in post-transcriptional changes. The significant alterations in isoenzyme patterns are perhaps an attempt to compensate for the interference observed in the glycolytic pathway (Maritz, 1986) at the post-translational level.

6.3.2 mRNA Expression of LDH isoforms in normal developing lung and the influence of maternal nicotine exposure

LD-M is expressed at a much lower concentration than LD-H in control rat lung. A comparison drawn between LD-5 (M-homotetramer) and LD-M sub-unit shows that between day 1 and day 49 the former decreases in % Density, whereas it increases in

the latter. Furthermore, LD-5 decreases significantly between day 7 and day 14, and then increases noticeably between day 14 and day 21. In contrast a significant increase is observed in LD-M between day 7 and 14, and day 14 and 21. Research shows that the half-life for LD-5 in rat is approximately 4 to 6 days (Lindy, 1974; Don and Masters, 1976). This might explain the delayed increase in LD-5 expression noted between day 7 and day 14. LD-M expression increases as the rat lung matures up until day 21 and then plateaus. In the nicotine exposed lung a similar trend is observed but on a greater scale.

LD-H is expressed at significantly higher levels than LD-M in nicotine exposed lung up until day 21, while at day 49 no statistical differences are found between the two. These results show that maternal nicotine exposure has resulted in the altering of both LD-M and LD-H mRNA expressions in developing rat lung. The data shows that under normal conditions the rat lung is generally in an oxidative state catalyzing the formation of pyruvate from lactate, however maternal nicotine exposure during pregnancy and lactation results in a shift in the metabolic state of the lung. By day 49 the lungs exposed to nicotine are expressing LD-H and LD-M sub-units at very similar levels, implying a greater need for anaerobic isoforms. This change at the transcriptional levels is reflected at the post-translational level in which LD-5 (consisting entirely of LD-M subunits) increases significantly in lungs exposed to nicotine during gestation and lactation, thus driving the formation of lactate from pyruvate. (Pyruvate $\longrightarrow$ Lactate) However, LD-1, the oxidative associated isoform remains significantly higher than any of the four LDH isozymes in the nicotine-exposed lungs. Thus the major impact that maternal nicotine exposure has on the reaction catalyzed by LDH is in the formation of pyruvate from lactate. (Pyruvate $\longleftarrow$ Lactate) This may partly explain the increased glucose turnover in the lungs of the offspring exposed to nicotine during pregnancy and lactation.

It is also clear from the results that both isoforms' mRNA levels remain higher in the nicotine-exposed lungs even after the pups are weaned. Thus even though nicotine is

no longer present in the tissue, mRNA expression is still significantly elevated when compared to control tissue. This implies that maternal nicotine exposure during gestation and lactation results in permanently higher transcript levels of LDH.

The data in this study clearly shows that neonatal rat lung exposed to nicotine during pregnancy and lactation is affected at transcriptional as well as post-transcriptional levels. The lung is over-expressing all the LDH isoenzymes from day 7 onwards as well as over-expressing the sub-units at the mRNA level. The glycolytic sub-unit and homotetramer isoform is particularly affected by maternal nicotine exposure. It is thus proposed that inhibition of the glycolytic pathway results in the over-expression of LDH at both the transcriptional and post-transcriptional levels in an attempt to rectify the hindrance caused by maternal nicotine exposure.

In a study by Maritz (1997), LDH activity in control lung tissue is compared to lungs exposed to nicotine during pregnancy and lactation. No differences were found between these groups at any age group investigated. Furthermore, a 21.59% decrease in activity was noted between postnatal days 1 and 49 (Maritz, 1997). Maternal nicotine exposure during pregnancy and lactation thus, essentially does not affect LDH activity in a developing neonatal rat lung. However, fetal lung generates the ATP and NAD^+ in the presence of phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Bhavnani, et al.1990). The high rate of anaerobic cellular respiration results in significant accumulation of lactate that can be transferred back to glucose via gluconeogenesis (Bhavnani et al., 1990). In humans and non-human primates the capacity for gluconeogenesis develops during the last trimester (Burd et al., 1975; Battaglia and Meschia, 1978). However, this pathway is quite limited in rodent fetal tissue and develops only after birth (Burch et al., 1963; Girard et al., 1975). This may explain the sharp decrease observed in LDH activity just after birth. These findings may not have provided the mechanism for damage observed in the lungs of neonates exposed to nicotine during pregnancy and lactation, but it has partially substantiated the findings by Maritz (1986). Furthermore, it has provided a partial explanation for the decrease in lactate

production observed in the lungs of pups exposed to nicotine during pregnancy and lactation (Maritz, 1986). This study thus demonstrates that the neonatal rat lung significantly up-regulates its expression of LDH both transcriptionally and post-transcriptionally under the influence of maternal nicotine exposure, even after the pups are weaned and nicotine is no longer present in the tissue.

CHAPTER 7

8. ANALYSIS OF EXPRESSION OF CYTOCHROME P450 1A1, 2A3 AND 2B1 AT THE mRNA LEVELS IN DEVELOPING RAT LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

7.1 INTRODUCTION

The respiratory system is constantly exposed to air-borne substances, but it also gets exposed to chemicals via the systemic circulation. This is particularly true during gestation when the developing fetal lungs are exposed to chemicals transferred from the maternal circulation across placental tissue into fetal circulation (Lee *et al.*, 2000) and during lactation when compounds are conveyed via the mother's milk (Luck and Nau, 1984).

Nicotine is an ideal example of a chemical that freely crosses the placenta (Van Gilder, 1997) because it is lipid-soluble (Lambers and Clark, 1996) and has been found in significant quantities in the milk of smoking mothers (Luck and Nau, 1984). Thus this substance can freely interact with the developing fetus and neonate.

The lung contains many enzymatic pathways with the ability to metabolize xenobiotics, however, general consensus is that the cytochrome (CYP) P450 superfamily of enzymes is its dominant means of metabolizing these exogenous substances (Hukkanen *et al.*, 2001).

