Nicotine and cotinine effects on fetal rat lung type II alveolar cells

by

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A Thesis submitted to the Faculty of Graduate Studies of

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ABSTRACT

The type II alveolar cell is the primary site for pulmonary surfactant synthesis, secretion, storage, and recycling. Pulmonary surfactant is composed of a complex mixture of lipids and proteins. It enables efficient ventilation of the lungs by reducing the surface tension at end expiration. Cigarette smoke is linked to a number of respiratory complications and nicotine is the major bioactive component of cigarette smoke. Cotinine is the major metabolite of nicotine and is far more stable than nicotine *in vivo* and thus may also be harmful. The purpose of this study was to examine the effects of nicotine or cotinine on fetal rat lung type II alveolar cells and on pulmonary surfactant metabolism. Fetal rat lung type II alveolar cells were isolated and used in the studies after reaching confluence over a period of 4-5 days. Structural changes on the fetal type II cell were observed through electron microscopy. Analysis of mitochondrial activity, DNA damage, and DNA synthesis were conducted through Formazan assay, Comet assay, and [^{3}H]thymidine incorporation respectively. To examine the effects of nicotine or cotinine on pulmonary surfactant metabolism, disaturated phosphatidylcholine (DSPC) synthesis, DSPC secretion, surfactant protein levels, and surfactant-related material quality were measured separately. DSPC synthesis and secretion were analyzed using [^{3}H]choline and Thin Layer Chromatography. Nicotine or cotinine treated samples were simultaneously labeled with [^{3}H]choline for DSPC synthesis whereas DSPC secretion studies required [^{3}H]choline prelabeling of fetal rat lung type II alveolar cells. Changes in protein levels and synthesis were measured using confocal microscopy, western blots, and [^{35}S]methionine labeling. The Capillary Surfactometer was used to measure surfacatant-related material quality. The results showed changes to the fetal rat lung type

II alveolar cells with signs of apoptosis. Mitochondrial activity increased from 1, 3, 6, and 12 hour time points in nicotine or cotinine treated samples compared to the controls ($p<0.01$). DNA damage was also observed in the fetal rat lung type II alveolar cells and the rate of DNA synthesis decreased. Nicotine or cotinine treatment to fetal rat lung type II alveolar cells altered pulmonary surfactant metabolism by significantly increasing ($p<0.05$) DSPC synthesis but decreasing DSPC secretion after 1-6 hours of treatment ($p<0.01$). Changes in protein radiolabelling levels were not detected but cellular protein secretion was altered showing a significant decrease ($p<0.01$) in cells exposed to nicotine, cotinine, phorbol ester and a combination of cotinine and phorbol ester samples. The evaluation of surfactant-related material quality showed a dysfunction of surfactant-related material where a significant decrease ($p<0.01$) in capillary tube patency was observed for the nicotine or cotinine-exposed samples. The effects of nicotine or cotinine on fetal rat lung type II alveolar cells showed structural changes and an alteration to the intracellular activities within the cell. DSPC synthesis increased but secretion decreased and the surface tension reducing properties declined significantly. Taken together these experiments suggest that these changes alter metabolism of surfactant-producing fetal rat lung type II alveolar cells. Future studies are required to determine the mechanisms behind the changes to the type II alveolar cell and pulmonary surfactant metabolism.

Introduction

The Respiratory System - Overview

The respiratory system is one of the most complex systems in the human body. It functions in air conduction, air filtration, and gas exchange. The conducting portions of the respiratory system consist of the mouth, nose, nasopharynx, oropharynx, larynx, trachea, the bronchi, and bronchioles. The bronchioles represent the terminal portions of the conducting passages. Gas exchange occurs in the respiratory portion and this consists of respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The alveoli are the terminal air spaces and are the sites for gas exchange between air and blood. The air-blood barrier located in the alveolar septum, provides a barrier for the lung where gas exchange occurs. The alveolar septum contains alveolar epithelial cells, endothelial cells, connective tissue elements as well as basal laminae of the alveolar epithelium and capillary endothelium. Gas exchange is crucial at the alveolar septum because both oxygen and carbon dioxide must cross this barrier.

The cellular makeup of the respiratory portion of the lungs consists of alveolar macrophages, Clara cells, fibroblasts, type I alveolar cells, and type II alveolar cells. The type II alveolar cells are of special interest in this study. Alveolar type II cells are found within the alveoli and produce a complex lipoprotein mixture known as pulmonary surfactant. Pulmonary surfactant is a substance that is used to decrease the surface tension within the lungs and prevent the lungs from collapsing. Components and metabolites of tobacco smoke alter fetal rat lung alveolar type II cell metabolism which in turn affects cellular replication and the synthesis and secretion of pulmonary surfactant

components. The focus of this study will be centered on the effects of nicotine and its metabolite, cotinine on pulmonary surfactant and type II alveolar cells, thus further details on these components will discussed.

2.1 Lung development

In the human, development of the prenatal lung begins on the 26th gestational day and is completed during postnatal life at 2-7 days [1]. Prenatal lung development is divided into five distinct phases. The early phases of lung organogenesis include the embryonic (26th day-7th wk) and pseudoglandular phases (5th-7th wk). The canalicular (16th-26th week), saccular (24th-38th week), and alveolar phases (36th week – 36 months postnatal), makeup the remaining stages of lung development. The embryonic phase is characterized by the formation of the major airways [1]. Embryonic cells cluster and both the mesenchymal and epithelial cells undergo widespread proliferation [2]. During the pseudoglandular phase, the epithelial cells lining the airways continue to expand and differentiate [1-3]. The airways go through repetitive dichotomous branching which results in the formation of the bronchial airway tree [1-3].

The transition into the subsequent canalicular phase begins with the development of the lung periphery. The epithelial cells located at the periphery of the airway tree give rise to the alveolar type I and alveolar type II cells. Shortly after their appearance the onset of surfactant synthesis and secretion begins [1, 2]. During this stage respiratory bronchioles appear, the airways continue to differentiate, the acinus is produced, the vascular network increases completely, and formation of the air blood barrier takes place [1-3].

In the saccular phase, the airspaces expand and develop into saccules. The interstitium between the airspaces decreases in size and the vascular network continues to expand [1, 2]. During the alveolar phase, the primary septae, consisting of connective tissue, fibroblasts, and surrounding capillaries, protrude into the terminal saccules giving rise to the alveoli. The lengthening of the septae occurs concurrently with surfactant secretion and an increase of lamellar bodies, the intracellular storage form of pulmonary surfactant [3]. In the microvascular phase the interalveolar septae are altered and the capillary bed is reorganized [1, 2].

2.2 Epithelium

2.2a) Clara cells

Clara cells are cuboidal to columnar epithelial cells, which are found in the bronchiolar epithelium and are present from the upper airways to the distal conducting airways. Within the Clara cells exist basal nuclei and apical secretory granules. There are no cilia or apical microvilli present in these cells. The function of the Clara cell has yet to be determined. Studies have suggested Clara cells produce surfactant proteins and secrete a substance similar to pulmonary surfactant in the human lung that may assist in preventing lung collapse [4, 5]. The surfactant proteins identified in Clara cells consist of surfactant proteins-A, -B, and –D. The SP-A and SP-D in Clara cells may play a role in host defense mechanisms but there is no evidence indicating that the SP-A and SP-D produced in the Clara cells have a direct role in the pulmonary surfactant function at the alveolar level [6].

2.2b) Alveolar macrophages

The alveolar macrophages are mobile cells derived from blood monocytes that develop in the lungs. These cells are located as free-lying on the epithelial surfaces of the lung and within the parenchymal interstitium. Alveolar macrophages represent the mononuclear phagocytic system and are considered to be the first line of defense against foreign material. As a result, alveolar macrophages play a critical role in maintaining the structure and function of the lungs [7]. These cells contribute to lung homeostasis through a variety of mechanisms. One mechanism is through the phagocytosis of microorganisms. Other ways they maintain lung homeostasis is by the removal of particulates and macromolecular debris [7]. Alveolar macrophages play a role in the immune response by acting as an accessory cell whereby they recruit and activate other inflammatory cells [8].

2.2c) Fibroblasts

Fibroblasts are elongated spindle-shaped cells. These cells are located in both the alveolar septum and in thick portions of the pulmonary interstitium [3]. An important function of the fibroblast cell is in their ability to produce triglycerides. Triglycerides play a role in the synthesis of surfactant phospholipids [9]. Growth factors found within fibroblasts such as keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) have been found to promote cell proliferation and DNA synthesis in rat alveolar type II cells [10]. During lung development, fibroblasts produce extracellular matrix structural proteins such as elastin and collagen. Fibroblasts may also store and metabolize retinoids [3].

2.2d) Alveolar type I cell

Alveolar type I cells are squamous cells having a broad, flattened appearance [11, 12]. The origin of the alveolar type I cell during fetal development has yet to be determined but studies have suggested that alveolar type II cells are the precursors to type I cells in late fetal life [11, 13]. The alveolar type I cells are terminally differentiated and cannot undergo cell division nor alter their phenotypic characteristics [11]. During lung injury damaged alveolar type I cells are sloughed off and replaced by type II alveolar cells which alter their phenotype and obtain characteristics similar to that of the alveolar type I cell [11] .

The main functions of the alveolar type I cells are provisions of the air-exposed side of the blood-air barrier and thus in gas exchange [11, 14] . Other important functions involve regulation of ion and fluid transport and in possible regulation of lamellar body secretion from alveolar type II cells [14, 15]. The presence of aquaporins in the alveolar type I cell creates high water permeability allowing for an environment that is active in ion transport. In effect, the transport of ions allows the alveolar type I cell to sustain alveolar fluid balance [14]. The regulation of lamellar body secretion is possible via intercellular communication between the alveolar type I cell and the type II cell. Studies conducted by Ashino *et.al.*, have found that during alveolar expansion alveolar type I cells will undergo $[Ca_{2+}]_i$ oscillations. The gap junctions present between the type I and type II cells transmit the $[Ca_{2+}]_i$ oscillations from the type I cell to the type II cell resulting in the exocytosis of the lamellar bodies. As a result, alveolar type I cells act as mechanotransducers which aid in regulation of the secretion of the lamellar bodies from the alveolar type II cells [15].

2.2e) Alveolar type II cells

Alveolar type II cells are small cuboidal cells with a diameter of 9μm located within the corners of the alveoli [16]. The alveolar type I and type II cells line approximately 99% of the internal surface area of the alveolar epithelium [17]. The alveolar type I cell constitutes >95% of the internal surface area of the lung whereas the alveolar type II cell makeup <5% [14]. Of the epithelial lining cells, 63% are the type II cells while 37% are the alveolar type I cells [18]. Alveolar type II cells are polarized cells containing a cell membrane that is divided into apical and basolateral domains. The apical membrane displays an abundant number of microvilli protruding toward the air space and overlying hypophase. A variety of molecules exist in the apical membrane that appear not to be present in the basolateral domain [16]. With respect to their intercellular components, the alveolar type II cells contain organelles found in most metabolically active cells including mitochondria, endoplasmic reticulum, polyribosomes, microperoxisomes, microtubules, and Golgi apparatus [16, 19]. A unique characteristic of the alveolar type II cells is the presence of lamellar bodies [20]. Lamellar bodies are the intracellular storage form of pulmonary surfactant. The surfactant phospholipids found within the lamellar bodies are stacked in layers within the lamellar bodies and are surrounded by a membrane [16]. The main functions of the alveolar type II cell include: the synthesis and secretion of pulmonary surfactant, xenobiotic metabolism, transepithelial water movement, and proliferation and differentiation after lung injury [16, 19, 20].

2.3 Pulmonary Surfactant

Pulmonary surfactant is a complex mixture of lipids and proteins. Synthesis and secretion of pulmonary surfactant occurs in the type II alveolar cells. The importance of this substance lies in its ability to reduce the surface tension within the lungs. Maintaining a low surface tension is crucial in preventing lung collapse at maximum exhalation [21]. The prevention of lung collapse is also essential in preventing the onset of respiratory distress syndrome in premature infants [22]. Surface tension is a measurement of force acting on the surface of a liquid. This force minimizes the surface area of the liquid [23]. The air-water interface in the alveoli contains a high surface tension. Therefore it is imperative that the surface tension be reduced by pulmonary surfactant to prevent lung collapse [21, 22].

2.3a) Molecular composition

The amount of lipids present in pulmonary surfactant is approximately 90% whereas proteins only makeup 10% [24, 25]. Phospholipids represent a majority of the surfactant lipids consisting of ~ 85 %. Among the phospholipids, 70-80 % of them are phosphatidylcholine and 60 % of the phosphatidylcholines are in the saturated form of dipalmitoyl phosphatidylcholine (DPPC) [25]. DPPC is the most significant phospholipid due to its high surface activity within surfactant [22]. Phosphatidylglycerol is another phospholipid present in surfactant and represents 12 % of the phospholipid component [25]. The most abundant neutral phospholipid present in pulmonary surfactant is cholesterol[25]. The proteins identified in surfactant consist of four different types which include surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C), and surfactant protein D (SP-D) [21].

The molecular composition of pulmonary surfactant is dependent on structural form and the respiratory cycle. The different structural forms of pulmonary surfactant consist of lamellar bodies, tubular myelin and the surface monolayer [21]. The lamellar bodies are vesicles located within the type II alveolar cells. These structures consist of several layers of surfactant phospholipids and proteins that are densely packed together [21]. The function of these vesicles is to store pulmonary surfactant. The lipid composition found in both the alveolar surfactant and the intracellular lamellar bodies is similar. However, the protein content differs by the absence of SP-D and a very small amount of SP-A present in lamellar bodies. Research has also identified that less than 1 % of SP-A is found within the lamellar bodies and approximately 50 % of SP-A is present in alveolar surfactant [21].

The exocytosis of lamellar bodies into the hypophase layer results in the formation of a complex lattice structure known as tubular myelin [21]. Tubular myelin has been identified as the source of the surface monolayer [21]. The molecular composition of tubular myelin has yet to be accurately assessed but SP-A has been found to exist at the corners of the tubular myelin lattice [21]. The surface monolayer found at the air-water interface consists of mainly DPPC [26, 27].

2.3b) Role of lipids

Phospholipids are amphipathic molecules containing polar head groups and non polar acyl chains [27]. In pulmonary surfactant, the phospholipid head groups consist mostly of choline and to a lesser degree glycerol, serine, inositol and ethanolamine [28]. The arrangement of the phospholipids within the air-liquid interface of the alveoli is based on their physical makeup. Strong interactions exist between the polar head groups

of the phospholipids and the aqueous phase in the alveoli, which enable these molecules to be closely associated with one and other [28]. On the other hand, the acyl chains do not reside in the aqueous phase due to their hydrophobic nature [28]. The organization of the phospholipids in the air-liquid interface plays an important role in lowering the surface tension. High surface tension in the alveoli occurs at 70 mN/m. In order to prevent lung collapse the surface tension must be reduced to a minimum of 25mN/m or less during expiration [27, 29]. Since DPPC makes up the majority of pulmonary surfactant, research has been able to identify that this phospholipid is primarily responsible for reducing the surface tension during compression at the air-liquid interface [26, 27].

The acidic phospholipids have been linked to enhancing the adsorption process of pulmonary surfactant [24, 27]. Mixtures of acidic phospholipids with DPPC tend to increase the rate of adsorption, which ultimately contributes to the reduction of surface tension [26, 27]. Strong interactions exist between the phosphatidylglycerol head group and calcium. These strong interactions result in the stabilization and also the improved function and organization of the surfactant system [27, 28].

Increased amounts of minor phospholipids have been suggested to decrease the activity of surfactant [24]. It has also been speculated that the minor phospholipids play a role in signaling events involved in surfactant metabolism [24]. The neutral lipids consisting mainly of cholesterol with small amounts of monoacylglycerol, diacylglycerol, triacylglycerol and palmitic acid, have been implicated in enhancing the adsorption rate of DPPC vesicles through an increase of fluidity within the hypophase. The increase in fluidity also helps in improving the respreading of the phospholipid film [24]. The

physiological effects of cholesterol in relation to pulmonary surfactant have not been established [24].

2.3c) Role of Proteins

2.3.c.i) Surfactant protein A (SP-A)

SP-A is the most abundant protein present in pulmonary surfactant. The expression of SP-A occurs in both the type II alveolar cell and Clara cells [22, 27, 28, 30, 31]. SP-A is an oligomeric glycoprotein that belongs to a group of proteins known as collectins [30, 32-34]. The primary structure of SP-A consists of four domains which include: a short N-terminal segment containing intermolecular disulfide bonds, a proline rich collagen-like domain, a hydrophobic neck domain, and a carbohydrate recognition domain (CRD) [26, 31, 33]. These structural domains play an important role in the binding and interactions of SP-A with various amphipathic lipids, carbohydrates, and cell surface receptors [22, 33, 34].

The role of SP-A within the alveolus involves maintaining the function and homeostasis of pulmonary surfactant [26]. SP-A is involved in the secretion of pulmonary surfactant, formation of tubular myelin, and in facilitating the surface tension lowering abilities of surfactant phospholipids [30, 32]. Homeostasis is maintained through the inhibition of phospholipases, protection of the surfactant film from protein inhibitors, and lung clearance of pathogenic organisms [26, 30, 32]

In respiratory distress syndrome (RDS), the lung injury that is caused by this disease results in a leak of plasma within the alveolar space [30]. The plasma proteins that are present in blood can alter or even inhibit the activity of pulmonary surfactant [30]. The presence of SP-A is crucial because of its ability to minimize the inhibition of

surfactant activity caused by plasma proteins [30]. SP-A also has important implications in the pulmonary innate immune response [28, 31, 33]. Lung particulates or bacteria that are present within the alveolus can cause serious infections [30, 34]. SP-A can bind to foreign material through its CRD or through the lipid structures of bacterial lipopolysaccharides [30, 34]. As a result, SP-A functions as an opsonin for lung pathogens, leading to the phagocytosis of pathogens through the stimulation of alveolar macrophages [30, 34].

2.3.c.ii) Surfactant protein B (SP-B)

Surfactant protein B is a homodimeric proteolipid [27, 28]. Expression of SP-B occurs in both type II alveolar cells and Clara cells [27, 35]. SP-B is stored in the lamellar bodies and secreted along with the surfactant phospholipids into the subphase of the lining fluid of the alveolus [26-28, 36]. SP-B is both amphipathic and hydrophobic which enables the protein to interact strongly with the anionic surfactant phospholipids [22, 26-28]. The physical structure of SP-B consists of disulfide-linked homodimers containing two of the 79 amino acid residue polypeptide chains[21]. These structural characteristics allow for a constant interaction between the phospholipids and SP-B [37].

The major role of SP-B is to improve the physical properties of surfactant phospholipids [38]. SP-B enhances the adsorption of surfactant phospholipids, thereby allowing the surfactant phospholipids to be rapidly inserted into the air-water interface [38, 39]. Other important functions of SP-B include: tubular myelin formation, respreading of films from collapse phase, reuptake of surfactant to type II cells, membrane binding and membrane fusion [28, 38, 39]. Recruitment of phospholipids to

the expanding film and the alteration of the film composition during compression and expansion of the lungs by SP-B, stabilizes the phospholipid layer [36].

The significance of this protein in RDS is that lethal RDS occurs when it is completely deficient in pulmonary surfactant [26, 36, 38]. Of the four proteins that have been identified in pulmonary surfactant, SP-B is the only protein required for postnatal function and survival [36]. Furthermore, the absence of SP-B leads to a decreased production of mature SP-C peptides and altered structure of the lamellar bodies [36].

2.3.c.iii) Surfactant protein C (SP-C)

Surfactant protein C is the only protein that is unique to pulmonary surfactant [26, 37]. The most notable characteristic feature of SP-C is its hydrophobic nature[21, 26, 36]. The expression and synthesis for SP-C has been detected exclusively in the lung and more specifically within the alveolar type II cells [26, 28, 37]. SP-C has been established as a surfactant specific protein and does not contain any other proteins of homologous structure [37].

The structure of SP-C contains a hydrophobic stretch which enables the peptide to span the phospholipid bilayer rich in DPPC [38, 40, 41]. Another unique physical characteristic of SP-C is the palmitoylation of two cysteine residues, which contributes to the hydrophobic nature of SP-C [38, 41]. Palmitoylation can strengthen the protein interactions with membranes, which may play a role in membrane fusion and orientation of the peptide [42]. The functions of SP-C in surfactant are similar to SP-B and these include: the promotion of phospholipid adsorption at the air-liquid interface, stabilization and ordering of the phospholipid monolayer film, respreading of films from collapse phase, and the reuptake of surfactant by type II alveolar cells [21, 26, 28, 36, 38].

2.3.c.iv) Surfactant protein D (SP-D)

Surfactant protein D is a hydrophilic glycoprotein and is the second collectin found in the pulmonary alveolus [22, 43, 44]. Synthesis and secretion of SP-D occurs in the alveolar type II cells, the non ciliated airway cells, and in the cells of the gastric mucosa [22, 28, 43]. Since this protein is not restricted to the lung and its role is not exclusively directed toward maintaining surfactant homeostasis, it has been suggested that SP-D is not a true surfactant protein [38]. SP-D is involved with the host defense mechanisms in the lung and there is little evidence indicating a direct role in the surface activity of pulmonary surfactant [21, 45]. SP-D does have the ability to bind to lipids, which may suggest a role in the reorganization and recycling of pulmonary surfactant [21]. Furthermore, mice deficient in SP-D develop alterations in surfactant metabolism and homeostasis [44]. The most recognized functions of SP-D involve activating alveolar macrophages, defending against any bacterial, viral, and/or fungal infections, along with a possible role in phosphatidylinositol metabolism [38, 46]. SP-D can bind with high affinity to alveolar macrophages and this induces the activation of the alveolar macrophages and the production of oxygen radicals both of which are important in the host defense properties of the lung [38, 45]. SP-D can bind to phosphatidylinositol although studies have yet to identify the physiologic importance of this interaction. It has been suggested that the binding of SP-D to phosphatidylinositol plays a role in intracellular lipid sorting and or signal transduction [38].

2.4 Surfactant Metabolism

2.4a) Surfactant Phospholipid Synthesis

Substrates required for Surfactant Phospholipid Biosynthesis

Surfactant lipid biosynthesis occurs primarily in the type II alveolar cells of the lung (refer to Figure 1). The substrates required for this process are obtained from the blood. Glucose and glycogen are precursors that become incorporated into the phospholipids during the early stages of lipid synthesis [47]. In fetal lung type II alveolar cells, glycogen is the major source for glycerol 3-phosphate in surfactant glycerolipid synthesis and it has also been suggested that glycogen may act as a precursor to fatty acids during the late fetal period [48-50]. Glucose is the main source of the glycerol backbone for surfactant glycerolipids in adult type II alveolar cells [48, 51]. Circulating glycerol is another precursor to glycerol 3-phosphate but its role as a substrate is limited [48, 51]. Fatty acids substrates can be obtained through circulation or via hydrolysis of phospholipids [51, 52]. Adult lung type II alveolar cells synthesize fatty acids *de novo* and these substrates are obtained through the circulation or via hydrolysis of phospholipids[51-54]. Polar head constituents such as choline and inositol of surfactant phospholipids are obtained from the blood [48]. Isolated type II alveolar cells can effectively take up choline and this is due to the abundance of choline kinase within these cells [55]. The synthesis of free choline does not take place in mammalian tissues and studies suggest that it is mostly derived from the diet [56].

Formation of surfactant phosphatidylcholine

Synthesis of phosphatidylcholine begins with the formation of phosphatidic acid [51]. Phosphatidic acid is derived from either glycerol 3-phosphate or dihydroxyacetone-

phosphate (DHAP). In the formation of DHAP, glucose and glycogen precursors are metabolized into the glycolysis intermediate [47]. DHAP will undergo reduction to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase or acylation into acyldihydroxyacetone-phosphate catalyzed by dihydroxyacetone-phosphate acyltransferase [51].