It is thus critical to this investigation to determine whether maternal nicotine exposure causes the induction of CYP P450 enzymes usually associa ted with the presence of nicotine. Since the CYP P450-hydroxylation pathways results in the formation of reactive intermediates (Newcomb, *et al.*, 2003; Coon, 2003), their formation may contribute to the interference of the glycolytic pathway observed. An increase in CYP P450 activity may also result in an increased glucose turnover reported earlier (Maritz, 1986).

The CYP enzymes predominantly involved in xenobiotic metabolism belong to the families designated CYP1, CYP2, and CYP3. The other families have essential roles in the metabolism of endogenous substances (Nelson, 1999). CYP 1-3 families metabolize a wide variety of xenobiotics, but some also possess the ability to metabolize endogenous compounds such as steroid hormones and arachidonic acid (Gonzalez, 1992; Capdevila *et al*., 2000). Approximately half of the 53 human CYP forms belong to the CYP 1-3 families (Nelson, 1999).

The liver has been shown to express the majority of CYPs both quantitatively and by CYP diversity (Oinonen and Lindros, 1998). Some extrahepatic tissues also express them but on a smaller scale (Raunio *et al*., 1995a); these include tissues such as lung, intestine, brain, skin, and placenta. (Oinonen and Lindros, 1998). Recent studies profiling 40 CYP P450 genes in mouse (Choudhary *et al.*, 2003) show that while the liver and kidney express most of them, the lung expresses a significant 24 of these genes. Since the liver acts as a port of entry for all ingested substances, the high concentration and diversity of CYPs present in relation to its function is self-explanatory. Tissues such as lung and skin also play critical roles as the first lines of defense against exogenous substances, and this partly explains the presence of the CYP enzymes, albeit at lower levels.

In humans, nicotine is extensively metabolized via C-oxidation to cotinine (Benowitz and Jacob III, 1987). Nicotine C-oxidation is predominantly catalyzed by CYP enzymes in which the 5' position is hydroxylated to yield an unstable intermediate, 5'-hydroxynicotine that exists in equilibrium with nicotine-1'(5')-iminium ion (Benowitz and Jacob III, 1994). Cytosolic aldehyde oxidase then catalyses the oxidation of the intermediate to cotinine (Brandlänge and Lindblom, 1979). Thus there is a strong possibility that maternal nicotine exposure will result in the induction of this CYP enzyme and thus result in the formation of the reactive intermediates.

In humans, 5'-hydroxylation of nicotine is predominantly catalyzed by CYP2A6 (Messina *et al.*, 1997). CYP2B6 has also been shown to be involved in nicotine C-

oxidation (Yamazaki *et al.*, 1999), particularly at high substrate concentration. Recently Hecht *et al.* (2000) showed that CYP2A6 also catalyzes 2'-hydroxylation of nicotine to yield substantial amounts of aminoketone (a lung cancer precursor). Both CYP2A6 and CYP2B6 play a major role in the activation of NNK (Hecht, 1998; Code *et al.*, 1998). It has been suggested that these two CYP2 isoforms are coordinately regulated in *Homo sapiens*, particularly since their respective genes occupy similar chromosomal locations (Gonzalez, 1992). CYP2A6 and CYP2B6 are expressed in lung tissue and are suspected of playing critical roles in tobacco-related biological effects in humans (Yamazaki *et al.*, 1999).

Also of interest to this investigation is a dominant extrahepatic CYP enzyme called CYP1A1 (Raunio *et al.*, 1995a) involved in the toxicity of a variety of carcinogens, particularly polycyclic aromatic hydrocarbons (PAHs) (Shimada *et al.*, 1996a; Shimada *et al.*, 2003). Essentially CYP1A1 is lowly expressed in extrahepatic tissues, but induction by aromatic (aryl) hydrocarbon receptor (AhR) ligands has been demonstrated in tissues such as lung, lymphocytes, mammary glands and placenta (Raunio *et al.*, 1995a). The environmental toxicant, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is its most potent known inducer (Whitlock, 1999). Real endogenous inducers of CYP1A1 have not yet been accurately determined (Murray *et al.*, 2001).

CYP1A1 is also highly inducible by cigarette smoke (Anttila *et al.*, 1991; Whitlock, 1999). Oxygenation by CYP1A1 induced by PAHs in tobacco smoke generates arene oxide that can create mutations leading to neoplastic tranformation (Whitlock, 1999). Thus there is the possibility that nicotine may be a compound within tobacco smoke that results in CYP1A1 induction.

Although CYP1A1 has been widely studied, many findings are still inconsistent with respect to its induction by nicotine. Studies by Wei *et al.* (2002) and Iba *et al.* (1998) report that CYP1A1 is induced by nicotine. In contrast, Le Gal *et al.* (2003) and Nakayama *et al.* (1993) have found that nicotine does not induce this cytochrome. These above-mentioned conflicting results make the analysis of CYP1A1 mRNA

levels under the influence of maternal nicotine quite critical. If CYP1A1 is indeed induced it may contribute to the mechanism for the glycolytic inhibition noted and may predispose the developing lung to a variety of disease states. If induction does not occur it will imply that CYP1A1-hydroxylation pathways is not activated by nicotine and therefore does not result in the formation of reactive species and/or does not have the potential to bio-transform nicotine.

In an attempt to elucidate the mechanism behind glycolytic inhibition and possibly understand the aetiology of respiratory disease with respect to nicotine, we investigated the expression of rat orthologs of the human CYP1A1, CYP2A6 and CYP2B6 at the mRNA level in the rat lungs. The objectives included a) analysis of the individual CYPs during normal lung development and b) investigating the effect that maternal nicotine exposure has on the expression of these CYPs and its possible implications.

7.2 RESULTS

8.2.1 Analysis of CYP P450 mRNA levels

8.2.1.1 CYP1A1 mRNA Expression

It is clear from the data obtained (Table 19) that maternal nicotine exposure during gestation and lactation has no effect on the expression of this major extrahepatic enzyme. Its mRNA levels increases by 3.94-fold ($P<0.001$) in control groups and by 3.83-fold in nicotine exposed neonatal groups between day 1 and day 49. No significant differences between these groups occur, except at day 1 where the CYP1A1/ß-actin ratio in the nicotine exposed group is 1.29-fold higher ($P<0.05$) than the controls. In both the control and nicotine-exposed groups the CYP1A1/ß-actin ratio increases as the animal ages (Fig.32). Maternal nicotine exposure has no effect on the expression on this CYP enzyme in the lungs of offspring.

Table 19: Influence of maternal nicotine exposure on CYP1A1 mRNA levels in neonatal rat lung (CYP1A1: ß-actin ratio).

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=6)	(n=6)	
1	0.33±0.03	0.43±0.02	P < 0.05
	(n=7)	(n=6)	P > 0.10
7	0.27±0.03	0.21±0.02	
1 vs 7	P > 0.10	P < 0.01	
	(n=9)	(n=8)	P > 0.10
14	0.43±0.05	0.48±0.09	
7 vs 14	P < 0.05	P < 0.05	
	(n=8)	(n=9)	P > 0.10
21	0.97±0.13	0.83±0.12	
14 vs 21	P < 0.01	P < 0.01	
49	(n=9) 1.31±0.14	(n=9) 1.64±0.27	P > 0.10
21 vs 49	P > 0.10	P < 0.05	

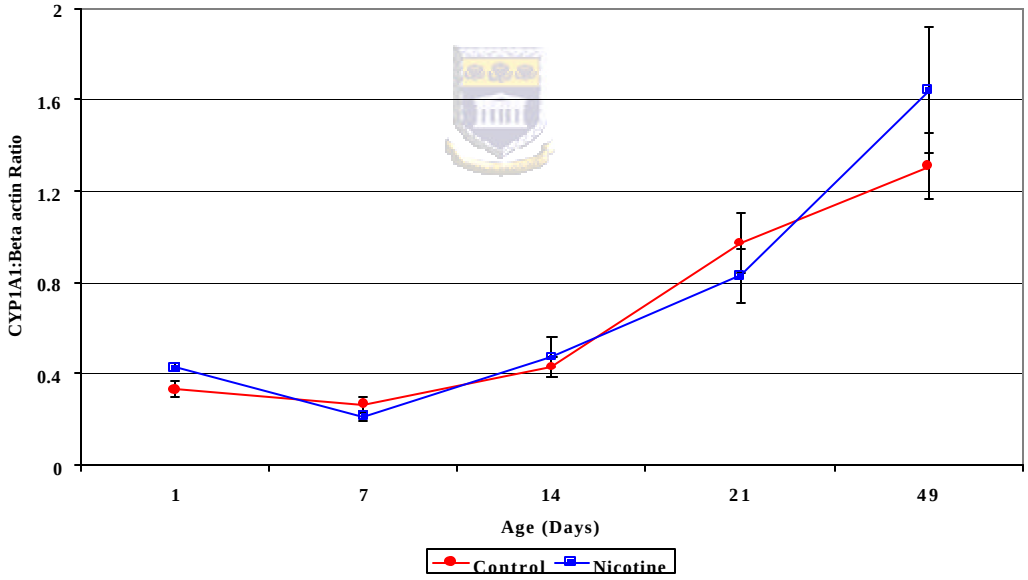


Fig.32. Expression of CYP 1A1 at the mRNA level in control developing rat lung and in the lungs of pups exposed to maternal nicotine.

7.2.1.2 CYP2A3 mRNA Expression

Data in Table 20 shows that maternal nicotine exposure results in the induction of CYP2A3 in neonatal rat lung after post-natal day 7. At day 1 CYP2A3/ß-actin ratio in control and nicotine-exposed rat lung are similar (0.29 ± 0.03 and 0.23 ± 0.01, respectively). At day 7 CYP2A3 mRNA levels are 1.93-fold higher ($P<0.05$) in control than in experimental animals. Although both groups display an increase from day 1 to day 7, the elevation to 0.96 ± 0.17 observed in controls is more profound than the increase to 0.50 ± 0.10 seen in experimental animals. Between days 7 and 14 maternal nicotine exposure causes a 6.1-fold ($P<0.001$) increase in CYP2A3 mRNA expression. Control tissue in contrast displays no changes ($P>0.10$) during the period of lung growth and development. At day 14 the CYP2A3/ß-actin ratio in experimental lung tissue is 2.3-fold higher ($P<0.01$) than that of controls. Between day 14 and day 21 a 2.3-fold increase ($P<0.01$) in expression is observed in control lung (1.31 ± 0.16 to 3.00 ± 0.52) whereas in nicotine-exposed tissue CYP2A3 only increases 1.5-fold from 3.031 ± 0.48 to 4.69 ± 0.46 ($P>0.10$). However, this enzyme is still expressed at significantly higher levels ($P<0.05$) in the latter at day 21 when compared to the former. CYP2A3 levels continue to increase in both groups up until day 49 where expression of this enzyme is still higher ($P<0.05$) in nicotine-exposed lung than in controls.

CYP2A3/ß-actin ratio in control lung significantly increases by 16.7-fold from day 1 to day 49. In nicotine-exposed lung the increase is by an astounding 27.7-fold. These findings indisputably show that CYP2A3 is induced in the lungs of neonatal rat pups when exposed to nicotine during gestation and lactation (Fig.33).

Table 20: Influence of maternal nicotine exposure on CYP2A3 mRNA levels in neonatal rat lung (CYP2A3: ß-actin ratio).