The first step in phosphatidic acid synthesis involves the acylation of glycerol 3-phosphate into 1-acylglycerol-3-phosphate by glycerol-3-phosphate acyltransferase which is located in the endoplasmic reticulum of type II alveolar cells [47, 51, 57]. The formation of 1-acylglycerol-3-phosphate can also occur through another pathway where dihydroxyacetone-phosphate acyltransferase catalyzes the acylation of DHAP into acyldihydroxyacetone-phosphate which undergoes reduction into 1-acylglycerol-3-phosphate by acyldihydroxyacetone-phosphate reductase [51]. The formation of phosphatidic acid proceeds through the acylation of 1-acylglycerol-3-phosphate by 1-acylglycerol-3-phosphate acyltransferase [57]. In type II cells, studies suggest that 60% of phosphatidic acid may be synthesized through DHAP acylation [58].

The formation of phosphatidic acid represents an important step in glycerolipid metabolism. At this point, the pathways for phosphatidylcholine, phosphatidylinositol, and phosphatidylglycerol synthesis begin to deviate [57]. In the lung, diacylglycerol and CDPcholine are the substrates used in the terminal step of phosphatidylcholine synthesis [57]. Diacylglycerol is formed though hydrolysis of phosphatidic acid catalyzed by phosphatidate phosphatase [51]. Cholinephosphotransferase catalyzes the final step in the synthesis *de novo* of phosphatidylcholine [57]. The CDPcholine required for PC formation is formed via the sequential action of choline kinase and choline-phosphate

cytidyltransferase [51]. Choline kinase is located in the cytosol and choline-phosphate cytidyltransferase can be found in the cytosol and endoplasmic reticulum [47, 59].

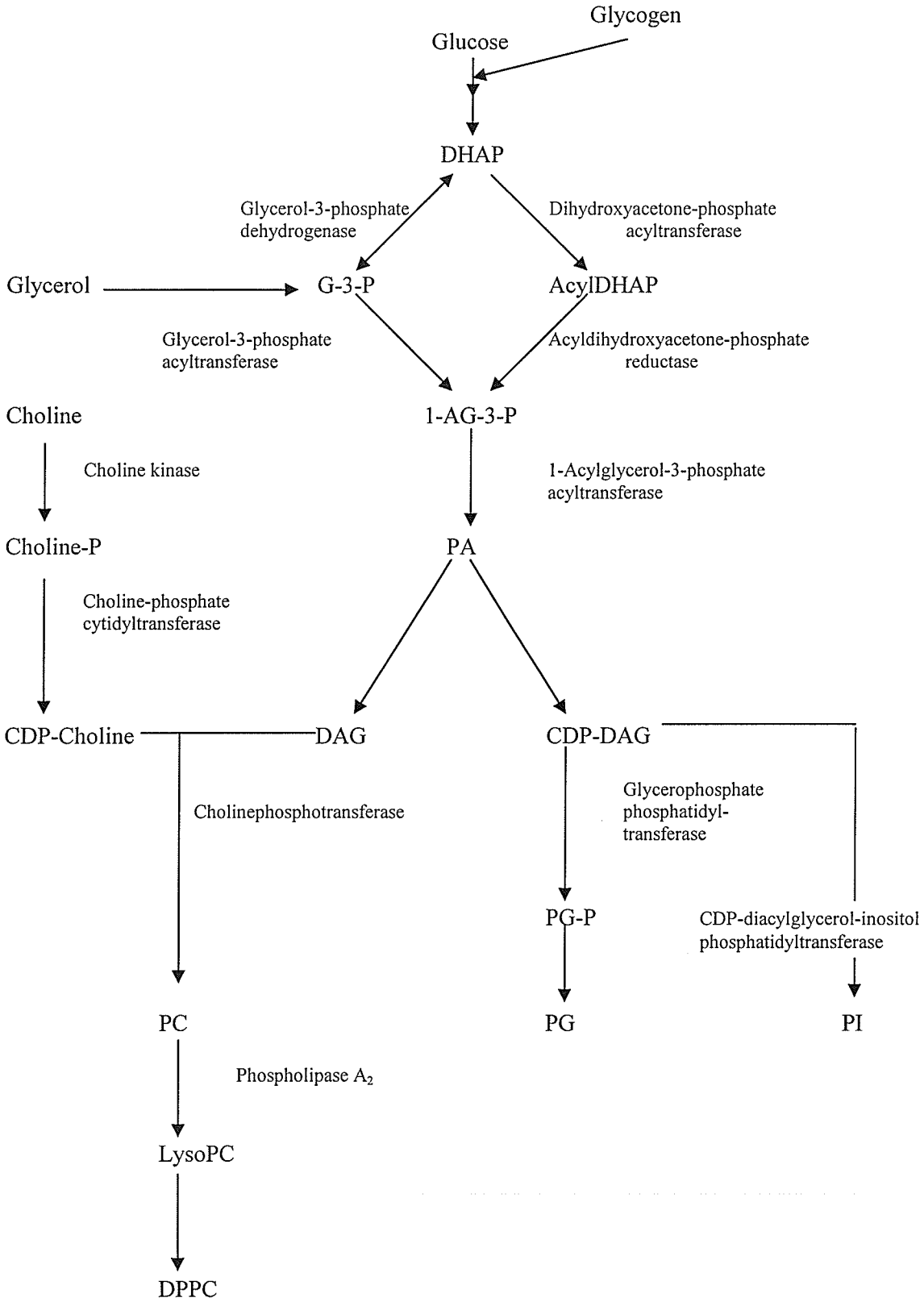
Formation of DPPC

The synthesis of DPPC can take place through two different pathways one being synthesis *de novo*, the second being remodeling of unsaturated phosphatidylcholine species [48]. It has been estimated that approximately 55% of DPPC formed is through remodeling and the remaining 45% is made via synthesis *de novo* [48, 51]. Pulse-chase experiments have also determined that under *in vitro* conditions 75% of disaturated phosphatidylcholine (DSPC) is formed through remodeling [60]. The remodeling process consists of two proposed mechanisms which include a deacylation-reacylation mechanism and a deacylation-transacylation mechanism [57]. In the deacylation-reacylation process, phospholipase A_2 catalyzes the deacylation at the 2-position of phosphatidylcholine forming lysophosphatidylcholine which is followed by reacylation of lysophosphatidylcholine by lysophosphatidylcholine acyltransferase into DPPC [51, 57]. The deacylation-transacylation begins with the removal of unsaturated acyl moiety followed by transacylation of lysophosphatidylcholine: lysophosphatidylcholine acyltransferase into the resultant DPPC [51, 57]. Studies suggest that the deacylation-reacylation process in whole lung, adult type II alveolar cell and fetal type II alveolar cell is the more important mechanism in DPPC synthesis [51].

Figure 1: Biosynthesis of surfactant phospholipids in lung type II alveolar cells [51, 57].

Abbreviations:

DHAP	Dihydroxyacetone-phosphate
G-3-P	Glycerol-3-phosphate
AcylDHAP	Acyldihydroxyacetonephosphate
1-AG-3-P	1-Acylglycerol-3-phosphate
PA	Phosphatidic acid
DAG	Diacylglycerol
Choline-P	Choline-Phosphate
CDP-choline	Cytidine 5'-diphosphate-choline
CDP-DAG	Cytidine 5'-diphosphate diacylglycerol
PG-P	Phosphatidylglycerol-phosphate
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
LysoPC	Lysophosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine


Glycogen
Glucose
DHAP
Glycerol-3-phosphate dehydrogenase
Dihydroxyacetone-phosphate acyltransferase
Glycerol
G-3-P
AcylDHAP
Glycerol-3-phosphate acyltransferase
Acyldihydroxyacetone-phosphate reductase
Choline
1-AG-3-P
Choline kinase
1-Acylglycerol-3-phosphate acyltransferase
Choline-P
PA
Choline-phosphate cytidyltransferase
CDP-Choline
DAG
CDP-DAG
Cholinephosphotransferase
Glycerophosphate phosphatidyl-transferase
PG-P
CDP-diacylglycerol-inositol phosphatidyltransferase
PC
PG
PI
Phospholipase A_2
LysoPC
DPPC

2.4b) Storage and Secretion

Surfactant phospholipids are stored in the lamellar bodies of type II alveolar cells and are secreted via exocytosis [22]. The majority of surfactant components are synthesized in the endoplasmic reticulum of the type II alveolar cell [51]. Surfactant lipids and proteins will undergo further processing in the Golgi before being transferred to the lamellar body [51]. The secretion of pulmonary surfactant from the type II alveolar cells can occur through constitutive or regulated pathways [61]. Constitutive secretion is a process where substances within a cell are continually being secreted. Regulated secretion involves the release of synthesized and stored substances in cytoplasmic organelles from a cell upon appropriate stimulation [61]. Currently, there is little evidence concerning the mechanisms of constitutive secretion; the majority of studies have focused on the regulated pathways of secretion. The intracellular secretion of surfactant occurs in response to a variety of humoral and mechanical stimuli [28]. Such stimulatory factors that have been identified *in vitro* include: phorbol esters, ATP, prostaglandins, arachidonic acid, and catecholamines [28, 62]. The *in vivo* stimulus for surfactant secretion is possible through mechanical ventilation induced stretch [16, 63].

Secretion of surfactant phospholipids occurs through three signal transduction pathways within the type II cells [22, 62]. One method involves the activation of adenylate cyclase [22, 62]. This pathway is triggered by the binding of β agonists to β adrenergic or adenosine receptors and leads to the formation of cyclic AMP and activation of cAMP – dependent protein kinase A [22, 62].

The second pathway involves the direct and indirect activation of protein kinase C [22, 62]. Direct activation of PKC is possible through the interaction of PKC with

permeable substances such as 12 – O – tetradecanoylphorbol – 13- acetate (TPA) and diacylglycerol [62, 64]. Both ATP and UTP indirectly activate PKC through the binding with purine receptors P2Y [62, 64].

The third signal transduction pathway involves the increase of intracellular Ca^{2+} levels [62, 63]. An increase in Ca^{2+} levels results in the activation of the Ca^{2+} calmodulin dependent protein kinase, which is necessary for surfactant phospholipid secretion [62]. Various factors such as the extracellular uptake of calcium via ionophores, Ca^{2+} transmission from type I alveolar cells, or the intracellular release of calcium following IP_3 hydrolysis all contribute to the increase of intracellular Ca^{2+} levels [62, 65, 66].

The major physiologic stimulus for surfactant secretion is ventilation[16, 62, 63]. Ventilation results in the stretching of type II alveolar cells, and this causes the release of arachidonic acid metabolites, ATP, UTP[16, 64]. Extracellular influx of calcium, and depolarization of the membranes also occur with ventilation and contribute to the secretion process [16].

Exocytosis of the surfactant phospholipids from the lamellar bodies to the alveolus is the final step in the secretion pathway [62]. Both the SP-B and SP-C proteins are found in the lamellar bodies and are secreted via exocytosis; however, the mechanisms behind the secretion of SP-A and SP-D have yet to be determined [22]. Transfer and fusion of the lamellar bodies to the membranes of the type II cells are carried out through the annexins[62, 67, 68].

Tubular Myelin Formation

Tubular myelin formation commences once the stored surfactant in the lamellar bodies has been secreted into the hypophase layer of the alveolus[27, 38, 62]. Factors

such as the pH levels, the presence of SP-A, and the Ca^{2+} concentration are all essential in the transformation process and in stabilizing the surfactant structure [27, 38, 62, 69]. During the initial stages of transformation, the concentric lamellae of the secreted lamellar bodies begin to lose their stability and are no longer tightly packed together [27]. The transitional membranes of the lamellar bodies begin to accumulate material and unravel leading to the conversion of lamellar bodies into tubular myelin[27].

The next step involves refining of the lining layer through SP-B, which results in an increase of DPPC function [62, 70]. The conversion of the surface-active surfactant into spent or inactive form is possible through the type II alveolar cell enzyme convertase [62, 71, 72]. Studies have found that tubular myelin is important in the onset of RDS, where patients lacking this structure develop severe respiratory dysfunctions [27, 38, 62].

2.4c) Adsorption

The adsorption of pulmonary surfactant is a process that involves the insertion of the surfactant phospholipids into the air-water interface within the alveolus [27]. During this process the amphipathic phospholipids arrange the hydrophilic polar head groups in the water of the air-water interface and the hydrophobic non polar tails are exposed to the air [27]. Adsorption generally occurs through three main steps that involve the transport of surfactant aggregates from the hypophase to the air-water interface, insertion of surfactant into the monolayer, and the removal of non-DPPC molecules from the interface [27, 73].

Transference from hypophase to air-water interface

Once formation of tubular myelin is complete, these large aggregates

must transfer from the hypophase to the air-water interface[74]. Since DPPC makes up the majority of pulmonary surfactant, adsorption of this phospholipid component is important. However, the gel-to-liquid transition phase temperature of DPPC occurs at 41°C and with a physiological temperature at 37°C, adsorption of DPPC is fairly slow [73, 75]. Therefore, unsaturated PCs, which are fluid at 37°C, aid in the adsorption of DPPC [73]. Furthermore, SP-B and SP-C interact directly with surfactant phospholipids, causing them to have a more profound effect on the adsorption of DPPC and thus, dramatically increase the rate of transference from hypophase to the interface [73, 76, 77]. SP-A on the other hand, does not have a significant effect on adsorption alone, but it may act in conjunction with the other surfactant proteins in increasing the adsorption rate [27]. Other factors that contribute to the adsorption process involve the molecular structure of the phospholipid head groups, lipid/protein ratio, pH, temperature, and the amount of lipid dispersion within the hypophase [27, 73].

The equilibrium surface tension of phospholipids within a surface film is approximately 25mN/m and the adsorption of phospholipids will only take place once the surface film reaches a surface tension greater than the equilibrium surface tension [27, 29]. Adsorption of surfactant phospholipids is continuous once the equilibrium surface tension is surpassed and will stop only when the surface tension drops back down to equilibrium [27]. Any material that is lost or desorbed during the adsorption process will be replaced with new surfactant material [27].

Insertion into the Monolayer

The next phase in adsorption is the insertion of surfactant material into the surface monolayer. At this point, surfactant bilayers are closely associated with the air-water

interface or with any preexisting monolayers present [74, 78]. Recent studies have identified that the surface film within the monolayer is not entirely made up of a single layer of phospholipids, rather it consists of several multilayer structures attached to it [26, 74, 78]. It has also been established that an abundant amount of surfactant aggregates interact with the air-water interface prior to its actual insertion [73].

Insertion of surfactant material to the monolayer occurs once an open air space is available which enables the surfactant phospholipids to form the surface monolayer and thus activate these surfactant components [73]. In the following respiratory cycles, more surfactant bilayers will transfer to the air-water interface and further contribute to the formation of the surface film [73].

SP- B and SP-C have both been found to enhance the insertion of phospholipids to the surface film while SP-A is involved in improving adsorption only in the presence of other hydrophobic surfactant proteins [73, 77]. The mechanism by which SP-C inserts surfactant phospholipids to the surface film involves the interactions of the phospholipid acyl chains and the α helix of SP-C [74]. As the surface pressure within the alveolus increases, the α helix of SP-C alters its configuration within the surface monolayer, causing the acyl chains of the underlying phospholipids, that are attached to the α helix, to be physically pulled into the air-water interface[74, 79]. As a result, the lipid content within the surface film can increase each time the surface pressure increases [74, 79]. SP-B functions like a bridge between adjacent bilayers and monolayers, which enhances the formation of surfactant reservoirs[26, 73, 80]. Surfactant reservoirs make up the entire interfacial film containing the phospholipids and proteins that eventually insert themselves into the air-water interface [73].

Compression and Selective Exclusion

Once insertion of the surfactant phospholipids has taken place, removal of non-DPPC components is the next phase in adsorption. This process occurs during expiration and results in the compression of the surface film [73]. Surface film compression is significant because it causes the release of materials from the film [27]. Compression of the surface film during expiration produces high surface pressures around 70mN/m and surface tensions of approximately 0-1mN/m [73, 81]. Therefore, only molecules that can withstand this surface pressure and still be able to produce low surface tensions can exist within the surface film.

The only surfactant phospholipid that is capable of sustaining and maintaining both high surface pressures and low surface tension is DPPC [73, 82]. DPPC contains saturated acyl chains and its gel-liquid phase transition temperature occurs at 41°C; in effect, this enables them to not only maintain their solid phase at 37°C but it allows the acyl chains to pack so tightly together that it would prevent the alveolus from collapsing [73]. To produce a surface film rich in DPPC, all the non-DPPC components must be removed through a process known as selective squeeze out [73, 83]. Some of the non-DPPC components collapse and are removed at lower surface pressures, but SP-B and SP-C can also further accelerate this process [73]. Both SP-B and SP-C can associate with the surfactant phospholipids in the fluid phases and can also be transferred into the air-water interface [26, 74]. The exclusion of SP-B from the interface occurs at 40mN/m and SP-C is squeezed out at approximately 55mN/m [26]. Studies have identified that at low surface pressures, SP-B has an affect on lipid packing while SP-C on the other hand, can remove 7-10 lipid molecules during its removal from the monolayer[26, 73].

Research has yet to identify the specific lipids that are removed alongside SP-C, which implies that there may be an alternate method of removal for the surfactant lipids. However, since SP-C is involved with the fluid phase of the monolayer, there is a good possibility that only non-DPPC molecules are being removed [26].

Surfactant Recycling

Some of the membranes found within the large network of membranes between the surface film and hypophase detach and form small vesicles of surfactant that will eventually be recycled [61, 73]. The surfactant phospholipids and surfactant proteins SP-A, SP-B, and SP-C, all end up in the type II alveolar cell, however, research has yet to establish the exact route for SP-D [61, 84-86]. Some of the surfactant material can also be removed through macrophages or by the airways and circulation [22]. Clara cells and other alveolar cells do not take part in the recycling process [22]. Once inside the type II cells, the lipids and proteins enter the multivesicular bodies and then either become degraded or incorporated into the lamellar bodies for resynthesis of pulmonary surfactant [84, 87].

2.5 Tobacco Smoke

2.5a) Composition

Cigarette smoke contains a variety of chemicals that are not only toxic to the lungs but to other areas of the body as well. The diseases induced by cigarette smoke include the increased risk of heart disease and several different forms of cancer such as oral, bladder, heart, pancreatic, ovarian and lung. It is estimated that 2000-4000 chemical agents make up cigarette smoke[88]. Of these components the polynuclear aromatic hydrocarbons (PAH), aromatic amines, and N-nitrosamines are the known carcinogens in

cigarette smoke [89]. Nicotine is known to be the most toxic alkaloid[90]. Microparticles are also found in cigarette smoke and cause further damage to the respiratory tract through their deposition in the bronchioles and alveoli [88].

Deposition of cigarette smoke components occurs through a variety of mechanisms and these involve: direct gas deposition, evaporative gas deposition, particle deposition, and particle deposition with diffusion [91]. The volatile agents of cigarette smoke exist in the vapor phase and all other non volatile or semi volatile agents are found in the particulate phase [89]. Mainstream smoke, the smoke exhaled by the smoker, contains approximately 400-500 gaseous compounds[89].

Nicotine is a major constituent of cigarette smoke. The amount of nicotine in a cigarette has decreased from 2.7mg in 1954 to 0.95mg in 1992 [89]. Several studies have identified the concentrations of nicotine and cotinine, its major metabolite in serum, breast milk, and urine in smoking mothers. However there are very few studies that have yet to determine the amount of nicotine and cotinine being transferred to the fetus. A study conducted by Luck *et.al.*, determined that nicotine concentrations in the human fetus were higher than the smoking mothers [92]. In this study the nicotine concentrations found in the placenta (range 3.3-28 ng/g), amniotic fluid (1.5-23 ng/ml), and fetal serum (range 0.5-25 ng/ml) were all higher than the corresponding maternal serum and amniotic fluid values [92]. Cotinine concentrations were either lower than or equal to the maternal serum in the amniotic fluid (range 5-188 ng/ml), placenta (range 10-131 ng/g) and fetal serum (range 15-233 ng/ml) [92].

Transfer of nicotine from a smoking mother to the fetus is possible via the breastmilk. In nonsmoking mothers, there are very low to non detectable levels of

nicotine and cotinine in the plasma or breast fluid [93]. Smoking mothers contained nicotine and cotinine in the plasma and breast fluid. The range of nicotine present in the plasma (30-50ng/ml) was similar to the amount in breast fluid whereas cotinine levels are higher in the plasma (300-400ng/ml) compared to the breast fluid (100-200ng/ml) [93].

2.5b) The effects of cigarette smoke on the developing respiratory system

The effects of cigarette smoke on lung development

Cigarette smoke metabolite exposure of the lungs of infants from smoking mothers has many adverse effects. Exposure of the fetus to cigarette smoke metabolites has been found to cause airway hyperesponsiveness, decreased lung function, and airway obstruction postnatally [94]. Infants of smoking mothers are known to have a higher occurrence of respiratory infections in the first year of life [95]. Several other studies have found reduced and abnormal lung growth *in utero* and postnatally [95]. In rat models, studies showed that pregnant rats exposed to smoke resulted in the fetuses experiencing a decrease in lung volume, reduction in the amount of enlarged alveoli, decreased parenchymal elastic tissue, increased density of interstitium, and underdeveloped elastin and collagen [94-97]. In the airways, cigarette smoke exposure disrupts mucociliary clearance and also causes hyperpnea and bronchoconstriction [95].

Interestingly, fetal exposure to cigarette smoke has also been found to accelerate lung maturation. This increase in lung maturation has been linked to a decreased incidence of neonatal respiratory distress syndrome [98]. An increase in adrenal cortisol production through cigarette smoke exposure may be responsible in the advancement of lung maturation [98]. Although the increase in lung maturity and decrease in the onset of

neonatal RDS may seem beneficial, the infants of smoking mothers may still experience lung abnormalities and respiratory illnesses [98].

The effects of cigarette smoke on alveolar cells

Cigarette smoke exposure may be detrimental to the proliferation, attachment, and detachment of alveolar epithelial cells [99]. Alveolar cells may experience DNA single strand breaks, decreased surfactant secretion, and collagen production may also be suppressed in the presence of cigarette smoke [99, 100]. The inability of type II cells to attach inhibits epithelial repair and causes a further decrease in cell proliferation [99].

The effects of cigarette smoke on pulmonary surfactant

The effect of cigarette smoke on the pulmonary surfactant system is largely unknown. Studies conducted by Subramaniam *et.al.*, have found that alterations in the biochemical makeup of surfactant occur when exposed to cigarette smoke during pre- and postnatal development [101]. However, the changes that occur to the surfactant system do not affect the overall surface tension reducing properties of pulmonary surfactant. Earlier studies have reported possible composition and surface activity changes in adult rats chronically exposed to cigarette smoke [101]. In adult rats exposed to cigarette smoke bronchoalveolar lavage (BAL) samples resulted in a decrease in the amount of surfactant associated phosphatidylcholines and there are some suggestions that the secretion of these surfactant associated phospholipids is impaired [102].

2.5c) The effects of nicotine on the respiratory system

Maternal nicotine exposure has been found to adversely affect lung development, lung growth, and lung function [103]. Maternal nicotine exposure interferes with the metabolic development, and elastogenesis of the offspring [103] which in turn compromises the overall structural development of the lungs and prevents proper expansion of the lungs [104]. The gas exchange functions of the fetus may be compromised through the thickening of the alveolar septa and associated blood-air barrier. Growth restriction which is observed in the fetus may be due to the inability of effectively transferring amino acids from the mother to the fetus via the placenta [105, 106]. The suppression of alveolarisation and glycolysis also occurs in the lungs when exposed to nicotine and this further contributes to the lack of lung development along with causing the lungs to be more susceptible to damage[107].

In vivo, alveolar type I cells are more vulnerable than alveolar type II cells to the toxic effects of nicotine [108]. In response to alveolar type I injury, alveolar type II cells will proliferate and differentiate into type I cells. Nicotine exposure appears to increase the number of alveolar type II cells which may be in response to the type I cell injury [109]. Within the alveolar type II cell, nicotine exposure causes an accumulation of lamellar bodies along with alveolar fenestrations, mitochondrial swelling and membrane blebbing [108, 110]. Protein phosphorylation which is required for surfactant secretion, is suppressed in rat pups exposed to nicotine [110]. With respect to surfactant, nicotine exposure results in reduced surfactant lipid content and causes variable changes in surfactant protein gene expression[111].

These studies cited above have examined the effects of cigarette smoke on the developing respiratory system using *in vivo* models. Fewer studies have examined whether cigarette smoke derived agents are harmful to both the type II alveolar cell and the surfactant system and their mechanism of action. This study was designed to examine the effects of nicotine, a major cigarette smoke component, and its metabolite cotinine, on the type II alveolar cell and on pulmonary surfactant metabolism using an *in vitro* model.

It is hypothesized that components and/or metabolites of tobacco smoke are detrimental to the fetal rat lung type II alveolar cell and alter pulmonary surfactant metabolism thereby affecting synthesis or secretion of surfactant components.

Materials and Methods

3.1 Materials

Time pregnant Sprague Dawley rats were obtained from the breeding facility at the Central Animal Care at the University of Manitoba, Winnipeg, Manitoba, Canada. Media, sera, antibiotics and other reagents were obtained from Gibco (Burlington, ON, Canada). Tissue culture flasks were from Corning Costar (Cambridge, MA).

Nicotine and cotinine were obtained from Sigma Chemicals (St.Louis, MO). The formazan assay kit and comet assay kit were obtained from Promega (Ontario, Canada) and Trevigen (Gaithersburg, MD) respectively. Thin layer chromatography plates were obtained from Fisher Scientific Canada (Edmonton, AB, Canada). Radioactive materials [^{3}H]choline chloride, specific activity 75 Ci/mmole, and [^{35}S]methionine, specific activity 5 Ci/mmole were obtained through New England Nuclear (Boston, MA, USA). Centrifugation steps were carried out using the Jouan CR3000, and the Beckman L8-80M ultracentrifuge. The Beckman centrifuge required rotor 80Ti. Scintillation counts were conducted using the Beckman L5801.

Reagents, molecular standards, protein assay kits, and nitrocellulose membranes used for protein electrophoresis and western blots were obtained from Bio Rad (Mississauga, ON). Goat polyclonal IgG, (200 μg/ml), surfactant proteins (SP-A, SP-B, SP-C, SP-D) and donkey anti-goat IgG secondary antibody, were obtained from Santa Cruz Biotechnology, Inc. (California, USA). Chemiluminescence reagents and HyperFilm ECL film were obtained from Amersham Life Science Inc. (Oakville, ON).

In the immufluorescenct microscopy experiments, paraformaldehyde was obtained from Sigma Chemicals (St.Louis, MO). Propidium iodide was purchased from

Molecular Probes (Eugene, OR). Fluorescein (FITC)-conjugated mouse, monoclonal anti-BrdU antibody was purchased from BD Biosciences (Missisauga, ON). SYBR® Green I nuclear stain was purchased from Trevigen (Gaithersburg, MD). Donkey Anti-goat IgG FITC conjugate was purchased from Santa Cruz Biotechnology, Inc. (California, USA). Prolong Antifade used for immunostaining was purchased from Invitrogen Canada Inc. (Burlington, ON).

3.2 Methods

3.2a) Isolation of fetal rat lung type II alveolar cells

Pregnant Sprague Dawley rats (time of mating designated as day 0) were euthanized on the 22nd gestational day (term = 23.5 days) with 2ml of Euthanyl (240mg/ml sodium pentobarbital) injected intraperitoneally. Fetuses were removed by hysterotomy and decapitated to minimize blood dispersion. The fetal bodies were placed in ice-cold, sterile Hanks Balanced Salt Solution (HBSS) (pH 7.1) and using sterile techniques, the fetal lungs were removed from the thorax with scissors and forceps. The fetal lungs were placed onto a Petri dish containing HBSS and the heart and trachea were removed and discarded. The lungs were cut into fine pieces with a Sorval Tissue Chopper. The lung tissue was trypsinized in a trypsinization flask containing 90ml of ice cold HBSS and 10ml of 10X trypsin/EDTA (0.5%) at 37^{0}C for 40 minutes. After trypsinization, the enzymatic reaction was stopped by adding 30ml of minimal essential medium (MEM) containing 10% of newborn calf serum (NCS). The solution was filtered using 3 layers of 150μm Nitex gauze and the filtrate was centrifuged at 250g for 10 minutes at 20^{0}C. The supernatant was discarded and the pellet was resuspended in 10ml of MEM with carbon stripped newborn calf serum (sNCS). The cell suspension was

plated onto two $75cm^2$ tissue culture flasks for approximately 1 hour in 5% CO_2 at 37^0C in order for the fibroblasts to adhere. The unattached cells in the medium was collected and used to determine the cell number through a Coulter cell counter. The cells were replated in either $150cm^2$ flasks at a density of $5.0x10^6$ cells/flask, $75cm^2$ flasks at $2.5x10^6$ cells/flask, $25cm^2$ flasks at $8.0x10^5$ cells/flask, 6 well plates at $1.25x10^6$ cells/well or in 96 well plates at $5.0x10^5$ cells/well. MEM with stripped newborn calf serum (sNCS) medium removes growth factors and is used to culture the fetal rat lung type II alveolar cells. The medium was changed after 24 hours and for every 48 hours afterwards. The type II alveolar cells reached confluence at approximately 4-5 days after isolation and were used for various experiments at this time.

3.2b) Formazan assay

The formazan assay is used to measure the amount of mitochondrial activity in cells. In this assay, a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) is bioreduced by cells into a formazan product. The conversion of MTS into formazan is possible through dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product was measured using the Celltiter 96® AQueous non-radioactive cell proliferation assay from Promega (Ontario, Canada). Fetal rat lung type II alveolar cells were seeded in 96 well tissue culture plates and were grown to confluence. The cells were exposed to nicotine and cotinine at concentrations of $10^{-7}M$, $10^{-6}M$, $10^{-5}M$, and $10^{-4}M$ in MEM with 10% sNCS at different time points (30min, 1hr, 3hr, 6hr, 12hr). After each exposure time 20µl of MTS solution was added to each sample and incubated for 1hr at 37^0C, 5% CO_2. The absorbance of formazan produced

by metabolically active cells was measured at 490nm using a Bio Rad Model 550 microplate reader.

3.2c) [^{3}H]thymidine incorporation assay

Fetal rat lung type II alveolar cells were seeded in 6 well tissue culture dishes and were grown to confluence. The fetal rat lung type II alveolar cells cells were washed 2X with Hanks balanced salt solution (HBSS) and were serum starved for 3 hrs to achieve cell synchronization [112]. The cells were incubated with [^{3}H]thymidine (1μCi/ml) overnight at 37^{0}C. The fetal rat lung type II alveolar cells were exposed to nicotine and cotinine at a concentration of 10^{-5}M and 10^{-4}M for 3 different time points (30min, 3hrs, 12hrs). Fetal rat lung type II alveolar cells were washed with ice cold HBSS and followed by two washes with 5% TCA to precipitate the DNA. Fetal rat lung type II alveolar cells were solubilized by adding 0.5ml 10.25M NaOH. Samples (0.5ml) were placed into scintillation vials containing 5 ml of Ready protein scintillation cocktail. The [^{3}H]thymidine levels were determined using the LS 5801 scintillation counter. Quench compensation was done by the method of H# using ^{137}Cs (Beckman, Polo Alto, CA).

3.2d) Comet assay

The Comet assay is a single cell gel electrophoresis assay that evaluates DNA damage in cells. The principle of this assay is based on the ability of denatured DNA to migrate under the influence of an electric field and therefore forming a "comet" tail shape within the gel. The tail shape and migration pattern may be used for the assessment of induced DNA damage. Fetal rat lung type II alveolar cells were seeded in 25cm^2 tissue culture flasks and were grown to confluence. Cells were treated with nicotine or cotinine at concentrations of 10^{-5}M and 10^{-4}M for 24hrs. After treatment approximately 1.0x10^5

cells/ml of each sample was used in the Comet assay. The Comet assay was done using the protocols of the single cell gel electrophoresis assay kit obtained from Trevigen (Gaithesburg, MD). Slides were stored at 4^0C until further use. Analysis of DNA damage was done using an Olympus IX70 inverted confocal microscope with Nomarski DIC optics. Images were analysed using Fluoview and Casp software.

3.2e) Electron Microscopy

Fetal rat lung alveolar type II cells were grown to confluence and were exposed to nicotine or cotinine at a concentration of 10^{-4}M for 12 hours. Fixation of the cells took place in 3% glut. in phosphate buffer for 1 hr followed by 1% OsO4 for 1 hr. The samples were dehydrated with alcohols, (no propylene oxide) and embedded in Epon 812.

3.2f) Capillary Surfactometer

The capillary surfactometer determines surfactant-related material quality by testing the ability of a sample to maintain capillary tube patency. Glass capillary tubes containing a short narrow section are used to simulate the terminal airways. Fetal rat lung type II alveolar cells were grown to confluence in 75cm^2 flasks. The cells were exposed to nicotine or cotinine at a concentration of 10^{-5}M and 10^{-4}M for 24 hours. ATP was used as a positive control and an established secretogogue [22]. Fetal rat lung type II alveolar cells were exposed to ATP at a concentration of 10^{-4}M for 24 hours. The media were collected in 1.5ml tubes and centrifuged at 100 000g for 1 hour in a Beckman L8-80M ultracentrifuge. The pellet was resuspended in MEM and the samples (0.5µl) were placed into the narrow section of the capillary tube and pressure was exerted at one end

of the tube. The results indicate the percentage in which the capillary tube remained open over a 120 second time period and is expressed as "% open"[113].

3.2g) [^{35}S]methionine labeling

Methionine labeling was used to determine the effects of nicotine, cotinine, or phorbol ester on protein secretion by fetal rat lung type II alveolar cells. Fetal rat lung type II alveolar cells were grown to confluence in 25cm^2 tissue culture flasks and were prelabelled with 1uCi/ml [^{35}S]methionine in MEM for 18hr overnight. The media containing [^{35}S]methionine was discarded and the cells were gently rinsed with Hanks balanced salt solution (HBSS). The cells (5 sets of 5 25cm^2 tissue culture flasks) were exposed to nicotine, cotinine, phorbol ester, or a combination of phorbol ester and cotinine at a concentration of 1.0x10^{-4}M for 6hr. The medium was collected into 15ml tubes and 1ml from each sample was placed into scintillation vials containing 5ml of Ready Protein scintillation cocktail (Beckman Instruments, Palo Alto, CA). Total protein levels were determined by measuring the amount of [^{35}S]methionine in each sample using the LS 5801 scintillation counter.

3.2h) Confocal Microscopy

Freshly isolated fetal rat lung type II alveolar cells were plated in 6 well tissue culture dishes containing glass coverslips. The glass coverslips were previously sterilized in acidified alcohol overnight, dried under UV light and ethanol dipped and flamed just before the coverslips were transferred into 6 well tissue culture plates. The cells were grown to approximately 95% confluence and treated with nicotine and cotinine for 24hrs at 1.0x10^{-4}M. The media was gently aspirated and rinsed twice with Cytoskelton buffer (CB).

Fixing cells

With the coverslips still in the culture plates, the cells were rinsed with 3% paraformaldehyde (PFA, pH 6.1) for 15 min at 20^0C. The cells on the coverslips were permeabilized using 3% PFA/0.3% Triton X-100 for 5 min at 20^0C. Coverslips were rinsed twice with cold CB buffer and stored in 1X Cyto-TBS at 4^0C in the parafilm sealed culture plates for up to 2 weeks.

Immunostaining

Individual coverslips were rinsed 2X with TBS and placed in blocking solution (1X Cyto-TBS / 1% BSA / 2% normal animal serum) for 30 min on a shaker at 20^0C. The animal serum used was based on the type of secondary antibody i.e. if the secondary antibody came from a donkey, then normal donkey serum was used in the blocking solution. The blocking solution was removed by rinsing the coverslips once with TBS. In a humidified chamber, each coverslip was inverted onto Parafilm-covered 6 well tissue culture covers and into 20-25µl of first antibody solution (1:200) in 1X Cyto-TBS. Samples were placed in a humidified chamber consisting of moistened tissue paper inside a large glass container sealed with saran wrap. Coverslips were either incubated with the 1^0 SP-A or SP-B antibody overnight at 4^0C or incubated for 1hr at 20^0C. After 1^0 antibody incubation was complete, the coverslips were carefully lifted from the Parafilm-covered plastic covers and placed back into the 6 well culture plates. The coverslips were immediately rinsed in 2mls of Cyto-TBST for 15 min (3X) and placed on a slow shaker at 20^0C. A fresh layer of Parafilm was placed onto the plastic covers and 20-25 µl of secondary antibody was spotted onto the surface. Samples were stored in the dark. The secondary antibody was a FITC-conjugated donkey anti-goat IgG (Santa Cruz

Biotechnology, Inc., California, USA) and was diluted 1:100 in PBS / 1% BSA / 0.1% Tween-20. Coverslips were placed cell side down onto the secondary antibody and left in the dark for 2-3 hrs at 20^0C. After secondary antibody incubation, the coverslips were placed back into the 6 well dishes and rinsed with Cyto-TBST, Cyto-TBS, and ddH_2O for the final wash. The coverslips were subjected to a nuclear stain by immersing in 500 µl of propridium iodide (Molecular Probes Eugene, OR) for 30 min and rinsing 3X with ddH_2O. Coverslips were mounted cell side down onto microscope slides containing 12-15µl of Prolong Antifade. Prepared slides were stored in the dark at -20^0C until they were viewed and photographed using a confocal microscope.

3.2i) Protein Quantification

Protein Assay

Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Mississauga, ON) which is based on the coomasie dye-binding protocol first described by Bradford (1976). Absorbance was measured at 595nm using a Beckman DU-640 Spectrophotometer and bovine serum albumin (BSA) was used as a relative protein standard. The Coomasie blue dye binds to the sample protein which produces a color change and the intensity is measured spectrophotometrically in comparison to the BSA standard.

Preparation of Cell Culture Lysates

The cells were grown to confluence over a period of five days and were exposed to nicotine or cotinine at a concentration of 10^{-5}M and 10^{-4}M for 3 hours (nicotine) or 12 hours (cotinine). The media was aspirated from the tissue culture flasks and the cells were washed 3X with 5ml of ice cold PBS. Ice cold solubilization buffer (500µl/75cm^2

flask) containing Tris (40mM, pH 8.0), NaCl (150mM), 1% IGEPAL (NP-40), 1% deoxycholic acid, 1mM PMSF, 5µg/ml leupeptin, 5µg/ml pepstatin, and 5µg/ml aprotinin was added to the cells and the cells were scraped in the buffer solution. Cell extracts were transferred to a tube and sonicated at 2-10s pulses. Protein samples were stored at -80^{0}C until further use.

Protein Electrophoresis: SDS-PAGE

A vertical slab mini-gel apparatus was used for all applications. Gels measured 8cm (W) x 7.3cm (H) and were 0.75mm in thickness. The resolution gels were 10% and contained 1.5M Tris-HCl (pH 8.8), 10% sodium dodecyl sulphate (SDS), 30% acrylamide bis. The composition of the staking gels consisted of 0.5M Tris-HCl (pH 6.8), 10% SDS, and 30% acrylamide bis. The amount of protein loaded into each well was 40µg. Proteins were fractionated at a constant voltage of 180V for 1 hr at room temperature. The running buffer used contained Tris base (25mM), glycine (0.192M), and SDS (0.1% w/v).

Coomasie Blue Stain

Gels were stained for total protein with Coomassie Brilliant Blue R 250. Staining of the gels occurred immediately after electrophoresis and took place in plastic containers containing staining solution (40% ethanol / 10% acetic acid / 1% Coomassie Brilliant Blue R250). The gels were heated for approximately 1 min in the microwave and were placed in the fumehood with saran wrap covering the containers for 1 hr. Gels were rinsed with ddH_2O and destained with 40% ethanol / 10% acetic acid until maximum resolution was obtained. Gels were dried on cellophane that was previously soaked in 10% glycerol along with the gels for 20 min. Gels were placed in between the cellophane

sheets and placed into a gel drying frame. The frame was placed in a fumehood and dried overnight.

Western blot analysis of protein homogenates

Protein samples were fractionated through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 8cm (W) x 7.3cm (H) mini-gels. Nitrocellulose, sponges, and filter paper was presoaked in transfer buffer (25mM Tris / 192 mM glycine / 20% methanol, pH 8.3) for 30 min. The gels were placed in the mini trans-blot cell apparatus and electroblotting of the samples took place at either 25 V overnight or at 100 V for 1 hour in transfer buffer at 4^0C.

Immunodetection

Blots were blocked using 10ml of 5 % non fat dried milk powder in TBS-Tween (10% Tween-20 / 10X TBS) for 1 hr at 20^0C. Blots were rinsed 3X with TBS-Tween and then 1X for 15 min and 2X for 5 min. Blots were incubated in primary antibody (SP-A, SP-B, SP-C, or SP-D goat polyclonal) diluted in 5% non fat dried milk powder / TBS-Tween (1:200) for 2 hrs at 20^0C. Blots were rinsed 5X for 5 min with TBS-Tween and were incubated with secondary antibody (donkey anti-goat IgG-HRP) that was diluted (1:3000) in 5% non fat dried skim milk / TBS-Tween for 2 hr at 20^0C. Blots were either rinsed 3X for 5 min with TBS-Tween or left overnight in TBS-Tween at 4^0C. Chemiluminescence for specific surfactant proteins was detected in 2ml of ECL Western blotting detection reagents (Amersham Life Science, Oakville, ON) for 1 min at 20^0C. Chemilumigrams were developed on Hyperfilm-ECL at exposure times ranging from 30s to 2 min.

3.2j) Synthesis of disaturated phosphatidylcholine (DSPC)

The effects of nicotine and cotinine on the synthesis of DSPC was determined by radioactively labeling DSPC with [^{3}H]choline. Once the type II alveolar cells reached confluence, the cells were treated with nicotine or cotinine (10^{-5}-10^{-4}M) and simultaneously with 1uCi/ml of [^{3}H]choline in Minimum Essential Medium (MEM) for 0, 3, 6, and 18 hours. After incubation, the medium was discarded and 4mls of methanol were added to each 25cm^2 tissue culture flask and stored in the 4°C fridge until further extraction.

3.2k) Secretion of DSPC

The secretion of DSPC was determined by prelabelling cells in 25cm^2 tissue culture flasks with 1uCi/ml of [^{3}H]-choline in Minimum Essential Medium (MEM) for 24 hours. The medium was discarded and the cells were exposed to nicotine (10^{-5}-10^{-4}M) or cotinine (10^{-5}-10^{-4}M) in MEM at three different time points (1, 3, 6 hours). The medium was collected in glass tubes and the cells were stored in the 4°C fridge until further use.

Isolation of DSPC

The cells were scraped from culture flasks using a cell scraper into glass tubes and a mixture of chloroform:methanol:potassium chloride (1:1:0.9) was added to the samples [114]. The top aqueous portion was removed with a Pasteur pipet and discarded. The bottom organic portion containing the organic soluble materials was dried under air using a SC-3 Sample Concentrator at 60°C overnight. A 1ml mixture of carbon tetrachloride and osmium tetraoxide (284ml CCl_4 / 1 ampule OsO_4) was added (1ml) to each sample, vortexed, and dried. The sample was resuspended in 100ul of chloroform. Single channeled thin layer chromatography (LK5D) plates were activated at approximately

60°C and 100ul of each sample was plated onto the TLC plates. A DSPC standard was prepared (40mg DSPC in 4mls chloroform/methanol, 20/1) and 4ul was plated onto every 2nd channel. The plates were run in a solvent system containing chloroform:methanol:water (65:25:4) until the solvent front reached the top of the plate. The TLC plates were dried and the standards were visualized by placing them in a glass chamber with an iodine saturated atmosphere. The standards were marked on the plates and scraped into scintillation vials. In the scintillation vials, 10ml of Ready Protein scintillation cocktail (Beckman Instruments, Palo Alto, CA) and 200µl of ddH_2O were added to the samples. The amount of [^{3}H]-choline was determined using the LS 5801 scintillation counter. Quenching was determined by the method of H# (Beckman) using ^{137}Cs as the standard and the graphs were produced through Microsoft Exel and Sigmaplot.

Results

Effects on fetal rat lung type II alveolar cells of exposure to nicotine or cotinine

To examine the effects of nicotine or cotinine, we used electron microscopy in order to identify changes in morphology of fetal rat lung type II alveolar cells. The control cells were grown in MEM with sNCS and their morphological appearance is shown in Figure 2. Both the nicotine and cotinine treated cells (10^{-4}M for 12 hours) displayed signs of apoptosis and thus revealed significant changes in their structure. The most recognizable change to the cells was the condensation of cytoplasm associated with the formation of cytoplasmic blebs. Cytoplasmic blebs are known also as apoptotic bodies and are characterized by membrane bound regions that exist along the periphery of an apoptotic cell [115]. Other key events that were observed within the cell involved swelling of mitochondria and the presence of lipid globules.

Since nicotine and cotinine caused morphological alterations to the fetal rat lung type II alveolar cells, we examined the ability of the cells to convert a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); MTS) into formazan as a measure of mitochondrial activity (Promega, Ontario, Canada). The formazan assay measures cellular mitochondrial dehydrogenase activity within a cell as a measure of cellular viability. In the control cells, mitochondrial dehydrogenase activity increased up to 12 hours (Figures 3 and 4). Exposure of fetal rat lung type II alveolar cells to nicotine resulted in an increase in mitochondrial activity after periods of 30 minutes, 1, 3, 6, and 12 hours at concentrations of 10^{-7}M - 10^{-4}M ($p<0.01$) (Figure 3).

Cotinine treatment of fetal rat lung type II alveolar cells produced similar trends showing an increase in mitochondrial activity after 30 minutes, 1, 3, 6, and 12 hours at concentrations of 10^{-6}M - 10^{-4}M ($p<0.01$) (Figure 4). However, cotinine exposure at 10^{-7}M for 30 minutes, 1, 3, 6, and 12 hours did not appear to alter mitochondrial activity. Exposure of fetal rat lung type II alveolar cells to nicotine or cotinine altered the mitochondrial function at specific doses. These increases in mitochondrial activity may reflect the ultrastructural observations of mitochondrial swelling shown in Figure 1.