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=5)	(n=5)	
1	0.29±0.03	0.23±0.01	P > 0.10
	(n=7)	(n=8)	
7	0.96±0.17	0.50±0.10	P < 0.05
1 vs 7	P < 0.01	P > 0.10	
	(n=9)	(n=6)	
14	1.31±0.16	3.03±0.48	P < 0.01
7 vs 14	P > 0.10	P < 0.001	
	(n=8)	(n=6)	
21	2.96±0.52	4.69±0.46	P < 0.05
14 vs 21	P < 0.01	P > 0.10	
49	(n=9) 4.86±0.34	(n=7) 6.41±0.62	P < 0.05
21 vs 49	P < 0.05	P > 0.10	

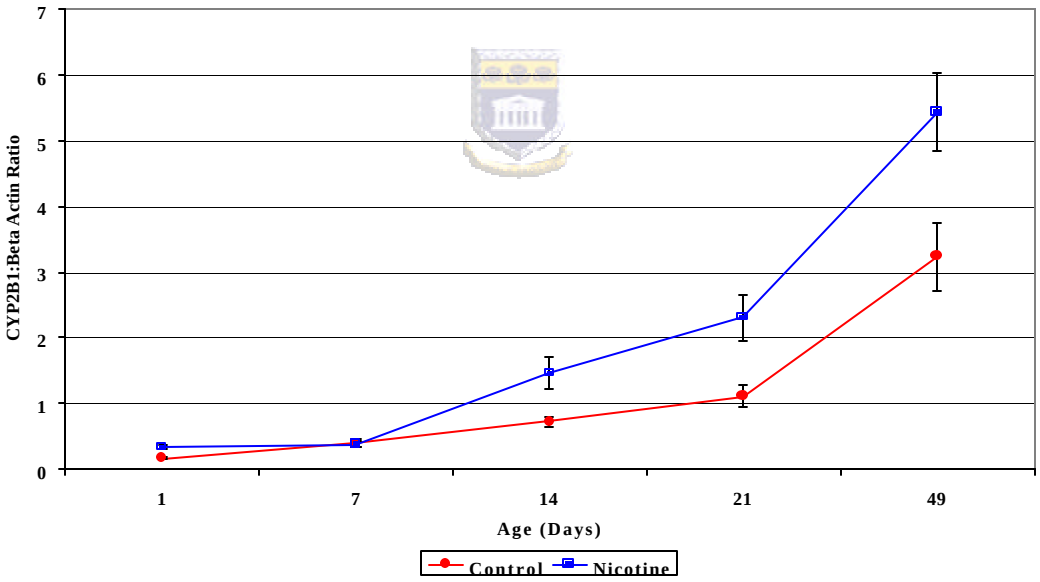


Fig.33. Influence of maternal nicotine exposure during pregnancy and lactation on the mRNA levels of CYP2A3 in developing rat.

7.2.1.3 CYP2B1 mRNA Expression

At postnatal day 1 (Table 21), CYP2B1/ß-actin ratio in controls is 0.17 ± 0.05 whereas it is 1.95-fold higher ($P<0.05$) in the nicotine-exposed animals (0.33 ± 0.03). At day 7 however, no change is detected in this CYP's expression levels in the latter but a marked elevation of 2.38-fold ($P<0.01$) is seen in the controls. When the groups are compared at day 7 no differences are found. Between days 7 and 14, control tissue shows an increase of 1.83-fold ($P<0.01$) in CYP2B1 levels. In the experimental tissue, the increase observed is 3.91-fold ($P<0.01$). When the groups are compared at day 14, CYP2B1/ß-actin ratio in nicotine-exposed tissue (1.47 ± 0.25) is 2.03-fold ($P<0.05$) higher than in controls (0.72 ± 0.082). Between day 14 and day 21, a 1.53-fold increase is seen in the control tissue, whereas CYP2B1 mRNA levels increases by 1.58-fold ($P<0.10$) in the lung tissue of nicotine exposed rat pups. At day 21 its levels in the latter group is still a significant 2.09-fold higher ($P<0.02$) than the former. Both groups show a marked increase (controls, 2.93-fold nicotine-exposed, 2.35-fold) from day 21 to day 49 ($P<0.01$). Statistical analysis indicates that CYP2B1/ß-actin ratio in nicotine-exposed lung tissue at day 49 is 1.68-fold higher ($P<0.05$) than in control lung tissue. The data clearly shows that induction of CYP2B1already started before birth. Statistical analysis between day 7 and day 49 indicate that maternal nicotine exposure results in a marked 14.5-fold increase compared to the significant 8.2-fold elevation observed in controls. Consequently the difference between control and nicotine-exposed animals becomes bigger as the lung ages (Fig.34).

Table 21: Influence of maternal nicotine exposure on CYP2B1 mRNA levels in neonatal rat lung (CYP2B1: ß-actin ratio)

Age (Days)	Control	Nicotine	Ctrl vs Nic
	(n=6)	(n=6)	
1	0.17±0.02	0.33±0.03	P < 0.01
	(n=6)	(n=6)	
7	0.40±0.06	0.38±0.04	P > 0.10
1 vs 7	P < 0.01	P > 0.10	
	(n=7)	(n=6)	
14	0.72±0.08	1.47±0.25	P < 0.05
7 vs 14	P < 0.01	P < 0.01	
	(n=6)	(n=8)	
21	1.11±0.16	2.31±0.35	P < 0.02
14 vs 21	P > 0.10	P < 0.10	
49	(n=8) 3.24±0.53	(n=7) 5.44±0.60	P < 0.05
21 vs 49	P < 0.01	P < 0.01	