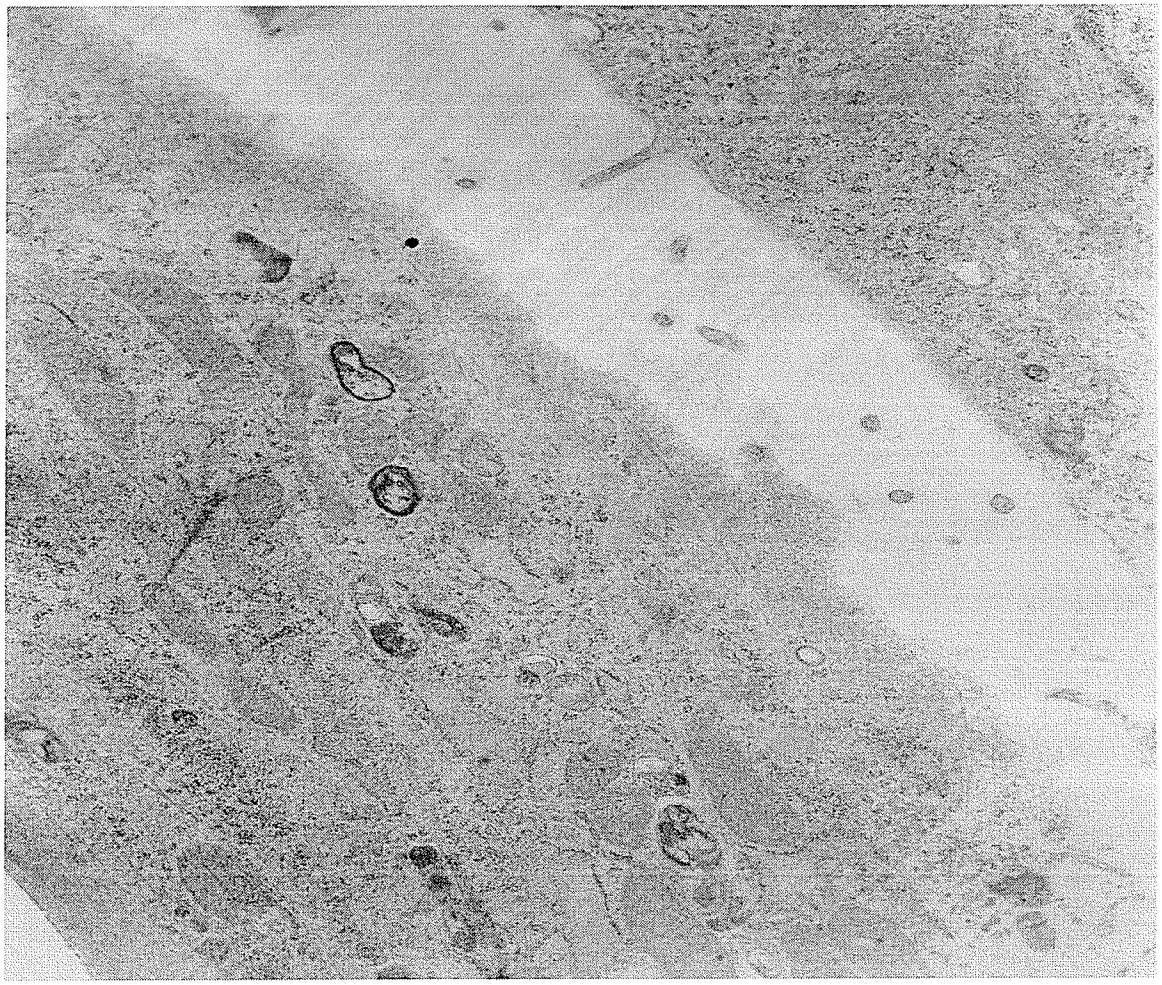
[^{3}H]thymidine incorporation is used as a measure of DNA synthesis levels by determining the amount of radioactively labeled thymidine incorporated into the cells during the S phase of the cycle. [^{3}H]thymidine incorporation by the fetal rat lung type II alveolar cells following nicotine exposure was generally reduced at 30 minutes, 3hours, and 12 hours at concentrations of 10^{-5}M and 10^{-4}M compared to the control samples (Figure 5). Similarly, cotinine exposure of the cells also resulted in a decrease in [^{3}H]thymidine incorporation after 30 minutes, 12 hours, and 24 hours at concentrations of 10^{-5}M and 10^{-4}M (Figure 6). The decrease in [^{3}H]thymidine incorporation after exposure of fetal rat lung type II alveolar cells to nicotine or cotinine suggests that the rate of DNA synthesis was reduced reflecting a reduction in cells entering S phase.

The comet assay (Trevigen, Gaithesburg, MD) was used to determine DNA damage within fetal rat lung type II alveolar cells (Figure 7). Quantitative analysis of DNA damage was determined by measuring the tail length and tail moment using CASP software available on the internet at http://casp.sourceforge.net/. Tail length is the distance of DNA migration from the middle of the nuclear core to the end of the DNA tail and it is used to evaluate the extent of DNA damage [116, 117]. The tail length of

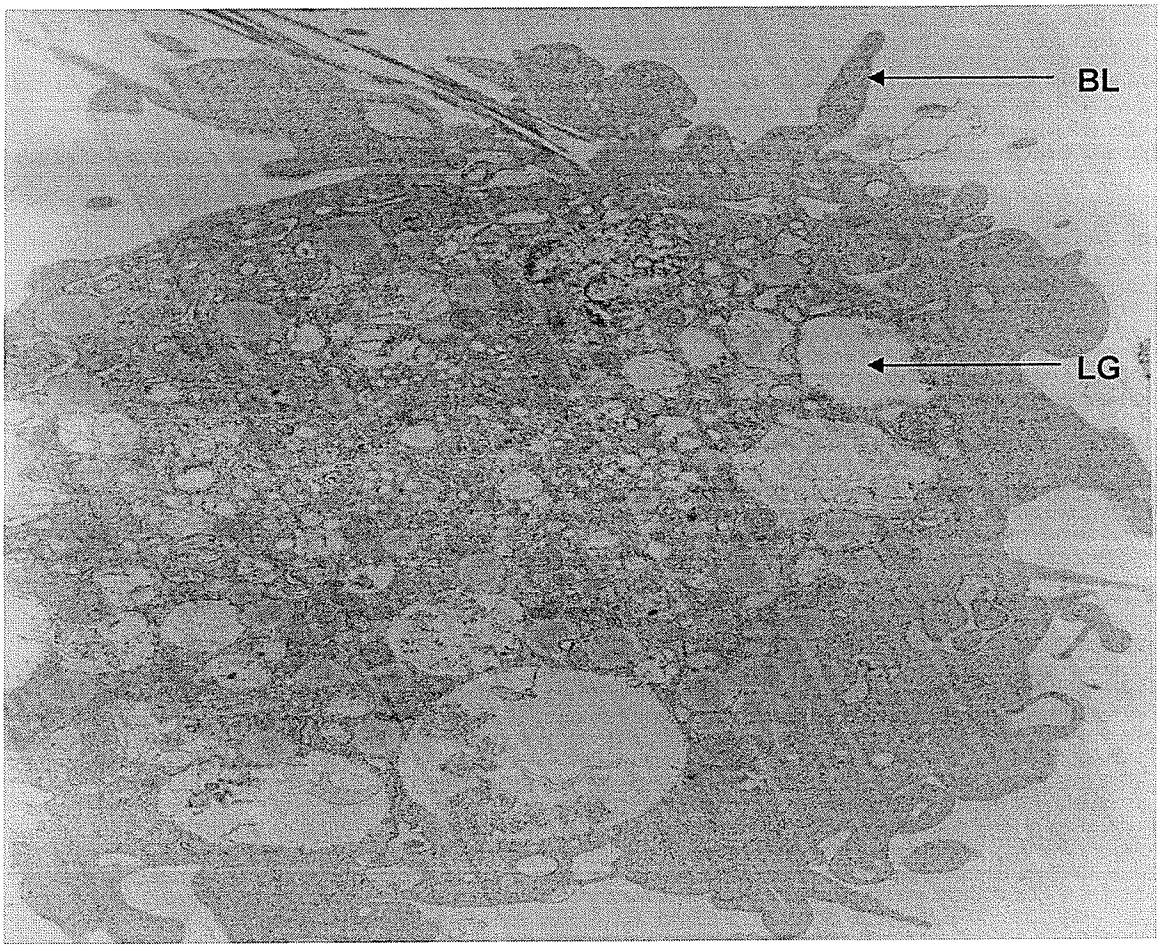
samples obtained from nicotine-exposed cells was approximately twice the value obtained from untreated cells ($p<0.01$) (Figure 8). Cotinine exposure for 24 hours at 10^{-5}M and 10^{-4}M resulted in an increase in tail length such that cells exposed to cotinine displayed mean tail lengths approximately three times greater than those of the untreated cells ($p<0.01$).

Tail moment is the product of tail length and the percentage of DNA in the tail of total DNA [116-118]. The mean tail moments for nicotine-exposed samples were four times greater compared to those of the untreated cells ($p<0.01$). The fetal rat lung type II alveolar cells that were exposed to cotinine displayed tail moments approximately eight times greater than those of the untreated cells ($p<0.01$) (Figure 9).

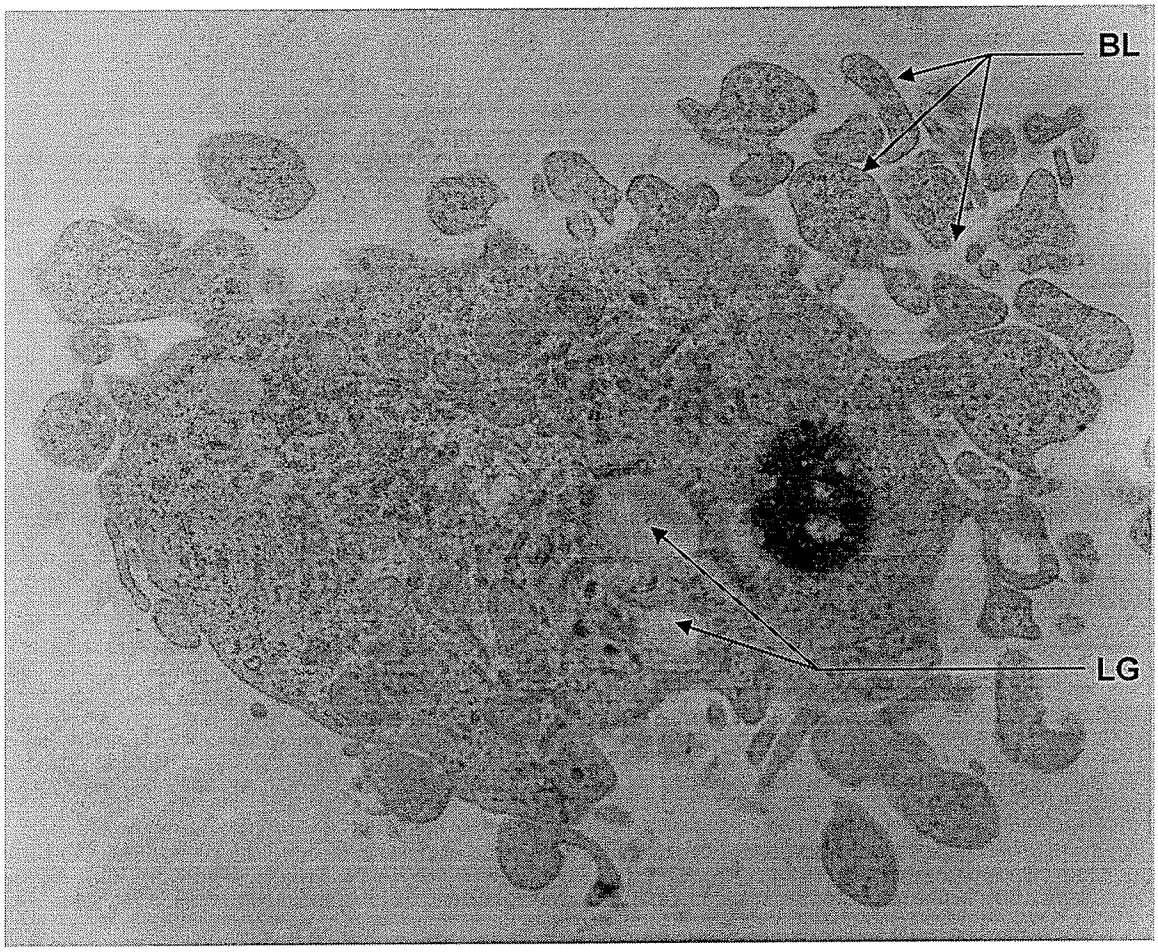
Figure 2: Electron micrographs of fetal rat lung type II alveolar cells following exposure to nicotine at 10^{-4}M for 12 hours. The control sample (A, x70000) showed distinct cellular organelles. The mitochondria and lamellar bodies were conspicuous. In the nicotine (B, x35000) and cotinine-exposed cells (C, x70000; D, x50000) cytoplasmic blebbing (BL), mitochondrial swelling (M), and the presence of lipid globules (LG) were observed.



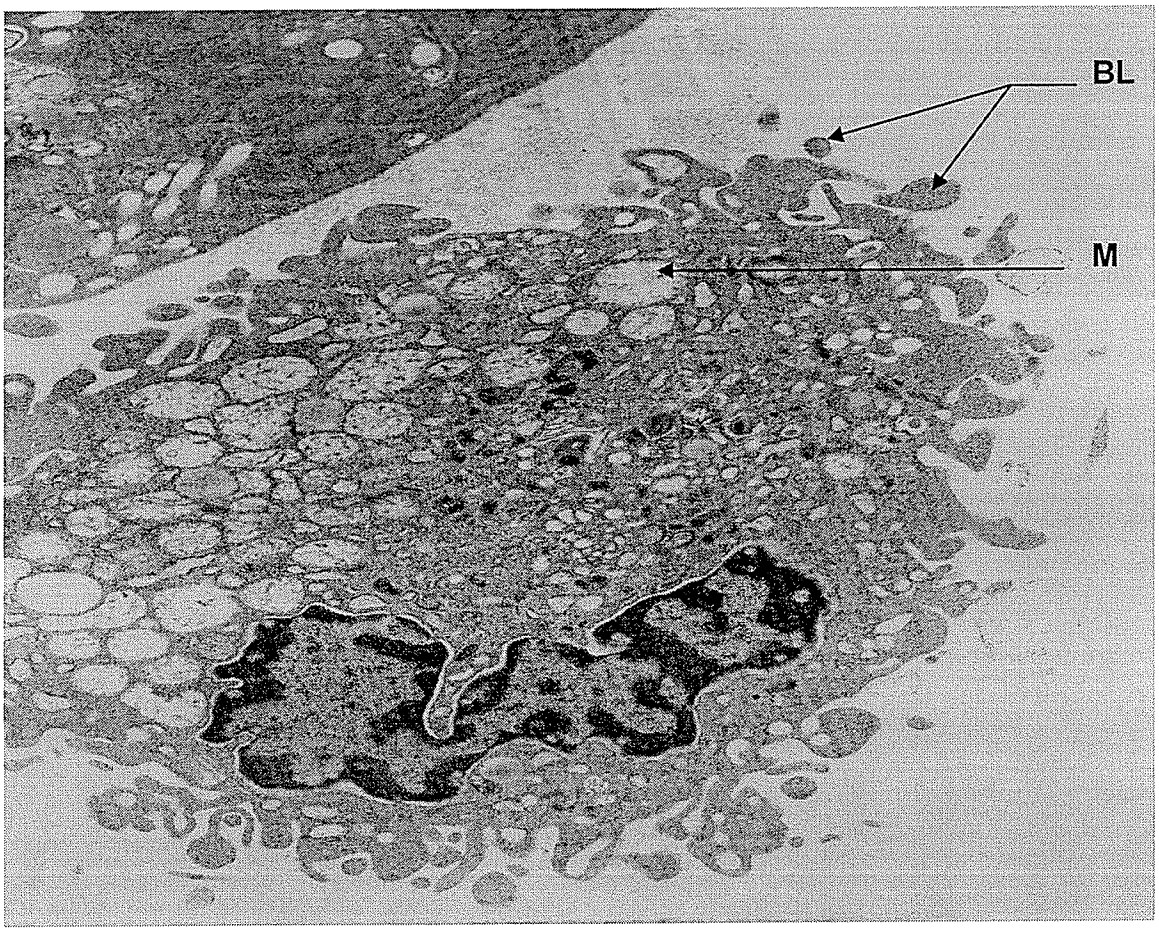
(A)



(B)



(C)



(D)

Figure 3: Absorbance values at 490nm reflecting mitochondrial dehydrogenase activity obtained using the MTS formazan assay in isolated fetal rat lung type II alveolar cells following exposure to nicotine at 10^{-7}M-10^{-4}M for 30 minutes, 1, 3, 6, and 12 hours. Fetal rat lung type II alveolar cells were isolated as described in the Materials and Methods. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Absorbance values were obtained using the Bio Rad Model 550 microplate reader. Results are expressed as the mean absorbance values +/- SD for 24 replicates at each time point. * indicates significantly different ($p<0.01$) between control values and cell groups exposed to nicotine.

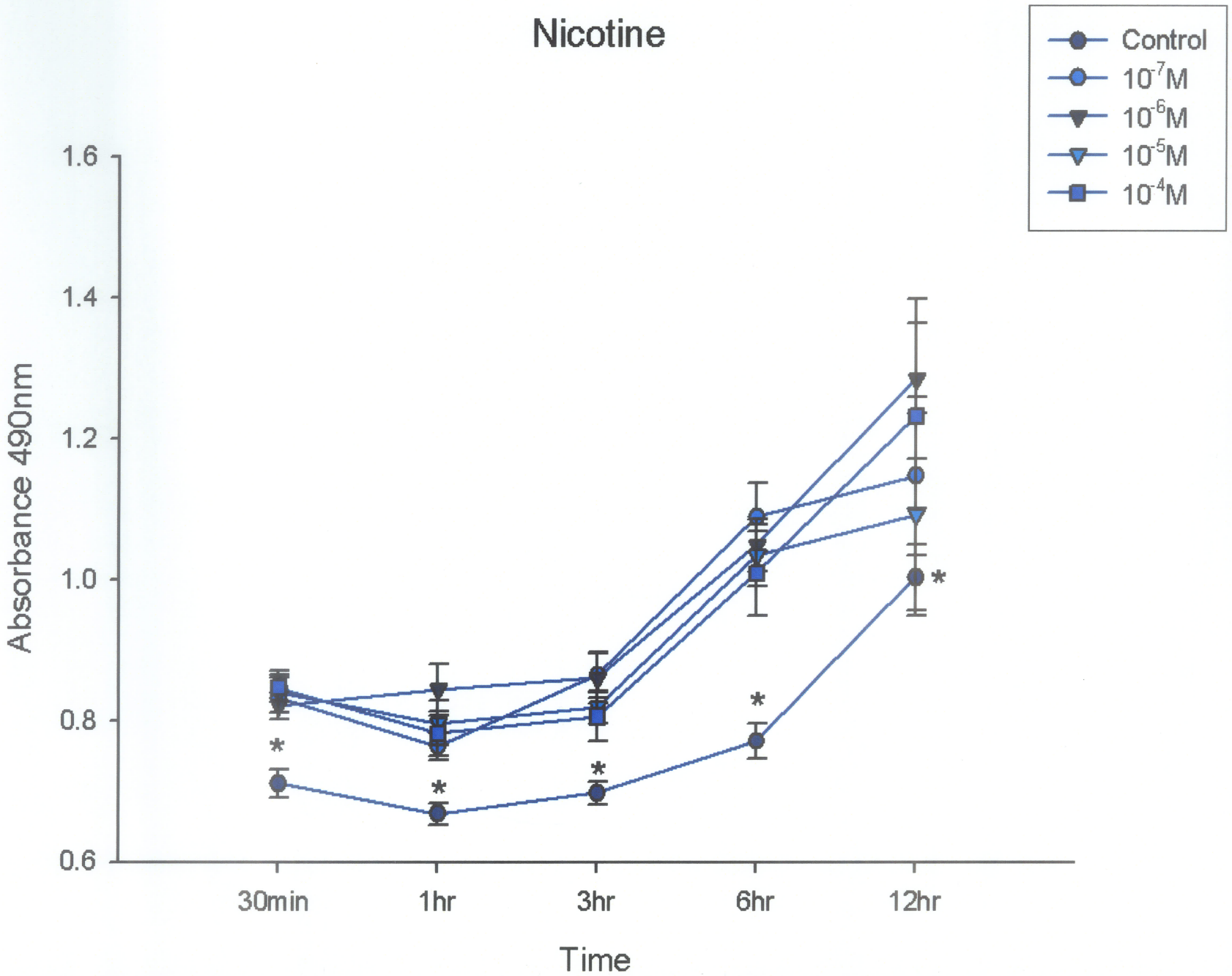

Nicotine
Control
10-7M
10-6M
10-5M
10-4M
Absorbance 490nm
1.6
1.4
1.2
1.0
0.8
0.6
30min
1hr
3hr
6hr
12hr
Time
*

Figure 4: Absorbance values at 490nm reflecting mitochondrial dehydrogenase activity obtained using the MTS formazan assay in isolated fetal rat lung type II alveolar cells following exposure to cotinine at 10^{-7}M-10^{-4}M for 30 minutes, 1, 3, 6, and 12 hours. Fetal rat lung type II alveolar cells were isolated as described in the Materials and Methods. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Absorbance values were obtained using the Bio Rad Model 550 microplate reader. Results are expressed as the mean absorbance values +/- SD for 24 replicates for each time point. * indicates significantly different ($p<0.01$) between control values and cell groups exposed to cotinine.

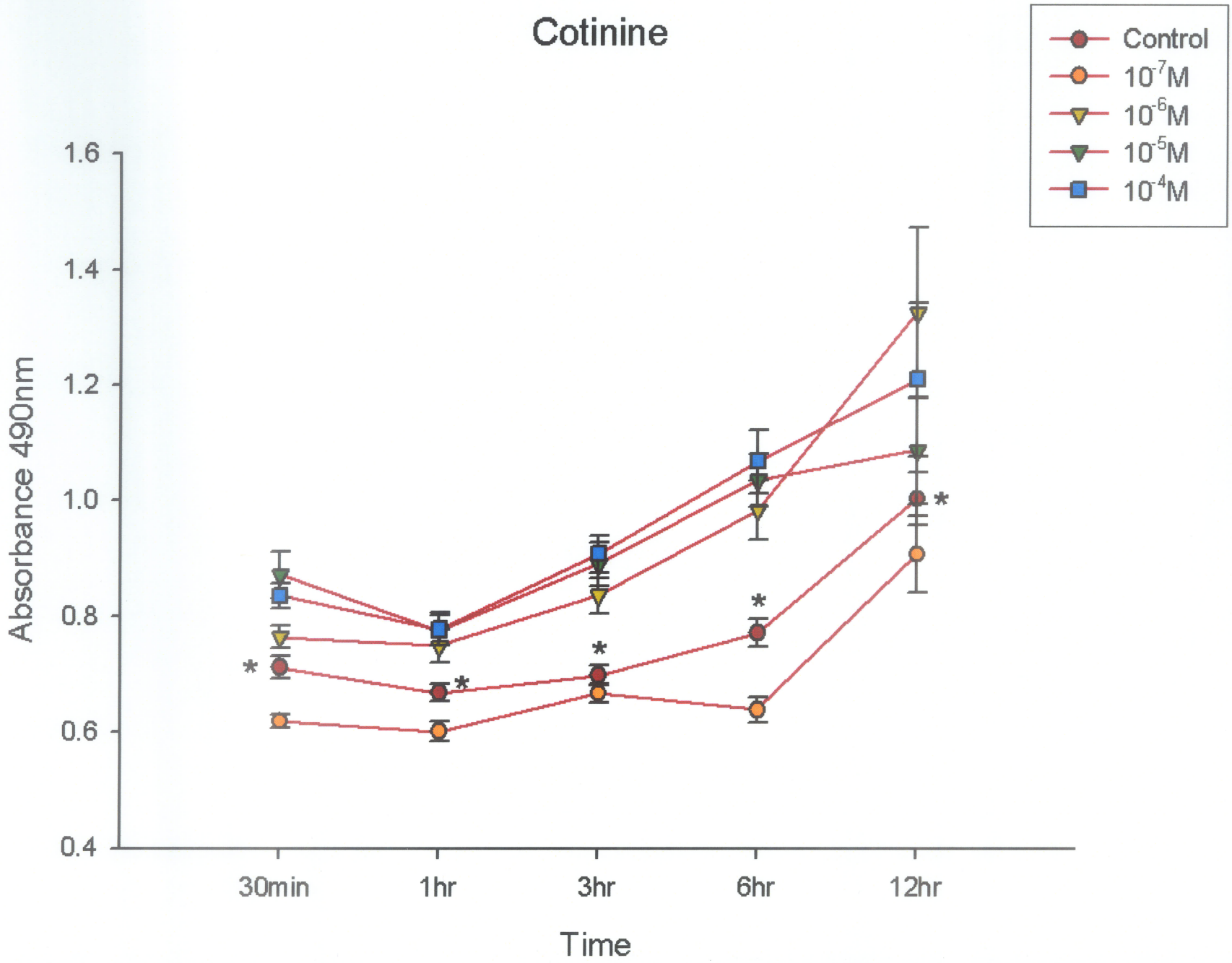

Cotinine
Control
10-7M
10-6M
10-5M
10-4M
Absorbance 490nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
30min
1hr
3hr
6hr
12hr
Time
*

Figure 5: [^{3}H]thymidine incorporation by isolated fetal rat lung type II alveolar cells following exposure to nicotine for 30 minutes, 12 hours, and 24 hours at concentrations of 10^{-5}M or 10^{-4}M. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Scintillation counts were obtained using the method of H# (Beckman LS501, Palo Alto, CA) for quench compensation. Results are expressed as mean disintegrations per minute +/- SD for 3 replicates.