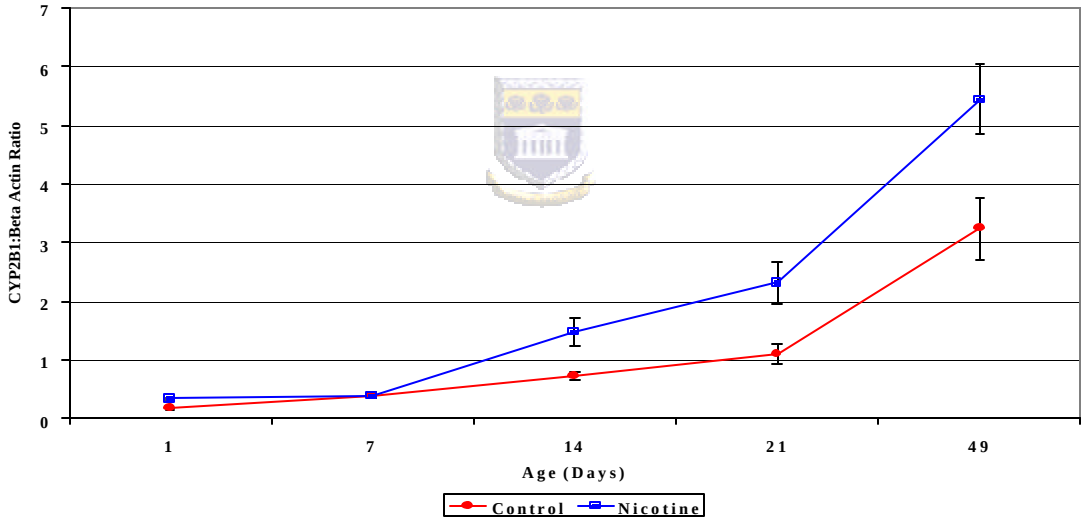


Fig.34. Transcript levels of CYP2B1 in the neonatal lungs of normal developing rat and in the lungs of neonates exposed to nicotine during gestation and lactation.

7.2.1.4 Comparison of CYP1A1, 2A3 and 2B1 mRNA Expression in Control Lungs and Lungs Exposed to Maternal Nicotine

A comparison made between CYP1A1, CYP2A3 and CYP2B1 in the lungs of control pups (Fig.35), shows that CYP2A3 is expressed at significantly higher levels ($P<0.01$, for both) from day 7 through to day 49 when compared to both CYP1A1 and CYP2B1. CYP1A1 levels remained relatively low compared to expression levels of CYP2A3 and CYP2B1. All the CYPs, however, displayed a significant increase from day 1 to day 49 in control lung tissue.

Figure 36 also illustrates changes in the levels of expression of the CYPs during maturation of the lung tissue of rats exposed to nicotine via the placenta and mother's milk. It is evident that CYP1A1 mRNA levels are not affected by maternal nicotine exposure. Although CYP2A3 still displays itself as the dominant enzyme when compared to CYP2B1, no significant differences could be detected between the two CYPs at day 49 (6.41 ± 0.62 and 5.44 ± 0.60, $P>0.10$). The expression of CYP2B1 increased 16.4-fold between post-natal day 1 and 49 while expression of CYP2A3 increased 27.9-fold. This implies that CYP2A3 is more profoundly affected by maternal nicotine exposure than CYP2B1.

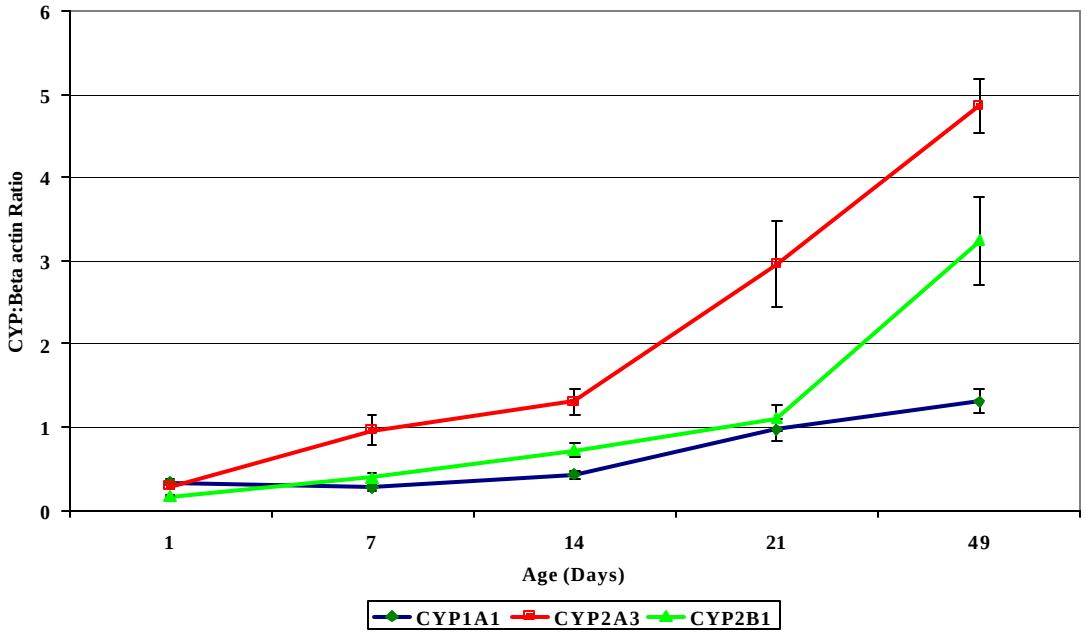


Fig.35. Graphical representation of CYP1A1, CYP2A3 and CYP2B1 transcript levels in normal developing neonatal rat lung.