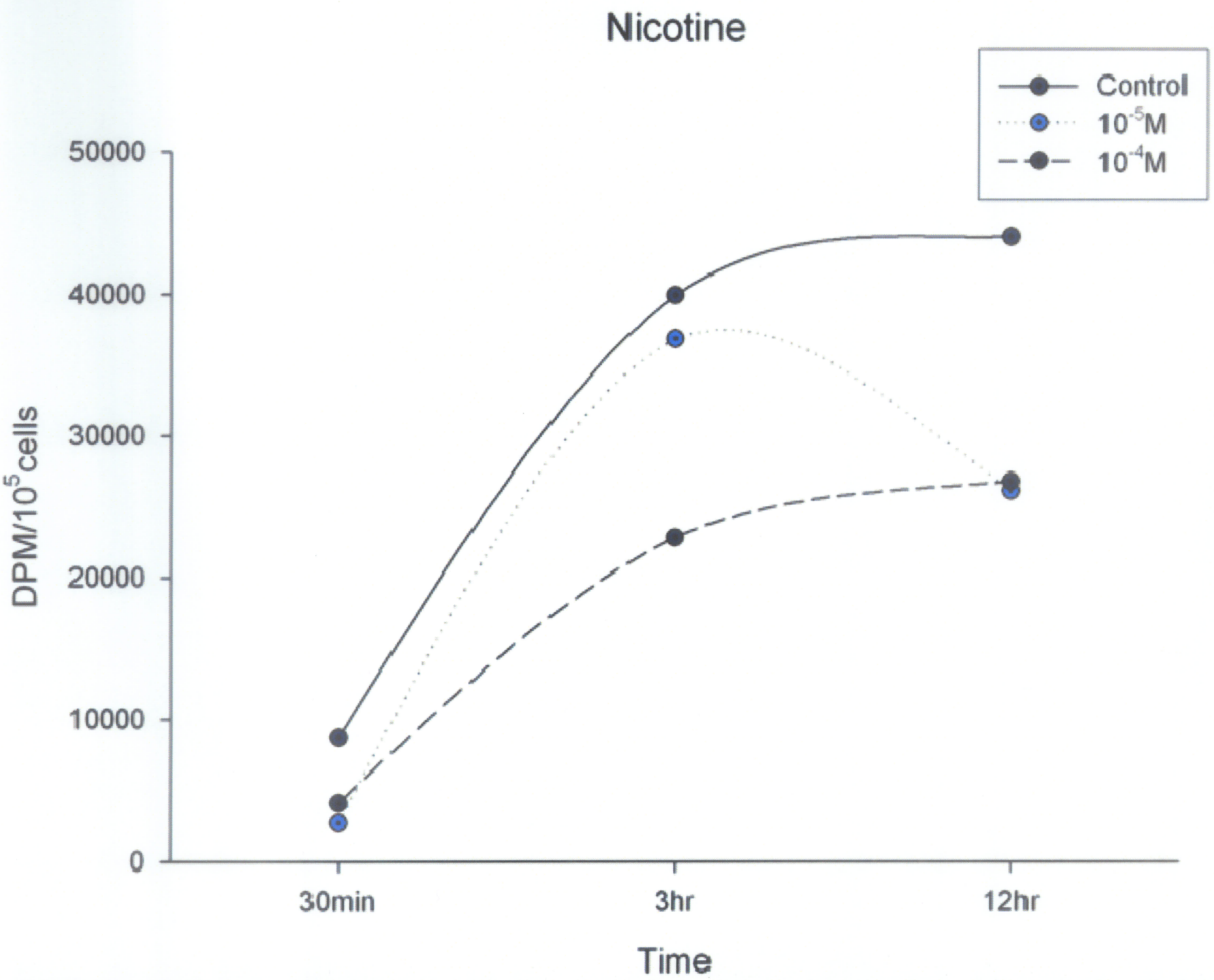

Nicotine
Control
10-5M
10-4M
50000
40000
30000
20000
10000
0
DPM/10^5 cells
30min
3hr
12hr
Time

Figure 6: [^{3}H]thymidine incorporation by isolated fetal rat lung type II alveolar cells following exposure to cotinine for 30 minutes, 12 hours, and 24 hours at concentrations of 10^{-5}M and 10^{-4}M. Isolation of fetal rat lung type II alveolar cells is described in the Materials and Methods section. Cultured fetal rat lung type II alveolar cells reached confluence after a period of five days. Radioactivity was obtained using the H# (Beckman LS501, Palo Alto, CA) for quench compensation. Results are expressed as disintegrations per minute +/-SD for 3 replicates.

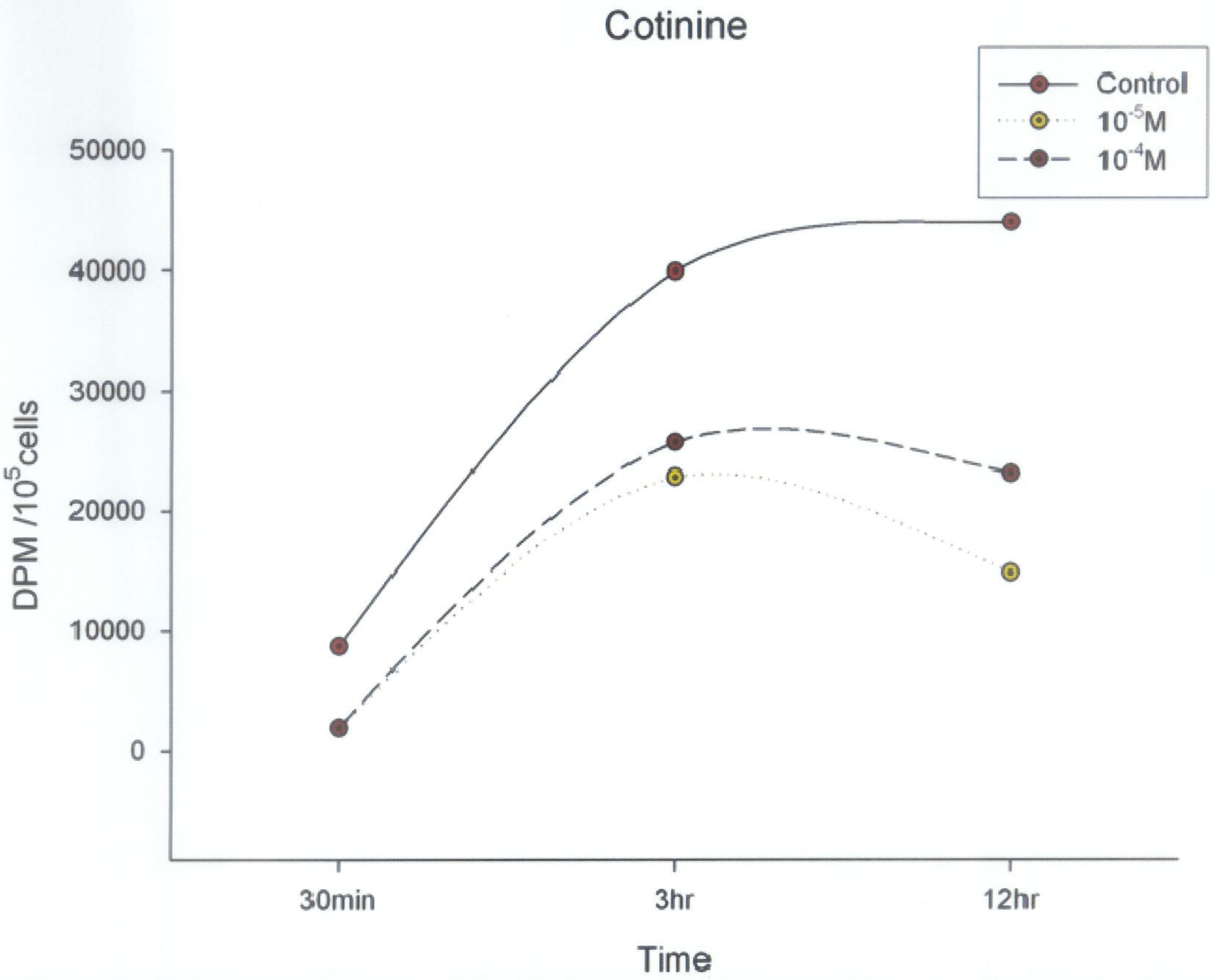
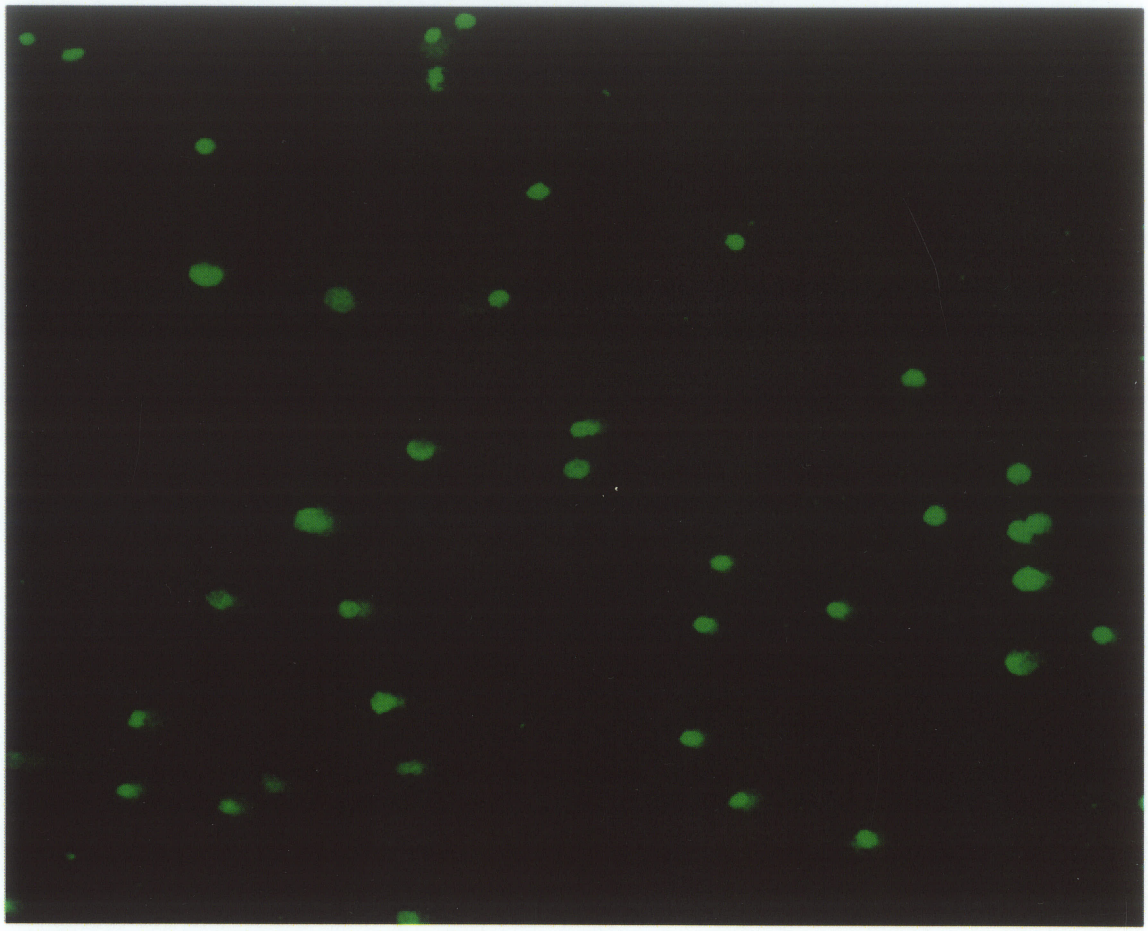
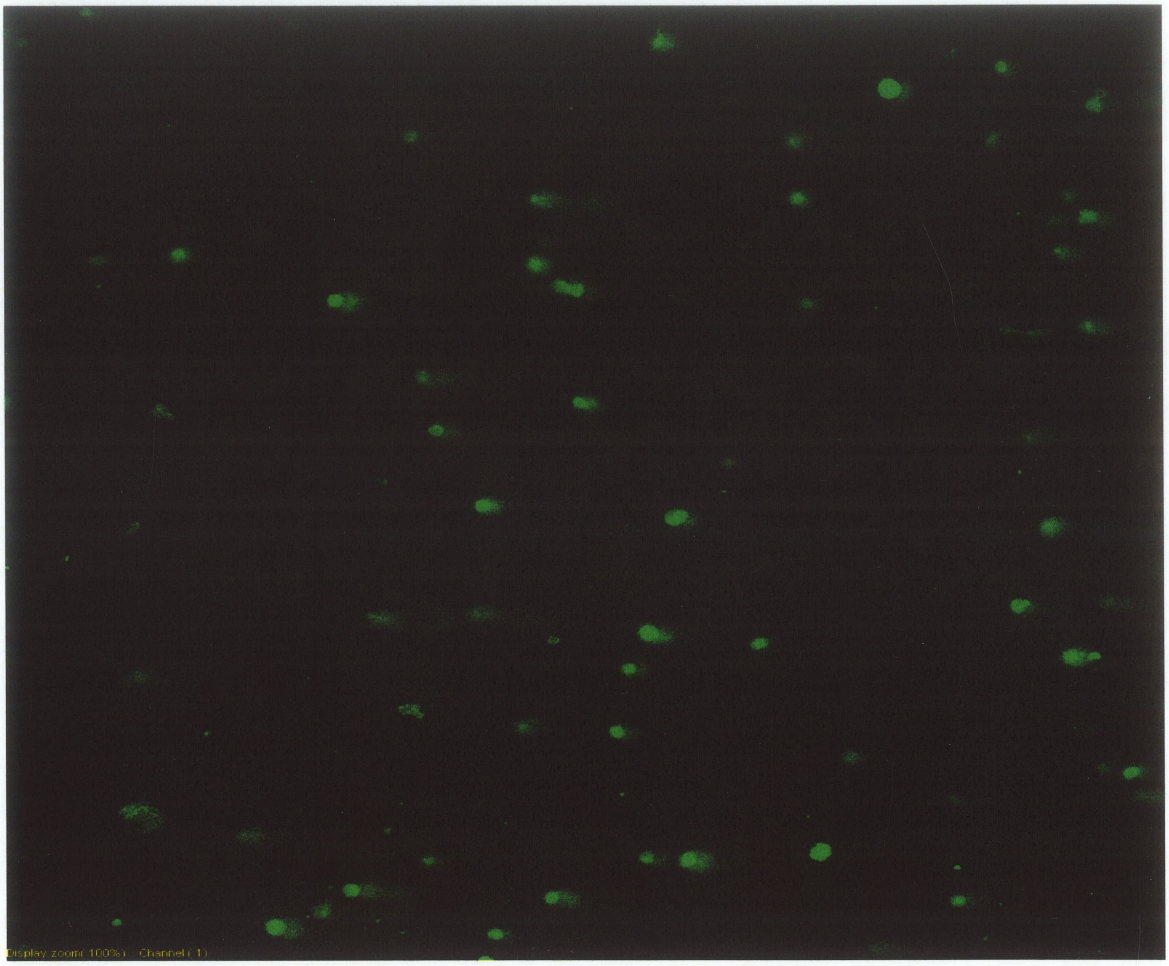

Cotinine
Control
10-5M
10-4M
50000
40000
30000
20000
10000
0
DPM /10^5cells
30min
3hr
12hr
Time

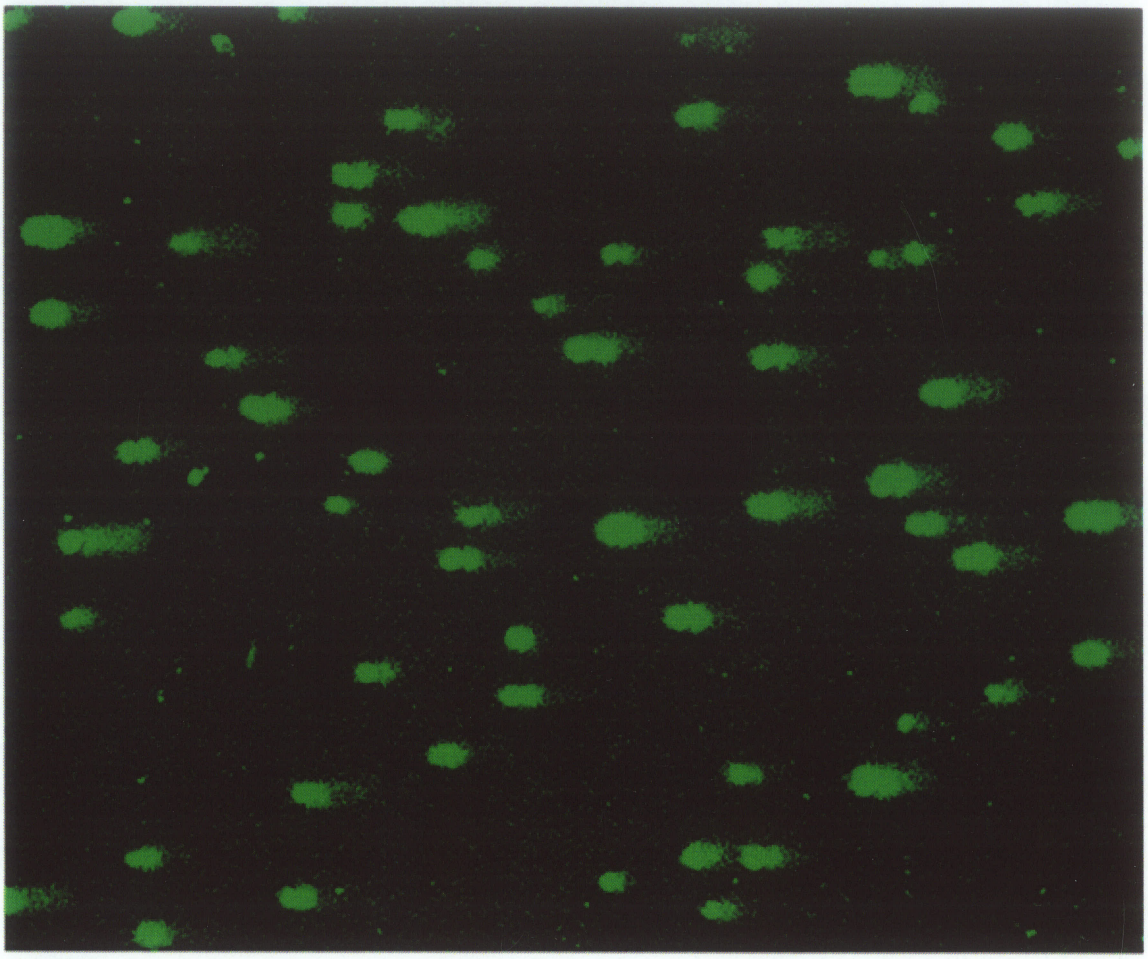
Figure 7: Confocal microscopic images of the control, nicotine, and cotinine treated samples at 10^{-4}M for 24 hours. SYBR® Green I nuclear stain was used to visualize the cellular DNA. The control (A, x10) shows minimal DNA migration. The nicotine (B, x10) and cotinine (C, x10) exposed samples induced the formation of "comets" and shows DNA migration from the nuclear core. Fluoview software and an Olympus IX70 inverted confocal microscope were used for analyses. Fetal rat lung type II alveolar cells were isolated as described in the Materials and Methods section. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days.



(A)



(B)



(C)

Figure 8: Tail length (number of pixels) of isolated fetal rat lung type II alveolar cells following exposure to nicotine or cotinine at concentrations of 10^{-5}M and 10^{-4}M for 24 hours. CASP software was used to determine mean tail length. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Results are expressed as the mean number of pixels +/-SD for six replicates ($1x10^5$cells/replicate). * indicates significantly different ($p<0.01$) between control values and cell groups exposed to nicotine or cotinine.