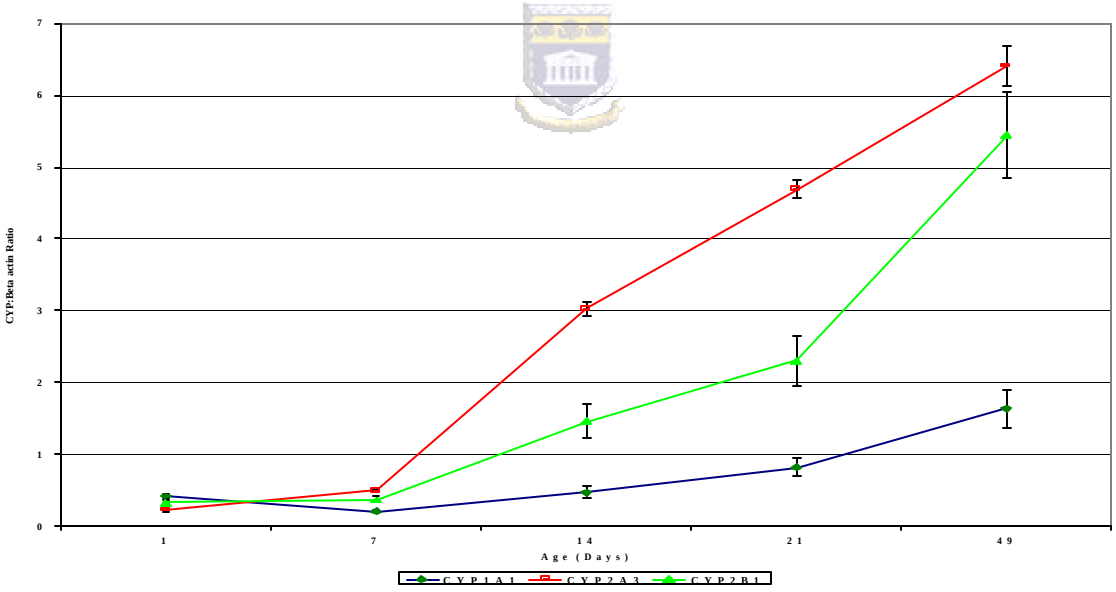


Fig.36. A comparison between CYP1A1, CYP2A3 and CYP2B1 mRNA in the lungs of neonates exposed to nicotine during gestation and lactation

7.3 DISCUSSION

7.3.1 CYP P450 mRNA Levels in Control Developing Lung

This study shows that the human orthologs of rat CYP1A1, CYP2A3 and CYP2B1 are transcribed in lung tissue of control neonatal rats. Furthermore, CYP1A1, CYP2A3 and CYP2B1 mRNA levels (expressed as a ratio with ß-actin), increase gradually after birth and continue to increase during the post-natal period. The results obtained for both CYP1A1 and CYP2B1 mRNA levels in control rat lung are consistent with the findings of Lee *et al.* (2000). Their study however did not analyze these CYPs at day 49 but the result of this study confirms their findings that the expression levels increases with age. To date, no work has been done on CYP2A3 mRNA levels in normal developing neonatal rat lung.

In conflict with the results of this study are the findings of Koskela *et al.* (1999) who suggest that CYP2A6 mRNA is not found in whole lung tissue at significant levels. Contrary to their findings, Miyamoto *et al.* (1999) demonstrated that CYP2A6 is present in human lung tissue. Furthermore, Yamazaki *et al.* (1999) report that CYP2A6 is expressed in lung tissue in which it is suspected of playing a critical role in tobacco-related biological effects in humans. CYP2A3 gene according to Ueno and Gonzalez (1990) is transcribed in rat lung (GenBank Acc. No. M33190).

The increase in CYP expression as a factor of age is perhaps closely related to the increasing capacity of the lung to inhale larger volumes of air from the atmosphere and consequently, the developing lung is exposed to increasing levels of pollutants and other foreign chemicals. Thus the increase in CYP expression may be a response to an increasing need of the tissue to protect itself against inhaled foreign chemicals.

7.3.2 Influence of Maternal Nicotine Exposure on CYP1A1 Expression

We also demonstrated that maternal nicotine exposure during gestation and lactation has no influence on the mRNA levels of CYP1A1 when compared to control lung

tissue. CYP1A1 plays a vital role in the metabolism of a variety of compounds including volatile hydrocarbons such as benzene, toulene, trichloroethylene, styrene and PAHs' found in tobacco smoke, many of which have mutagenic and carcinogenic potentials (Nakajima, 1997; Whitlock, 1999). The CYP1A1 pattern observed in both control and nicotine exposed animals displays a definite increase in mRNA expression between day 1 and day 49 which may clearly be attributed to the changes in the developmental needs in the lungs of the growing rat pup. Nakajima (1997) reported that although the expression of CYPs' is low during fetal development in most animals, it does increase rapidly soon after birth. The findings of this study support this theory since the CYP1A1 mRNA levels, although low just after birth display a progressively clear increase in its expression as the lungs of the neonate matures and develops.

Iba *et al.* (1998) and Wei *et al.* (2002) both report that nicotine induces CYP1A1 in lung tissue. Contrary to these findings, Nakayama *et al.* (1993), Yamazaki *et al.* (1999) and Le Gal *et al.* (2003) propose that CYP1A1 in not inducible by nicotine. The results obtained in my study support the finding of Nakayama *et al.*, Yamazaki *et al.*, and Le Gal *et al.* Closer examination of the data obtained by Wei *et al.* (2002) shows large standard deviations which they attribute to extensive inter-individual variability. These significant statistical variances may to a certain extent, compromise their findings. However, the present study's results show that maternal nicotine exposure does not result in the induction of CYP1A1 and therefore the activity of this enzyme does not contribute to the possible increased levels of oxidants that may exist in these lungs.

7.3.3 Influence of Maternal Nicotine Exposure on CYP2A3 and 2B1 Expression

A major catalyst in the oxidative metabolism of nicotine in humans is CYP2A6 (Messina *et al.*, 1997; Hecht *et al.*, 2000) and to a lesser extent CYP2B6 which also plays a role in C-oxidation of nicotine (Yamazaki *et al.*, 1999). The critical issue concerning organ specific chemical toxicity and carcinogenicity is whether the

targeted tissue has the ability to activate or efficiently inactivate chemicals. If during the functional reactions reactive intermediates are produced these unstable compounds may interact with cellular macromolecules, which could result in cellular toxicity and carcinogenicity (Nelson and Pearson, 1990). Furthermore, since the CYP P450-hydroxylation pathways result in the formation of reactive intermediates (Newcomb, *et al*., 2003; Coon, 2003), their formation may contribute to the interference of the glycolytic pathway observed by inhibiting oxidant-sensitive enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Substances like nicotine are highly lipophilic and have been shown to have longer retention times and higher local doses in pulmonary epithelium than less lipophilic substances (Gerde *et al.*, 1998). One of the most important tobacco carcinogens, (methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK) has been shown as a by-product of 2'-nicotine hydroxylation catalyzed by CYP2A6 (Hecht *et al.*, 2000). Research also shows that NNK is activated by both CYP2A6 and CYP2B6 (Hecht *et al.*, 1998; Code *et al*, 1998). Consequences of these CYPs being induced in neonatal rat lung as it matures and develops are as yet unknown.

In the present study the lungs of the fetal and neonatal rats were exposed during all the phases of lung development. The phase of rapid alveolarisation in rats is between days 4 and 13 (Burri, 1974). From the results of this investigation it is clear that expression of CYP2A3 and CYP2B1 in the lungs of nicotine exposed animals only increased after post-natal day 7. Since both of these CYPs are major catalysts in the oxidative metabolism of nicotine (Messina *et al.*, 1997; Hecht *et al.*, 2000; Yamazaki *et al.*, 1999), the consequences of this late induction, is not clear. However, since alveolar formation only occurs after postnatal day 4 and the epithelial cell numbers in the lower respiratory tract are relatively low prior to this, it may partly explain why significant changes in transcript levels are only detected after day 7. Furthermore, interference with nicotine metabolism may result in an extended half-life of this alkaloid in fetal and early neonatal lung (Klimek, 1990). This might contribute to the compromised state of the tissue observed in recent studies by Maritz and Windvogel (2003). They demonstrate that exposure of rat pups to nicotine via the mother's milk

from the onset of the phase of rapid alveolarisation, results in the interference of lung development to the same extent as those animals that are exposed to nicotine during all the phases of lung development.

This investigation also shows that CYP2A3 is dominantly expressed in neonatal rat lung, followed by CYP2B1 and the lowest levels shown by CYP1A1. All the CYPs displayed an increase in expression as the animals aged. It is interesting to note that CYP2B1 in control lung tissue remained significantly lower than CYP2A3, however in the experimental lung, there are no differences between the two by day 49. These results demonstrated how inducible CYP2B1 is by nicotine. However, the similarities in mRNA levels observed at day 49 between CYP2A3 and 2B1 are partly expected, since it has been suggested that these two CYP2 isoforms are coordinately regulated in *Homo sapiens*, particularly since their respective genes occupy similar chromosomal locations (Gonzalez, 1992). These results thus support the suggestion that the human ortholog CYP2A6 is a major enzyme involved in nicotine metabolism and that CYP2B6 is also linked to the metabolic reactions.

It is interesting to note that the higher levels of CYP2A3 and CYP2B1 are maintained after weaning despite the fact that the animals were not receiving any nicotine after post-natal day 21. Since the half-life of nicotine is approximately 90 minutes in the circulation, it is conceivable that at 49 days after weaning no nicotine will be present in the blood and the tissues of the offspring. Despite this the CYP2A3 and 2B1 mRNA levels remain higher after weaning in nicotine-exposed lungs when compared to control tissue. Thus, the continued higher expression levels may result in abnormally higher levels of oxidant radicals.

It is therefore plausible that inhibition of glycolysis in the lungs of offspring by maternal nicotine exposure may be as a result of the high oxidant sensitivity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Maritz (1997) showed that maternal nicotine exposure causes inhibition of GAPDH activity during the critical phase of alveolarization. The consequences of a permanent increase in these CYP levels on lung development and the maintenance of the structure of the lungs as the

animal ages is not known. However, as it is true that the increased level of CYP expression results in higher CYP activity and an increase in oxidant tissue levels, it is possible that it will result in a decrease in GAPDH activity and thus glycolysis permanently. Since type-I pneumocytes that contribute approximately 90% of the alveolar surface (Naimark, 1977) are dependent on glycolysis, it is conceivable that it may cause the observed deterioration of the lung parenchyma in the long term, despite the absence of nicotine. However, my study also shows that GAPDH expression is only altered in the presence of nicotine. Since no nicotine is present in lung tissue at postnatal day 49, it is unlikely that the increase in CYP enzyme expression causes the inhibition of GAPDH and thus glycolysis in the long term. This implies that the site of permanent inhibition of glycolysis lies elsewhere.

The increase in CYP2A3 and 2B1 transcript levels in the lungs of offspring exposed to nicotine during gestation and lactation may be a reflection of post-translational levels, implying that there is an increased need for reduced NADP from the Hexose monophosphate pathway by the CYP P450 enzymes. In order to provide these reduced molecules, the glucose-6-phosphate has to be supplied by the glycolytic pathway (Fig.1) and thus this would result in an increase in glucose turnover in the offspring's lungs. Thus if maternal nicotine exposure during pregnancy and lactation results in increased CYP P450 activity levels it could provide the mechanism for the increased glucose turnover in the nicotine-exposed lungs even though glycolysis is inhibited.

In conclusion, this study indisputably shows that CYP1A1, CYP2A3 and CYP2B1 are transcribed in neonatal rat lung and that their mRNA levels increase from day 7. CYP1A1 is not induced by maternal nicotine exposure during gestation and lactation. It is also clear from this investigation that the induction of CYP2A3 and 2B1 in the lungs of the offspring by maternal nicotine exposure is irreversible and thus “programmed”. Furthermore, if the increased transcript levels reflect increases in activity, it implies an increased need to utilize energy by these CYP enzymes and therefore may explain the increase in glucose turnover observed in the lungs of offspring exposed to nicotine during gestation and lactation.