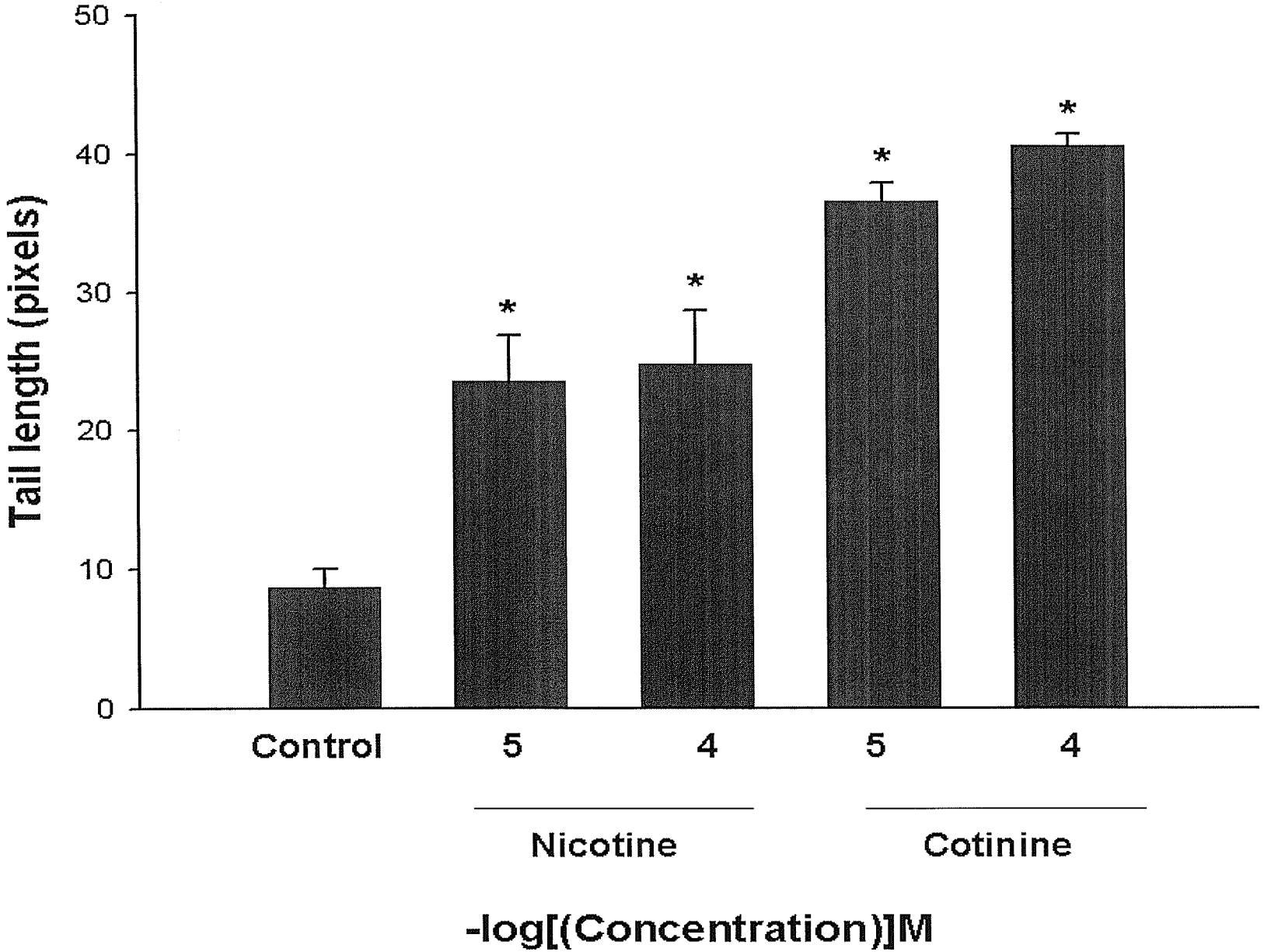
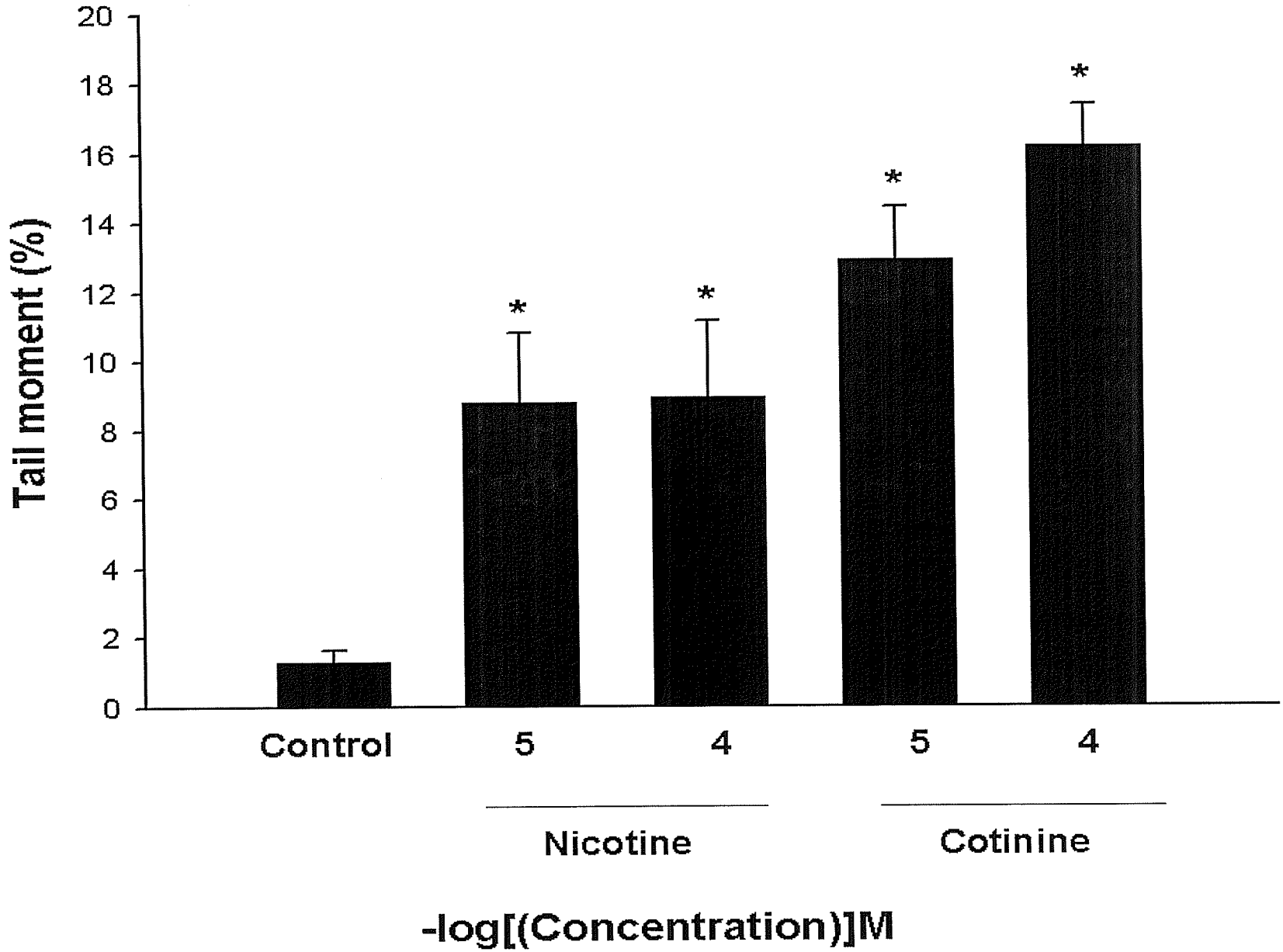

50
40
30
20
10
0
Tail length (pixels)
*
*
*
*
Control
5
4
5
4
Nicotine
Cotinine
-log[(Concentration)]M

Figure 9: Tail moment (percentage) of isolated fetal rat lung type II alveolar cells following exposure to nicotine or cotinine at concentrations of 10^{-5}M and 10^{-4}M for 24 hours. CASP software was used to determine mean tail moment. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Results are expressed as the mean tail moment +/-SD which represents the ratio of tail length and the fraction of total DNA in the tail for six replicates ($1x10^5$cells/replicate). *indicates significantly different ($p<0.01$) between control values and cell groups exposed to nicotine or cotinine.


Tail moment (%)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Control
5
4
5
4
Nicotine
Cotinine
-log[(Concentration)]M

Effects of nicotine or cotinine on surfactant metabolism

To examine the effects of nicotine or cotinine on cellular protein secretion, fetal rat lung type II alveolar cells were exposed to these agents after preincubation with [^{35}S]methionine. The fetal rat lung type II alveolar cells were treated with nicotine, cotinine, phorbol ester, or the combination of phorbol ester and cotinine at 10^{-4}M for 6 hours. Phorbol ester is an established secretagogue for surfactant phospholipids [119]. Control cells were not exposed to any of these agents (Figure 10). Exposure of fetal rat lung type II alveolar cells to nicotine, cotinine, phorbol ester, and the combination of cotinine and phorbol ester resulted in a significant decrease ($p<0.01$) in mean [^{35}S]methionine release which suggests these agents induced a reduction in release of protein in the cells.

Confocal images were used to determine qualitative changes in SP-A and SP-B levels in fetal rat lung type II alveolar cells after exposure to nicotine at a concentration of 10^{-4}M for 24 hours. The untreated cells were grown in MEM with sNCS. The results indicated little observable difference in SP-A or SP-B levels after nicotine or cotinine exposure compared to the control cells (Figure 11).

Western blot analysis was conducted to compare pulmonary SP-A, -B, -C, and –D expression in nicotine or cotinine treated cells and to compare these expression levels to those of untreated cells. Fetal rat lung alveolar type II cells were exposed to nicotine or cotinine at a concentration of 10^{-5}M or 10^{-4}M for 3 hours (nicotine) or 12 hours (cotinine). The control cells were grown in MEM with sNCS. The results suggest no clear differences occurred in surfactant protein expression for all four surfactant proteins after exposure to nicotine or cotinine (Figure 12).

DSPC the major surface-active phospholipid of pulmonary surfactant was used as a reflection of cellular phospholipid metabolism of surfactant-related materials. Thin layer chromatography (TLC) was used to isolate DSPC and to examine changes in [^{3}H]choline incorporation following exposure of the fetal rat lung alveolar type II cells to nicotine or cotinine at a concentration of 10^{-4}M for 0, 3, 6, and 18 hours. The control cells were grown in MEM with sNCS but were not exposed to nicotine or cotinine. Incorporation of [^{3}H]choline into DSPC by the control cells showed a gradual increase from 3 to 18 hours (Figure 13). Exposure of fetal rat lung type II alveolar cells to nicotine resulted in an increase in mean [^{3}H]choline incorporation into DSPC compared to the control samples at 6 hours. The cotinine treated samples showed a similar trend to the nicotine exposed samples in which [^{3}H]choline incorporation increased for 3 to 18 hours. Cotinine-exposed samples displayed significantly greater ($p<0.05$) [^{3}H]choline incorporation compared to the corresponding control cells after 6 hours of exposure. The increase in [^{3}H]choline incorporation by fetal rat lung type II alveolar cells after nicotine or cotinine exposure suggests cellular DSPC synthesis is increased by these agents after 6 hour exposure.

To measure DSPC secretion by fetal rat lung type II alveolar cell after exposure to nicotine or cotinine at a concentration of 10^{-4}M for 1, 3, 6, hours, release of [^{3}H]DSPC into culture media was determined. [^{3}H]DSPC was isolated from the medium and cells as described in the Materials and Methods. Secretion was defined as:

$$[^3H]DSPC_M / ([^3H]DSPC_M + [^3H]DSPC_{C)})X\ 100$$

where M = medium and C = cellular [^{3}H]DSPC content [120]. The control cells were not treated with nicotine or cotinine. DSPC secretion as a proportion of cellular DSPC in

untreated cells declined from 1 hour to 6 hours (Figure 14). Exposure of fetal rat lung type II alveolar cells to nicotine or cotinine resulted in a significant decrease ($p<0.01$) in DSPC secretion after 1 hour. Exposure of fetal rat lung type II alveolar cells to nicotine or cotinine for 3 or 6 hours did not alter the proportion of secretion of cellular DSPC compared to the control samples.

The electron microscopic images determined qualitatively the effect on fetal rat lung type II alveolar cells of exposure to nicotine or cotinine. Exposure to these agents produced similar results where nicotine or cotinine treated fetal rat lung type II alveolar cells showed an increase in frequency of lamellar bodies within the cells (Figure 15).

Evaluation of surfactant-related material quality

Surfactant-related material collected at 100,000g from isolated fetal rat lung alveolar type II cells was used to estimate surfactant-related material quality using a capillary surfactometer (Calmia Medical, Toronto, Ontario). The results represent the ability of surfactant-related material to maintain capillary tube patency and are expressed as the mean percentage of time (120 seconds) in which the capillary tube remained open (% open). The fetal rat lung type II alveolar cells were exposed to nicotine or cotinine at concentrations of 10^{-5}M or 10^{-4}M for 24 hours. ATP which stimulates surfactant secretion [64], was used as a positive control. The control cells were not exposed to nicotine, cotinine, or ATP. The ATP-treated cells showed an increase in mean capillary tube patency compared to the control cells. The nicotine-exposed samples showed a significant decrease ($p<0.01$) in capillary tube patency at 10^{-5}M and 10^{-4}M in comparison to the untreated and ATP treated cells (Figure 16). Similar results were observed for the cotinine treated samples where a decrease in capillary tube patency occurred following

exposure of the fetal rat lung alveolar type II cells to cotinine at 10^{-5}M or 10^{-4}M. The decrease in capillary tube patency suggests an alteration in the ability of surfactant related material to reduce surface tension.

Figure 10: [^{35}S]methionine secretion by fetal rat lung type II alveolar cells following exposure to nicotine (NIC), cotinine (COT), phorbol ester (PE), and the combination of cotinine and phorbol ester (PE & COT) for 6 hours at a concentration of 10^{-4}M. Fetal rat lung type II alveolar cells were prelabelled with 1uCi/ml [^{35}S]methionine in MEM for 18hours. Scintillation counts were determined using the method of H# (Beckman LS501, Palo Alto, CA) for quench compensation. Isolation of fetal rat lung type II alveolar cells is described in the Materials and Methods. Cultured fetal rat lung type II alveolar cells reached to confluence after a period of five days. Results are expressed as mean disintegrations per minute +/-SD per 10^5 cells. *significantly different ($p<0.01$) compared to the corresponding control samples.

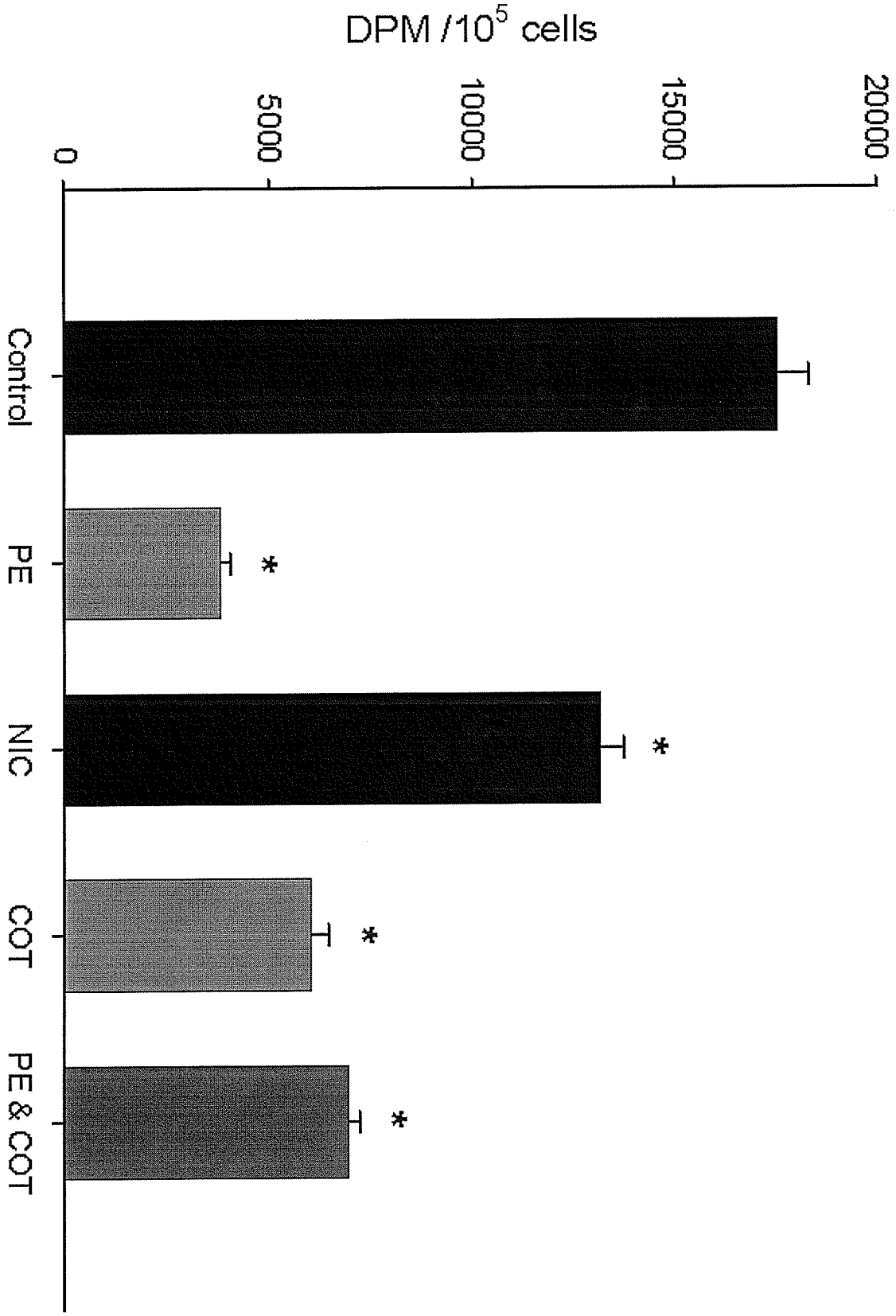
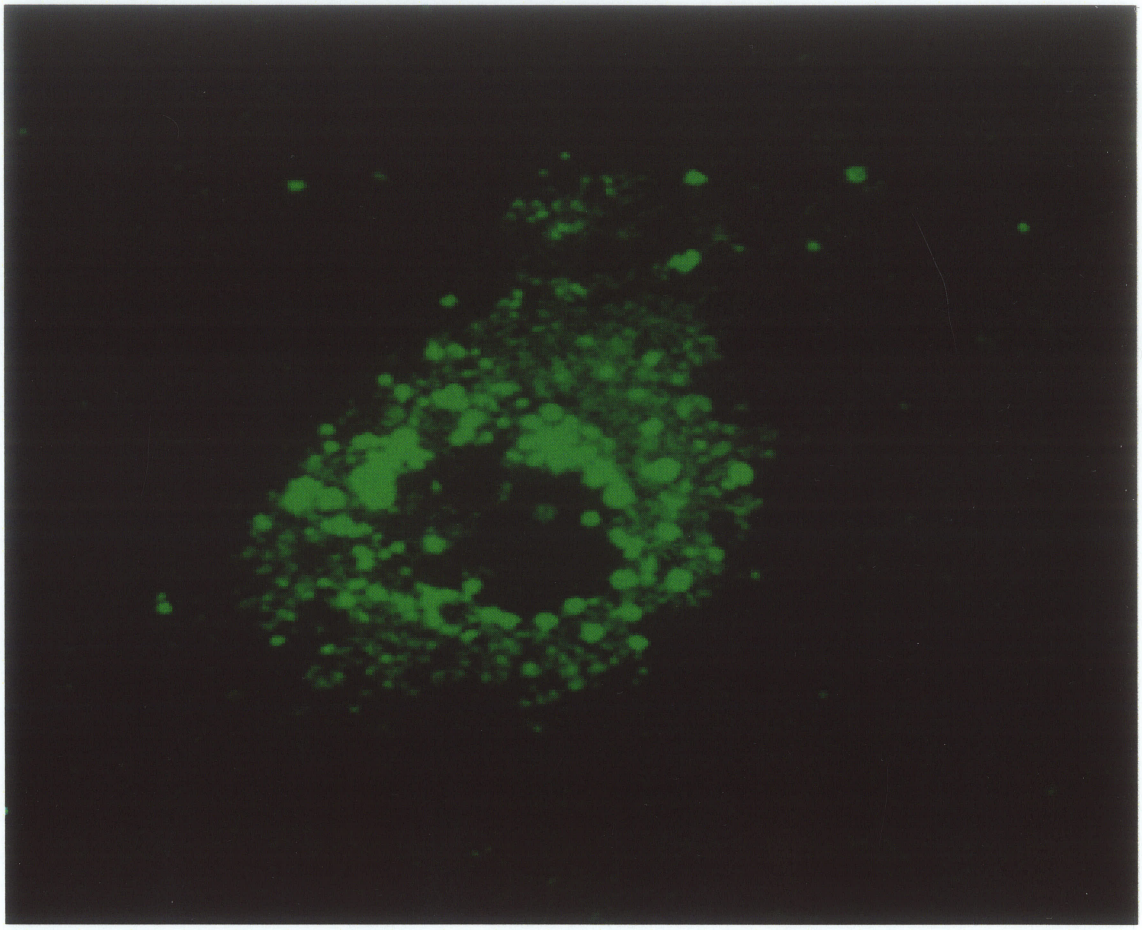
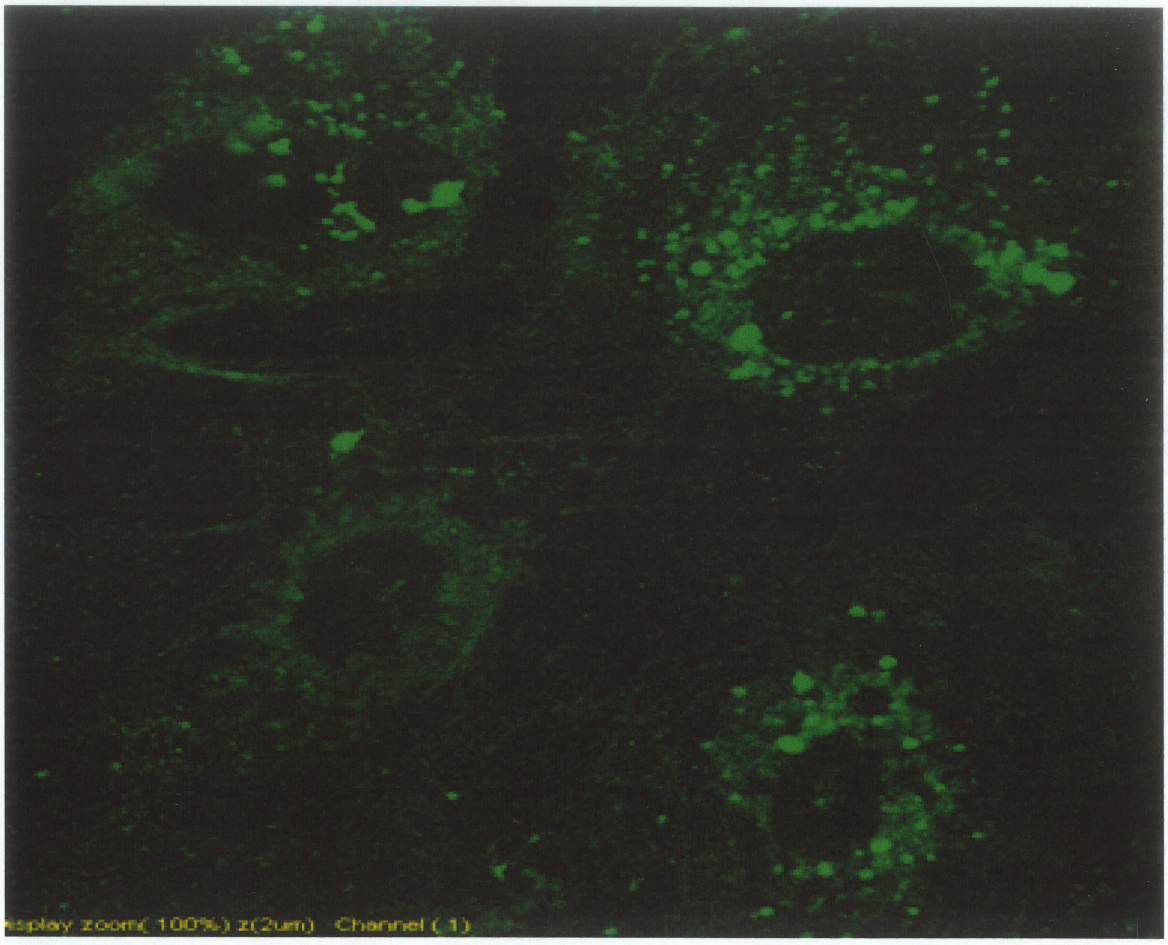

DPM /10^5 cells
0
5000
10000
15000
20000
Control
PE
NIC
COT
PE & COT
*
*
*
*

Figure 11: Confocal image of fetal rat lung alveolar cells exposed to nicotine at 10^{-4}M for 24 hours. Control SP-A (A, x10), nicotine treated SP-A (B, x10), control SP-B (C, x10), nicotine treated SP-B (D, x10). Cultured fetal rat lung type II alveolar cells reached confluence after a period of five days. The secondary antibody used was a FITC-conjugated donkey anti-goat IgG. Fluoview software and an Olympus IX70 inverted confocal microscope was used for analysis.