CHAPTER 8

8. FUTURE PERSPECTIVES

Interference with carbohydrate metabolism will have adverse effects on the structural and functional integrity of a developing lung (Tierney and Levy, 1976; Gilden *et al.*, 1977; Maniscalco *et al.*, 1978; Bourbon and Jost, 1982). Maternal nicotine exposure during gestation and lactation results in many structural (Maritz and Woolward 1992; Maritz, *et al.*, 1994; Maritz and Najaar, 1995; Maritz, *et al.*, 2000; Maritz and Windvogel, 2003) and biochemical alterations (Maritz, 1983; Maritz, 1988, Maritz and Burger, 1992; Maritz, 1997) in developing neonatal rat lung. To date, very little information exists that may provide a mechanism for the structural damage observed or the changes at biochemical levels observed.

8.4 Elucidating the Mechanism of Glycolytic Inhibition in Developing Rat Lung

The findings in this study have a pivotal role in the elucidation of the mechanism involved in the inhibition of glycolysis as a result of maternal nicotine exposure (Maritz, 1983). Exposure of nicotine during pregnancy and lactation results in over-expression of GAPDH at the mRNA level (Chapter 5) during the critical phase of alveolarization. This finding substantiated the inhibition in GAPDH activity during alveolarization observed in neonatal lung exposed to maternal nicotine (Maritz, 1997). Thus the marked increase in GAPDH transcript levels may be an attempt to return activity levels to normal.

However, studies show that GAPDH is oxidant sensitive (Knight *et al.*, 1996; Molina *et al.*, 1992; Stamler, 1994; McDonald and Moss, 1993; Mohr *et al.*, 1996). During myocardial reperfusion it is shown that GAPDH's activity is inhibited and this inhibition is thought to be attributed to covalent modification, possibly as a result of reactive oxygen species (Knight *et al.*, 1996). Furthermore, studies conclusively demonstrate that the inhibition observed is consistent with a

non-competitive form of inhibition or a decrease in the amount of enzyme (Knight *et al.*, 1996). Moreover, the presence of nicotine in the lung may elicit a chain of reactions by neutrophils and alveolar macrophages that may further contribute to increased oxidant levels (Zingarelli *et al.*, 1996). It has also been shown that metabolism of nicotine by specific cytochrome P450 enzymes (CYP2A3 and 2B1) via hydroxylation pathways will also result in significant oxidant levels in the tissue (Newcomb *et al.*, 2003).

Thus the oxidant levels need investigating to determine whether their presence could be the reason for the inhibition in activity and over-expression at the mRNA level of GAPDH in developing rat lung. These oxidants include nitric oxide, super-oxide, hydrogen peroxide and peroxynitrite (which are formed by the simultaneous production of nitric oxide and super-oxide). Furthermore, the glutathione/reduced glutathione ratio should be assessed. This would be a direct indication of the oxidant stress the cell may be under. Other enzymes involved in the metabolism of reactive oxidant species, such as super-oxide dismutase should also be investigated.

Oxygen radicals are known to cause damage to DNA molecules (Liu and Martin, 2001) and this causes the activation of poly (ADP-ribose) polymerase (PARP), a nuclear DNA-repair enzyme. $PARP_1$ catalyzes the addition of ADP-ribose units from NAD^+ to GAPDH and renders it inactive (Du *et al.*, 2003). Furthermore, when GAPDH undergoes poly (ADP-ribosyl)ation it probably occurs with nuclear translocation.

Therefore, if there are abnormal levels of reactive species in the tissue, it could cause DNA damage and therefore result in $PARP_1$ activation. Thus it would be important to determine whether maternal nicotine exposure causes DNA fragmentation and furthermore to determine if $PARP_1$ is activated. It would also be beneficial to analyze cellular localization of GAPDH possibly using immunohistochemical techniques, since the inactivation of GAPDH by $PARP_1$ is accompanied by nuclear translocation.

It is obviously also critical that the outstanding glycolytic enzymes not tested for in this study be analyzed, both at transcriptional and post-translational level.

8.5 Effect of CYP P450 enzyme activity

In chapter 7 it is demonstrated that maternal nicotine exposure during pregnancy and lactation results in the induction of CYP2A3 and 2B1 and that the transcript levels remain high even after the pups are weaned and are no longer exposed to nicotine via the mother's milk. Studies show that CYP activity may result in significant levels of oxidants (Newcomb *et al.*, 2003). It can not be directly assumed that an increase in transcription will result in an increase in activity and therefore the activities of selected CYP P450's involved in nicotine metabolism should be investigated. Studies show, however, that many CYP P450 enzymes, including CYP1A1 is regulated at the transcriptional level. Thus CYP P450 content and activity should be analyzed.

An interesting aspect to CYP P450 metabolism is that CYP2A3 utilizes a 2'-hydroxylation pathway as well as a 5'-hydroxylation pathway when metabolizing nicotine (Hecht *et al.*, 2000). The former can result in the formation of a lung pre-carcinogen, aminoketone. The implications of this pathway in developing lung, is not known. Thus if the presence of nicotine results in the formation of carcinogens, it would be imperative to test for mutagenicity of nicotine. It would also be important to determine if amino-ketones are produced in the lungs of neonates exposed to nicotine during pregnancy and lactation.

8.6 The use of Functional Biology

Modern high-throughput molecular biology technologies eg. microarrays, proteomics, and metabolomics enable the simultaneous measurement of metabolic processes of an entire cell. The computational biology techniques required to identify entire cellular networks that are activated or inactivated due to any

environmental change, including nicotine or its metabolites are now commonplace. In South Africa, there is a growing number of high-throughput biology platforms along with the bioinformatics capacity required to interpret the data. The power of using these technologies is that possibly many non-obvious mechanisms playing a role in the adverse effects of nicotine exposure may simultaneously be uncovered, expediting the completion of the proposed studies.

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9.1 CHAPTER 1: INTRODUCTION

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9.6 CHAPTER 6: ANALYSIS OF LACTATE DEHYDROGENASE ISOENZYME PATTERNS AND TRANSCRIPT LEVELS IN DEVELOPING NEONATAL LUNG: INFLUENCE OF MATERNAL NICOTINE EXPOSURE

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