(A)



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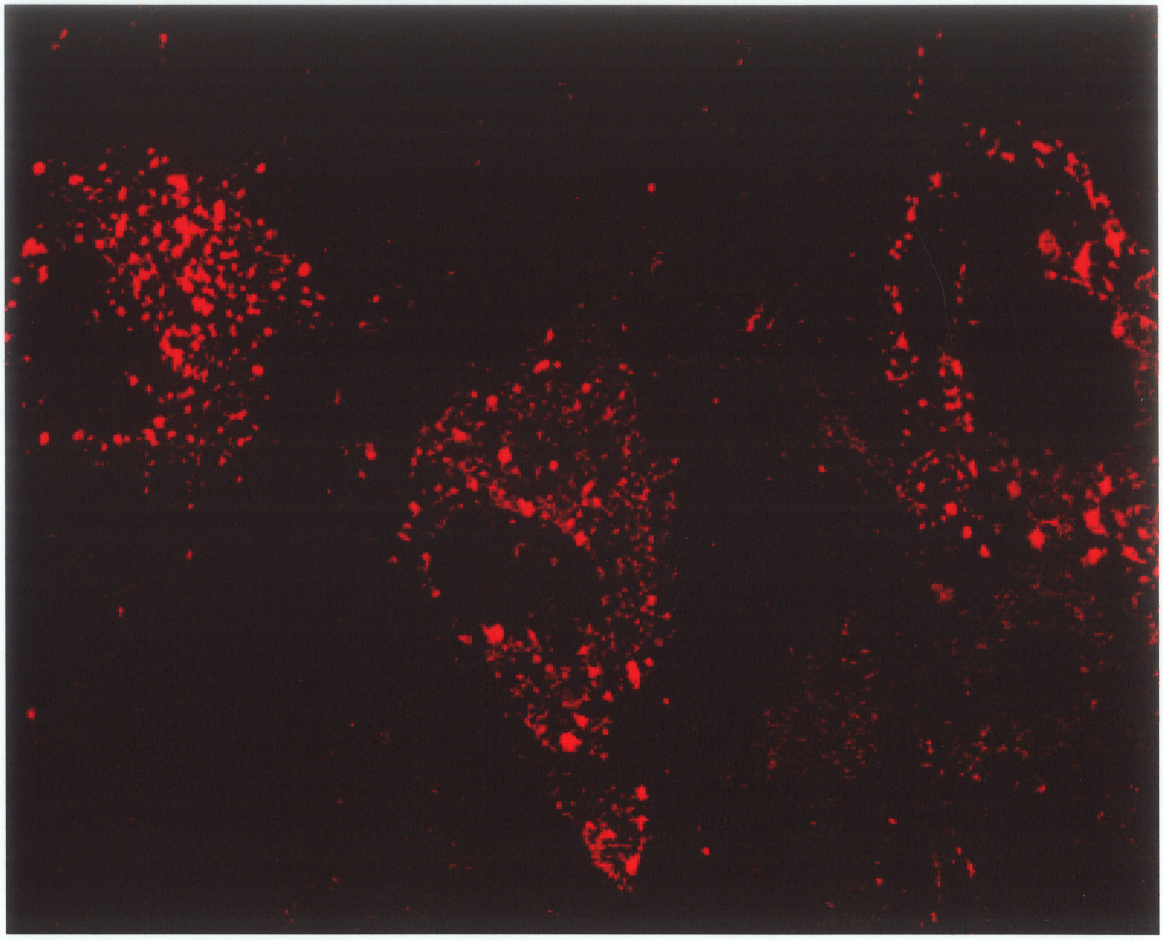
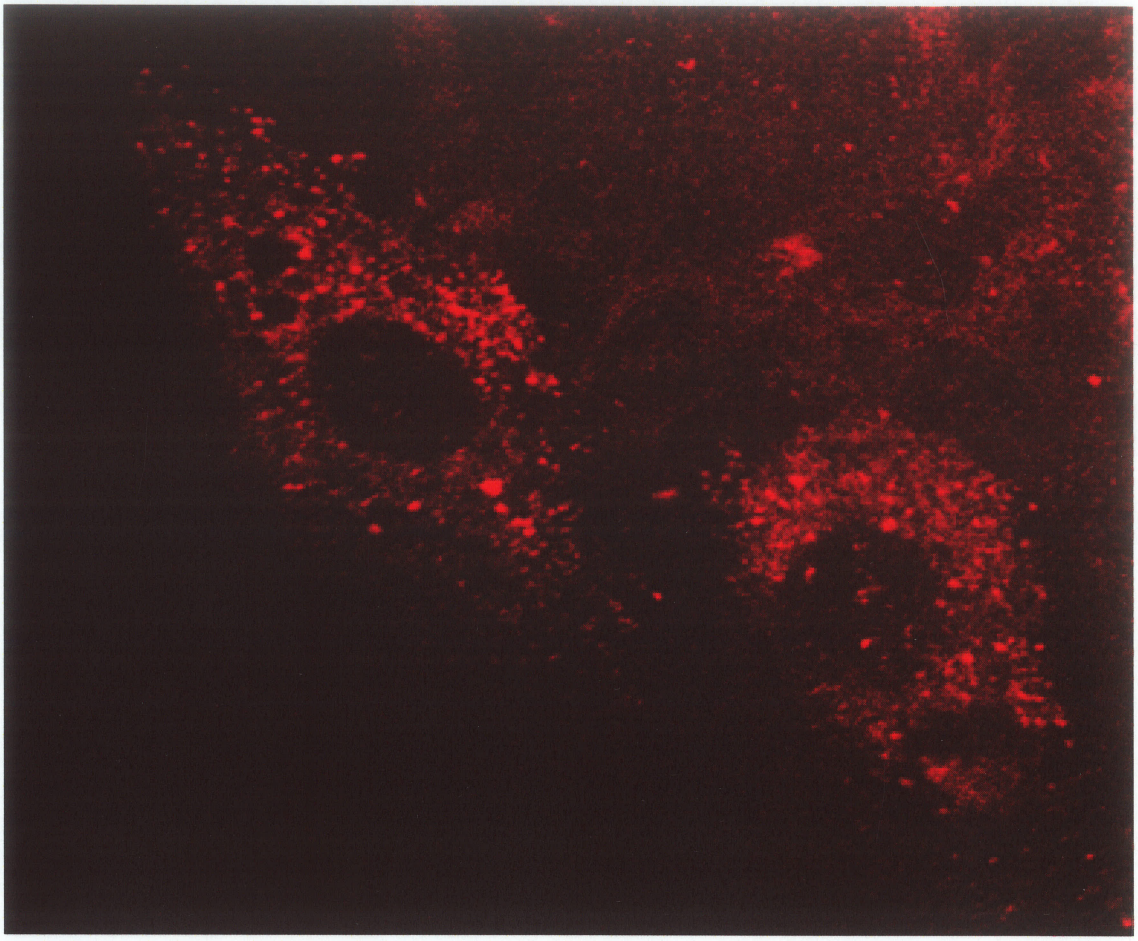
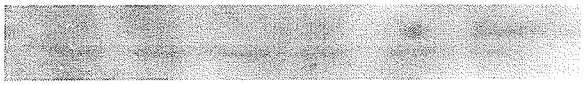


(C)



(D)

Figure 12: Western blot analysis of SP-A (A), SP-B (B), SP-C (C), and SP-D (D) expression in fetal rat lung alveolar type II cells following exposure to nicotine or cotinine at a concentration of 10^{-5}M and 10^{-4}M for 3 (nicotine) or 12 (cotinine) hours. The primary antibodies used were SP-A, SP-B, SP-C, or SP-D goat polyclonal (1:200). The secondary antibody was a donkey anti-goat IgG-HRP (1:3000). Cultured fetal rat lung type II alveolar cells reached confluence after a period of five days. Chemilumigrams were developed on Hyperfilm-ECL. 1 – WI-38 (positive control), 2 – untreated cells, 3 – nicotine treated cells at 10^{-4}M, 4 – nicotine treated cells at 10^{-5}M, 5 – cotinine treated cells at 10^{-4}M, 6 - cotinine treated cells at 10^{-5}M.



1 2 3 4 5 6

(A)

1 2 3 4 5 6

(B)

1 2 3 4 5 6

(C)

1 2 3 4 5 6

(D)

Figure 13: [^{3}H]choline incorporation to DSPC by fetal rat lung type II alveolar cells following exposure to nicotine or cotinine for 0, 3, 6, 18 hours at a concentration of 10^{-4}M. Cells were treated with nicotine or cotinine at a concentration of 10^{-4}M and simultaneously incubated with 1uCi/ml of [^{3}H]choline in MEM for 0,3,6, and 18 hours. DSPC was isolated as described in the Materials and Methods. Scintillation counts were determined using the method of H# (Beckman LS501, Palo Alto, CA) for quench compensation. Cultured fetal rat lung type II alveolar cells reached confluence after a period of five days. The results are expressed as mean disintegrations per minute +/-SD in DSPC per 10^{5} cells. * significantly different ($p<0.05$) compared to the corresponding control samples.

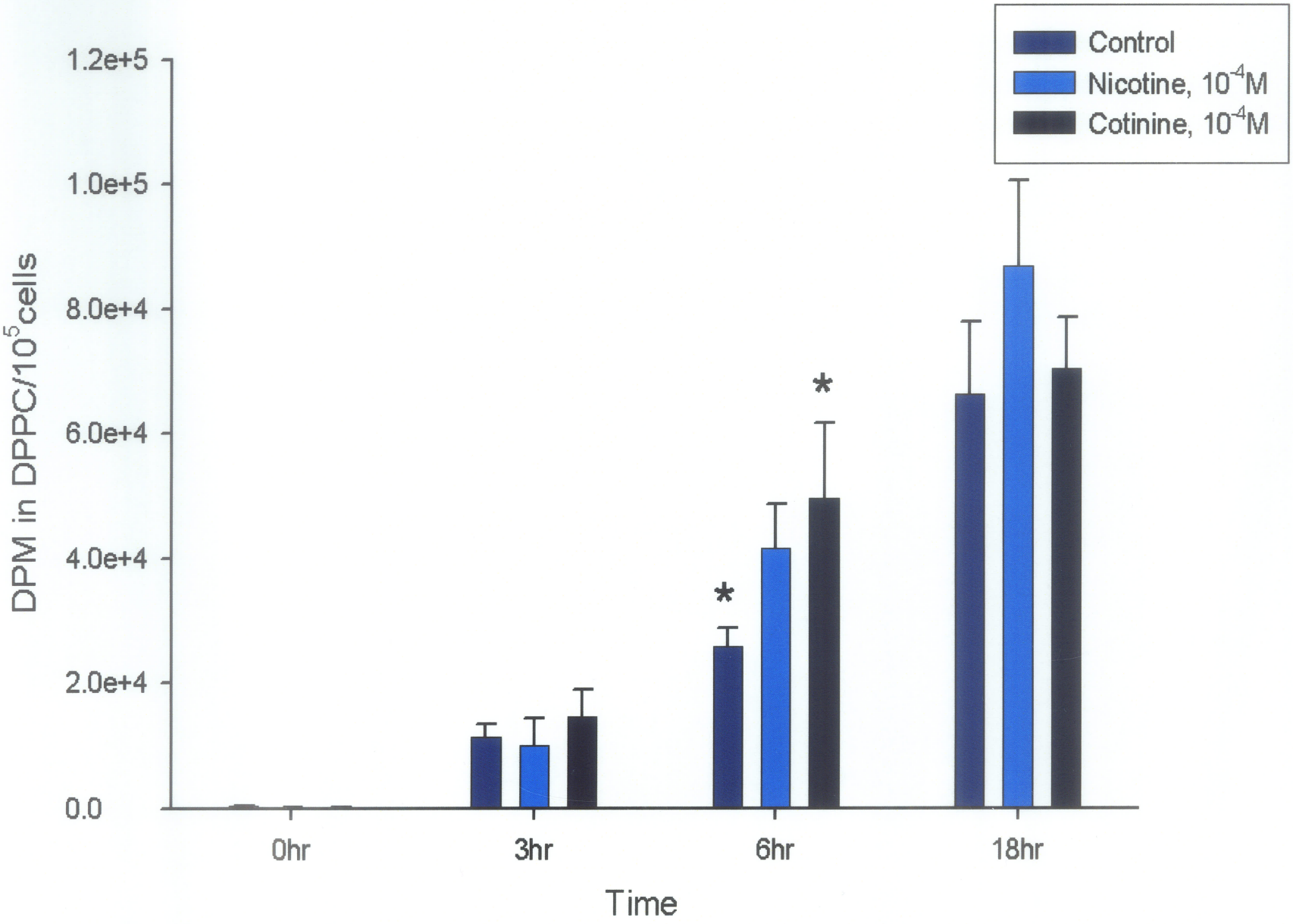

Control
Nicotine, 10-4M
Cotinine, 10-4M
DPM in DPPC/10^5cells
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
*
*
0hr
3hr
6hr
18hr
Time

Figure 14: DSPC secretion in fetal rat lung type II alveolar cells after exposure to nicotine or cotinine for 1, 3, 6 hours at 10^{-4}M. Cells were prelabeled with 1uCi/ml of [^{3}H]choline in MEM for 24 hours. The medium was discarded and the cells were exposed to nicotine (10^{-5}-10^{-4}M) or cotinine (10^{-5}-10^{-4}M) and in MEM for three different time points (1, 3, 6 hours). DSPC secretion was determined according to the formula: [^{3}H]$DSPC_M$ / ([^{3}H]$DSPC_M$ + [^{3}H]$DSPC_{C)}$ X 100 where M = medium and C = cellular [^{3}H]DSPC content [120]. Scintillation counts were determined using the method of H# (Beckman LS501, Palo Alto, CA) for quench compensation. Cultured fetal rat lung type II alveolar cells reached confluence after a period of five days. The results were expressed as mean disintegrations per minute +/-SD in DSPC per 10^5 cells.

* significantly different ($p<0.01$) compared to the corresponding control samples.

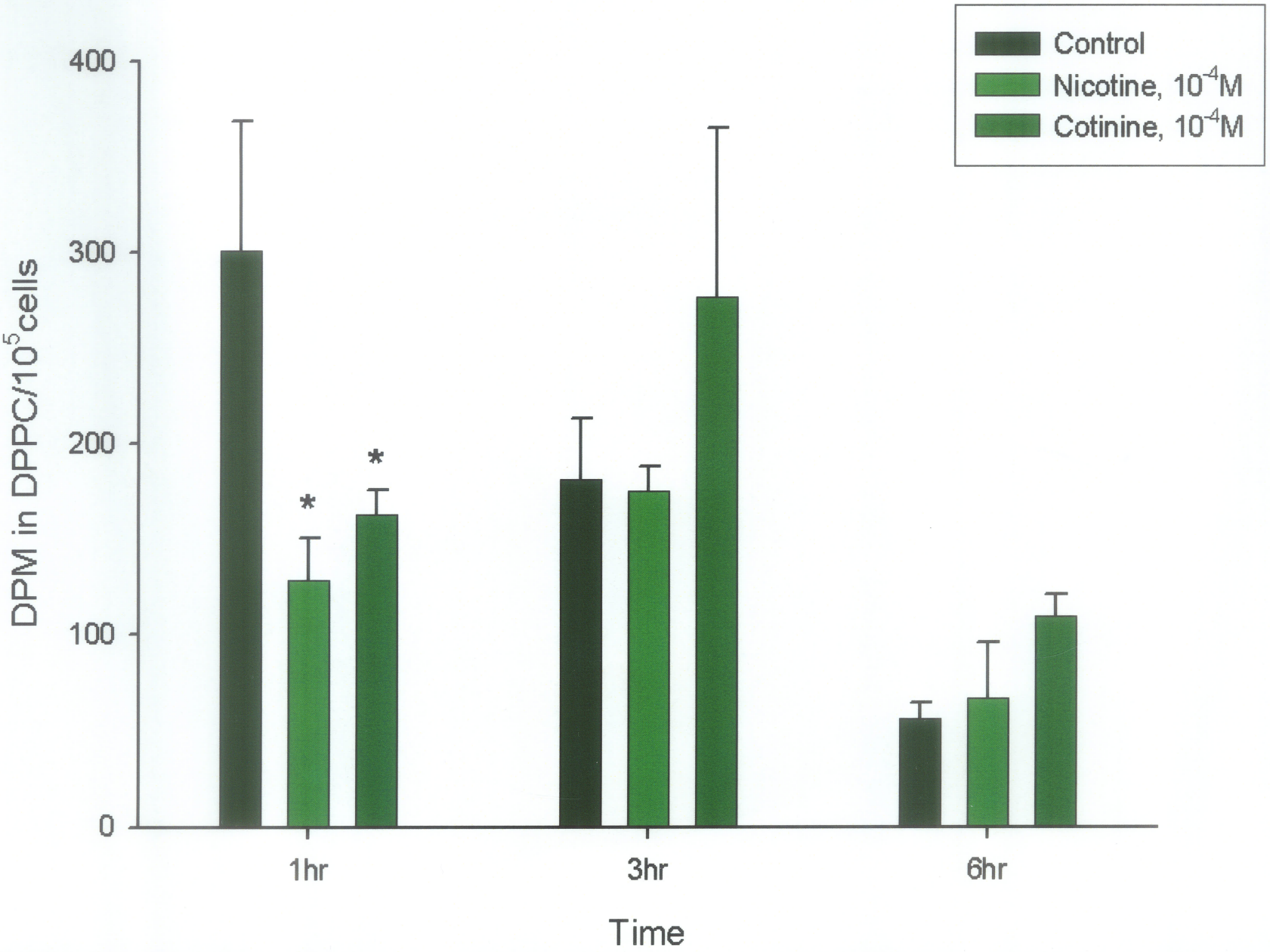
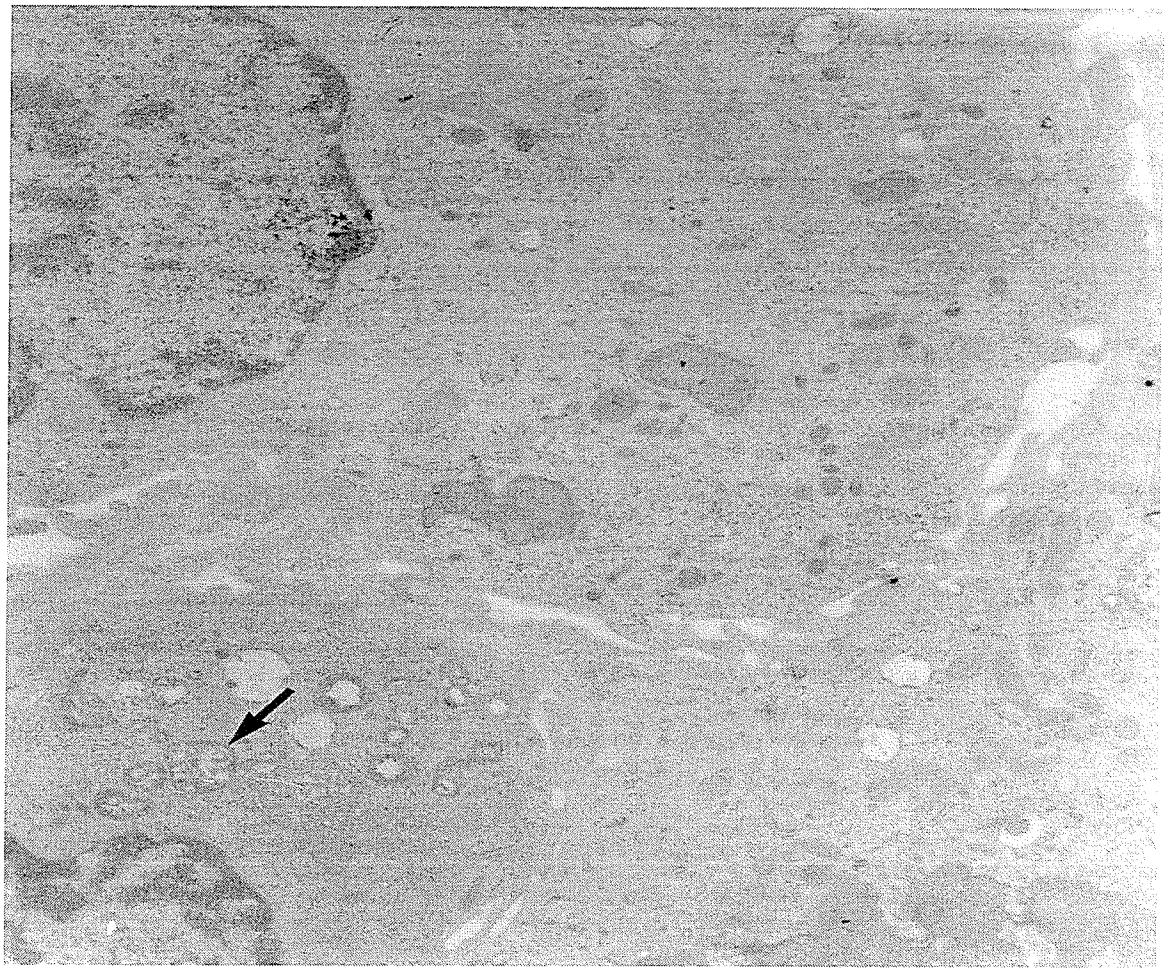
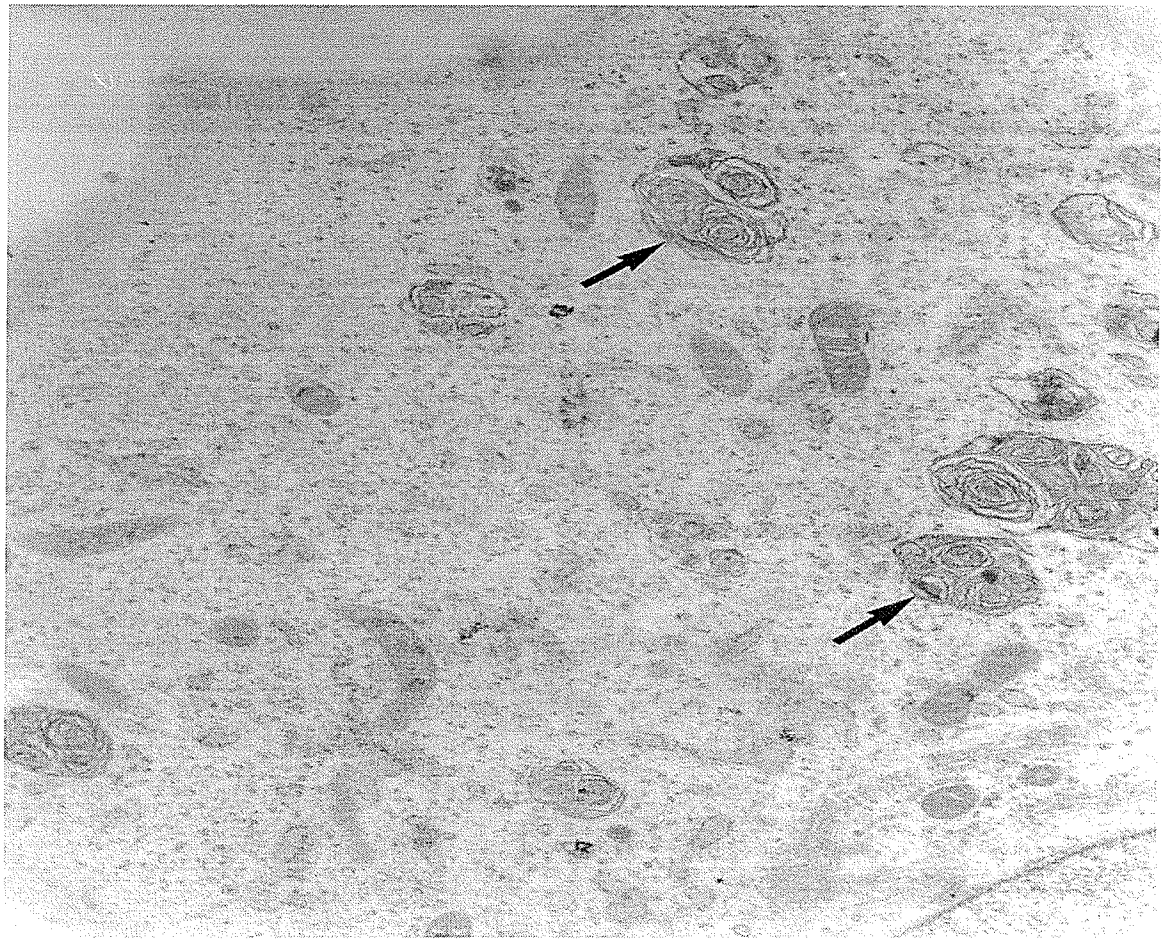

Control
Nicotine, 10^{-4}M
Cotinine, 10^{-4}M
DPM in DPPC/10^5cells
400
300
200
100
0
*
*
1hr
3hr
6hr
Time

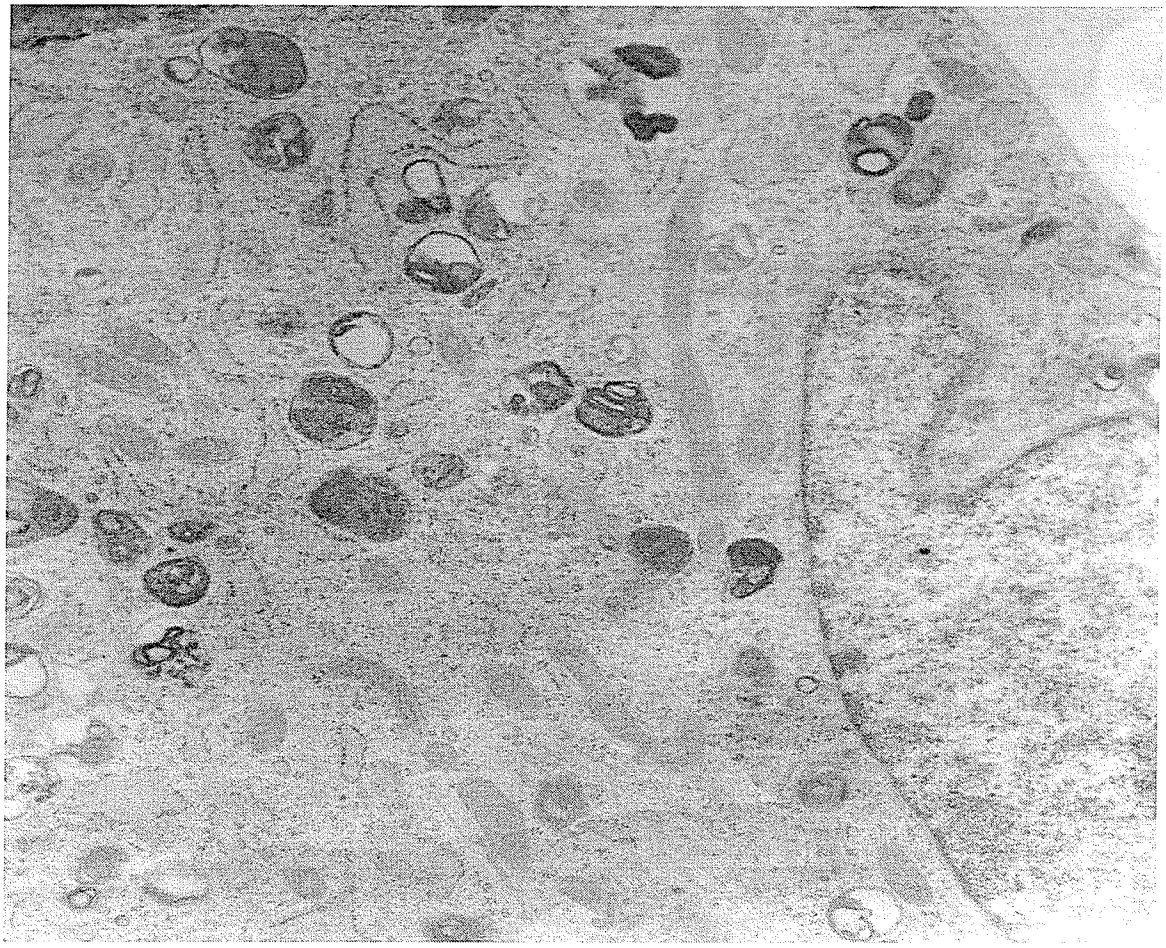
Figure 15: Electron micrographs of fetal rat lung type II alveolar cell following exposure to nicotine or cotinine for 24 hours at 10^{-4}M. Control (A, x50000). The nicotine exposed (B, C x50000) and cotinine exposed cells (D,E x50000) showed a qualitative increase in lamellar body numbers. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Arrows indicate Lamellar bodies.



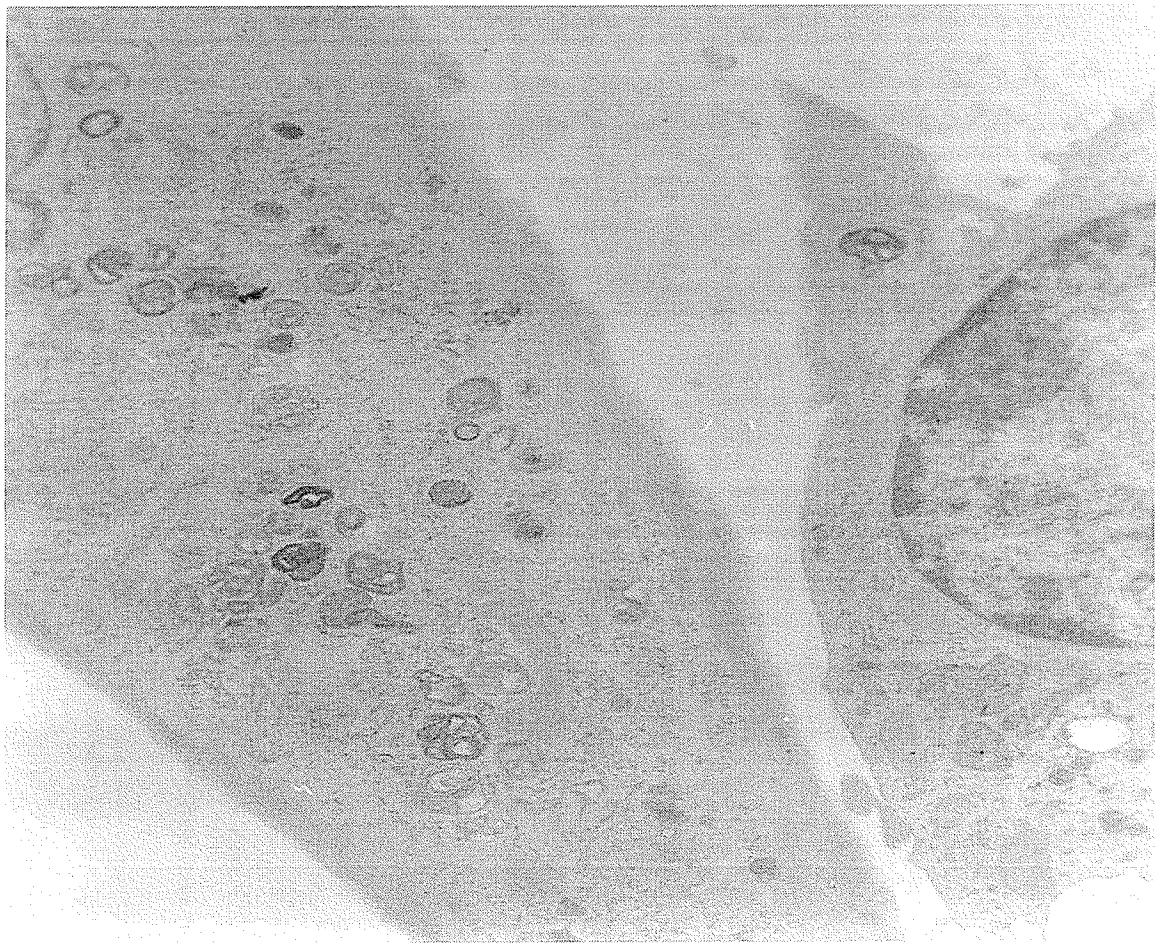
(A)



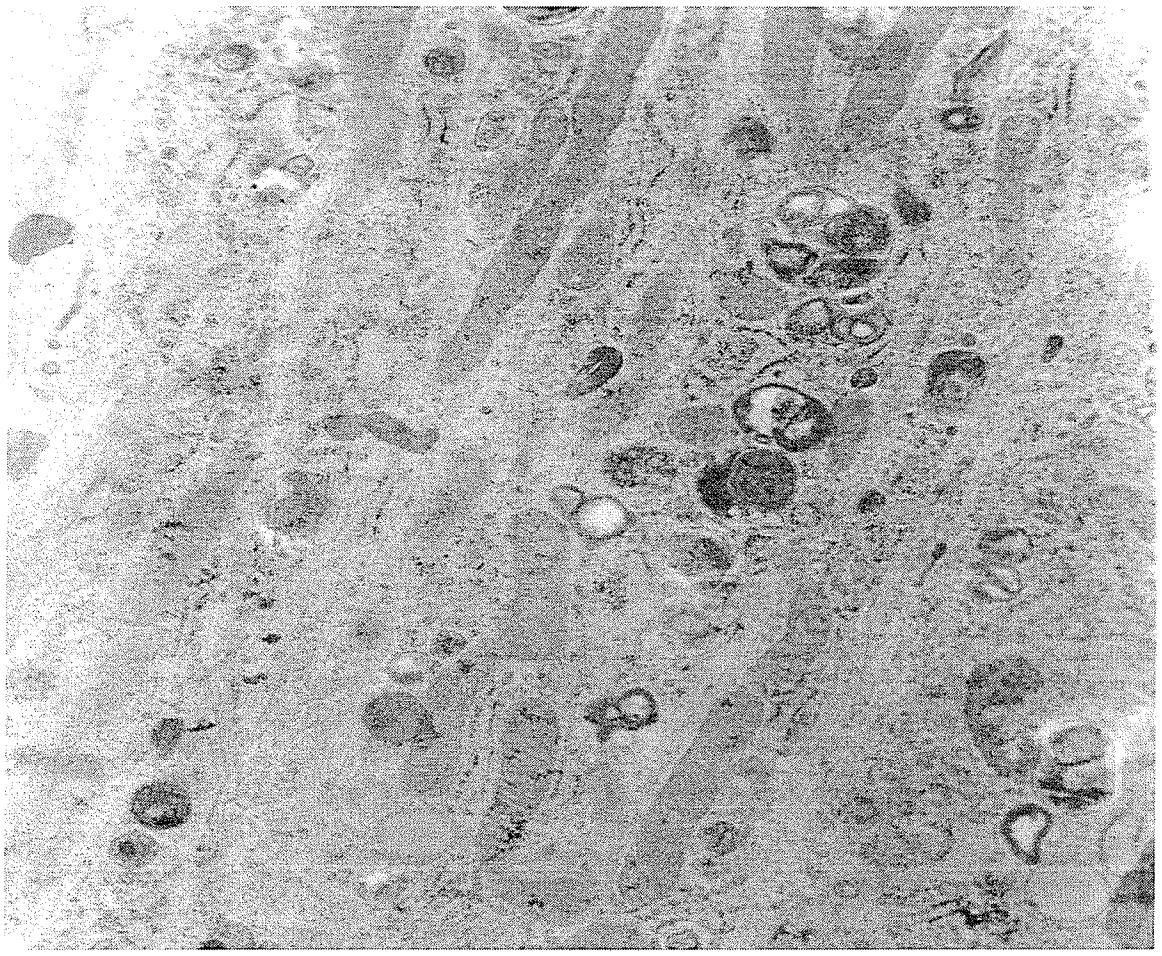
(B)



(C)

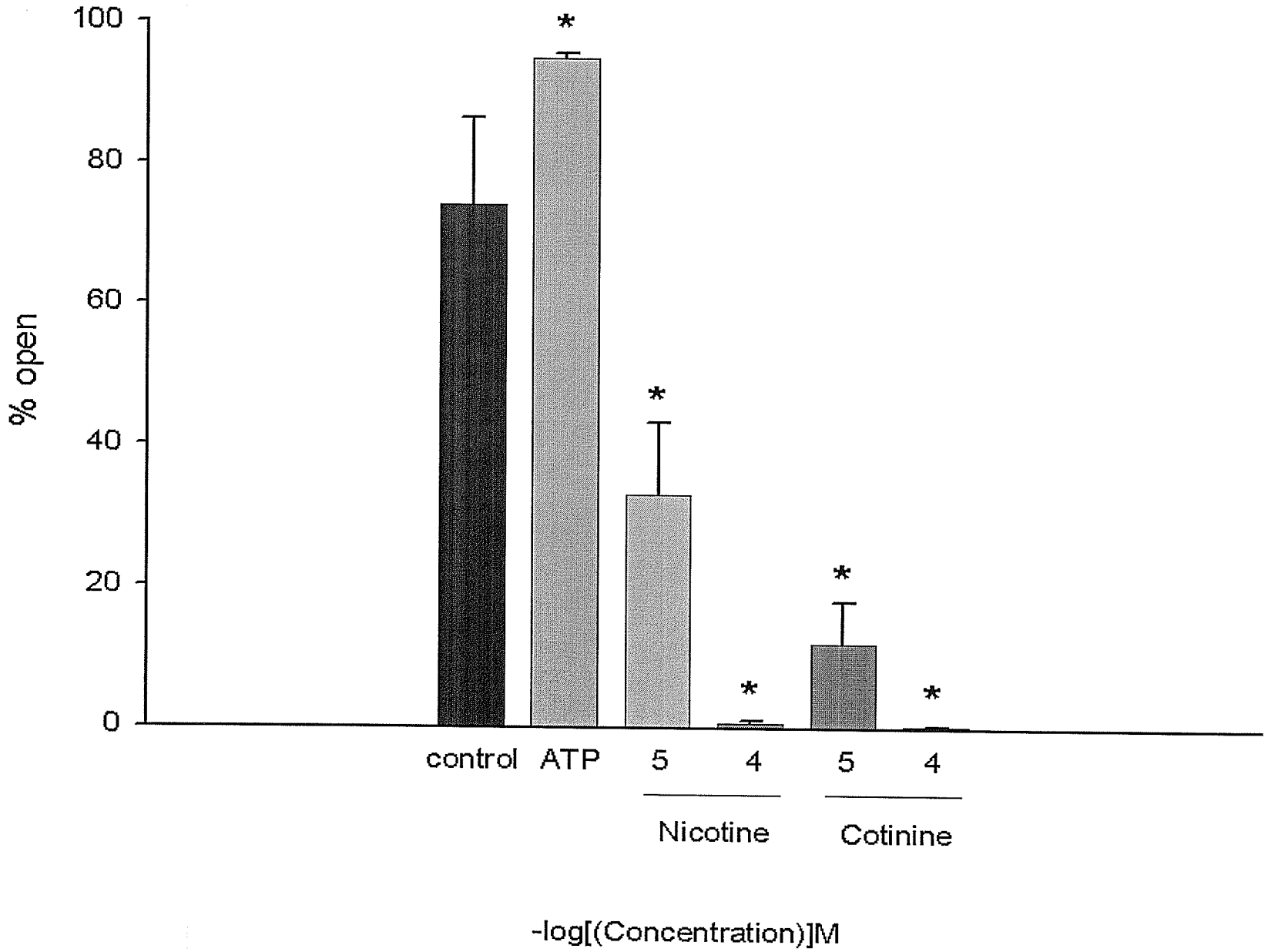


(D)



(E)

Figure 16: Capillary tube patency in fetal rat lung alveolar type II cells following exposure to ATP (10^{-4}M), nicotine, or cotinine at a concentration of 10^{-5}M and 10^{-4}M for 24 hours. The capillary surfactometer was used to determine airway patency. Cultured fetal rat lung type II alveolar cells were grown to confluence over a period of five days. Results are expressed as the mean percentage +/-SD of capillary patency (% open) for 5 replicates. * significantly different ($p<0.01$) compared to the corresponding control samples.


100
80
60
40
20
0
% open
*
*
*
*
*
control
ATP
5
4
5
4
Nicotine
Cotinine
-log[(Concentration)]M

Discussion

Cigarette smoking accounts for nearly 500,000 deaths each year in the United States and Canada [121]. Cigarette smoking alone increases the risk of cardiovascular and respiratory diseases [122]. In the lung, cigarette smoke is associated with a variety of abnormalities such as lung cancer, respiratory infections, abnormal lung growth, reduced lung volume, hypernea, and bronchoconstriction [95]. Ironically there exist some evidence that cigarette smoking may reduce the risk of neonatal RDS and increase lung maturation in the fetuses. On the other hand alveolar epithelial cells may be induced to undergo apoptosis following exposure to cigarette smoke [98, 123].

Nicotine is the major alkaloid found in cigarette smoke [90]. The absorption of nicotine by the fetus in smoking mothers is rapid and transfer occurs via the placenta [103]. The concentration of nicotine is generally higher in the fetus than the smoking mother and this is due to the accumulation of nicotine in the amniotic fluid [103]. Nicotine induced adverse affects on lung development have only received preliminary examination and in most cases only using retrospective epidemiological evidence [124]. Chen *et.al.*, have identified that maternal nicotine exposure results in variable changes in surfactant protein gene expression and a reduction in surfactant lipids [111]. Cotinine is the major metabolite of nicotine for most mammalian species [90]. The metabolism of nicotine to cotinine takes place in the liver where approximately 80% of nicotine is converted to cotinine [125]. Nicotine concentrations in the arterial blood range from 20-60ng/ml whereas cotinine concentrations are much higher ranging from 250 to 300ng/ml for cigarette smokers [126, 127]. The metabolism of cotinine is also very slow and the half life of cotinine on average is 16 hours whereas nicotine is only 2 hours [126, 128].

As a result, cotinine is far more stable in the body than nicotine but there is little research on what effects cotinine has on the developing respiratory system.

The synthesis and secretion of pulmonary surfactant occurs in the alveolar type II cells. In fetal rat lung development, type II alveolar cells appear by the 21st day of gestation [129]. Injury to type II alveolar cells not only alters lung function and surfactant metabolism, but it also may cause the lungs to be more susceptible to diseases related to environmental pollutants [110]. Proper lung function requires a layer rich in surfactant situated between the air-water interface of the alveolus. This layer consists mostly of DPPC and creates an environment low in surface tension which prevents the lungs from collapsing. Maintaining the structure and function of alveolar type II cells is essential in surfactant quality and function.

The chemical composition of cigarette smoke is very broad consisting of approximately 2000-4000 chemical agents [88]. The majority of studies have focused on the effects of cigarette smoke on the lungs and alveolar epithelial cells *in vivo* but there is little information regarding what effects the specific components of cigarette smoke have on the respiratory system. Studies have also yet to address what effect nicotine or cotinine has on surfactant synthesis and secretion *in vitro*. The purpose of this study was to focus more specifically on the major component of cigarette smoke - nicotine and its metabolite cotinine - and their effects on fetal rat lung alveolar type II cells and pulmonary surfactant metabolism *in vitro*.

The effects of nicotine or cotinine on the fetal rat lung type II alveolar cell

Alveolar type II cells are essential in the synthesis and secretion of pulmonary surfactant. Damage to these cells is detrimental to lung homeostasis and surfactant

metabolism. Previous studies have detected cell surface blebbing in adult rat type II alveolar cells after exposure to cigarette smoke condensates [99]. Our results clearly show an alteration in morphology of the fetal rat lung type II alveolar cells after exposure to nicotine or cotinine (Figure 1). The structural changes that occurred to the fetal rat lung type II alveolar cells involved cytoplasmic blebbing, mitochondrial swelling, and the formation of lipid globules. These effects were also demonstrated in rat pups exposed to nicotine where swollen mitochondria and the disruption of mitochondrial cristae were detected [110]. The structural changes that occurred within or around the type II alveolar cells in the adult, postnatal, and fetal rats suggest that cigarette smoke, nicotine or cotinine induced apoptosis in the cells. Maternal nicotine exposure has been shown to inhibit the glycolytic pathway resulting in a decrease in the amount of mitochondrial adenosine triphosphate (ATP) [108, 110]. ATP energy derived from the glycolysis pathway is required for type II alveolar cells to function properly. A deficiency in ATP energy will not only disrupt type II alveolar cell viability but also cause significant damage to lung alveolar development and integrity [110]. These results support the notion that nicotine or cotinine is detrimental to the fetal rat lung type II alveolar cell.

The glycolysis pathway is also essential in maintaining the structural integrity of the type I alveolar cell [130]. Inhibition of this pathway results in a loss of or an increased susceptibility to damage for the type I alveolar cell. Type II alveolar cells undergo an increase in cell proliferation after type I alveolar cell injury. An increase in type II alveolar cell proliferation results in higher mitotic activity [131]. The formazan assay, is a colorimetric method used to determine the number of viable cells in assays measuring proliferation and cytoxicity. The conversion of MTS into formazan is possible

through the mitochondrial dehydrogenase enzymes. Since mitochondrial dehydrogenase enzymes are responsible for the conversion into formazan, we interpreted the results as a measure of mitochondrial activity rather than cell viability. The formazan assay as a measure of cell viability does not specify any changes within a cell; therefore, by focusing on mitochondrial activity, this provided a more accurate depiction of the intracellular changes occurring within the fetal rat lung alveolar type II cells. The results for the formazan assay demonstrate an increase in mitochondrial activity after nicotine or cotinine treatment to the fetal rat lung alveolar type II cells (Figure 2). The increase in mitochondrial activity was also shown *in vivo* for fetal rat lung type II alveolar cells exposed to maternal nicotine [132]. The importance of our results was that we were able to show an increase in mitochondrial activity after treating the fetal rat lung type II alveolar cells with nicotine or cotinine. The increase in mitochondrial activity may be interpreted as an increase in cell proliferation potential since type II alveolar cells increase their proliferation *in vivo* during type I alveolar cell injury [16, 19]. However, the formazan assay was conducted using isolated fetal rat lung type II alveolar cells and any type I alveolar cells that may be present in cell culture would have minimal effects in inducing type II alveolar cell proliferation. Therefore these observations may suggest a direct non-type I cell mediated effect of these agents on the fetal rat lung type II alveolar cells.

Since detrimental effects were shown on the structural morphology and an alteration in mitochondrial activity after fetal rat lung alveolar type II cells exposure to nicotine or cotinine, the next area we examined was the changes that might occur at a DNA level. The techniques used to examine changes at the DNA level involved

[^{3}H]thymidine incorporation and the Comet assay. Thymine is a pyrimidine base that forms the pyrimidine nucleoside thymidine or deoxythymidine. Thymidine is important in the biosynthesis of DNA and is also used in preserving and transferring genetic information. Thymidine is phosphorylated into its nucleotide form deoxythymidylate which is one of the major constituents of DNA [133]. As a result, [^{3}H]thymidine incorporation was a valuable tool in measuring DNA synthesis. Our results demonstrated a decrease in [^{3}H]thymidine incorporation after exposure of fetal rat lung alveolar type II cells to nicotine or cotinine which suggests a decrease in DNA synthesis. The decrease in DNA synthesis may also be interpreted as a decrease in cell proliferation. The decrease in cell proliferation may be due to the inhibition of the glycolysis and glycogenolysis pathways within the fetal rat lung type II alveolar cells [110, 134]. Nicotine has been found to inhibit both the glycolysis and glycogenolysis pathway which decreases the production of ATP and glycogen [110]. ATP and glycogen are essential components in cell proliferation and a decrease in these molecules may decrease the rate of cell proliferation [110].

The comet assay provided further support to the [^{3}H]thymidine incorporation results. In the Comet assay, damage of DNA content within cells is detected when any denatured or cleaved DNA fragments migrate out of a cell after being exposed to an electrical field via electrophoresis. The migration of DNA results in the formation of a comet tail and DNA damage is based on the extent to which the DNA fragments have migrated. Currently, there are no studies that address the effects of nicotine or cotinine on fetal rat lung type II alveolar cells at the DNA level. DNA single strand breaks have been found in cultured cells after exposure to cigarette smoke [100]. However, we were

able to demonstrate that nicotine and cotinine caused DNA damage within fetal rat lung type II alveolar cells using the Comet assay. Cotinine had a more detrimental effect on the DNA of the fetal rat lung type II alveolar cells than nicotine and this may be related to the longer half life and stability of the metabolite. Furthermore, the DNA damage that was shown in the Comet assay could further explain the decrease in [^{3}H]thymidine incorporation which suggests a decrease in cell proliferation.

The effects of nicotine or cotinine on surfactant metabolism

Pulmonary surfactant synthesis and secretion is a complex process. The synthesis of surfactant occurs within type II alveolar cells and the surfactant is stored in the lamellar bodies. In order to create a surface film rich in DPPC and prevent lung collapse, it is important that these synthesis and secretion steps are not compromised in any way. In type II alveolar cells, the majority of the DSPC that is formed is comprised of DPPC [51]. To analyze the effect of nicotine or cotinine on surfactant related phospholipid synthesis we analyzed the incorporation of [^{3}H]choline into DSPC. Choline is an essential substrate in phosphatidylcholine (PC) synthesis. DSPC is synthesized from diacylglycerol and choline. Choline reaches the biosynthetic pathway for PC by sequential conversion to choline phosphate by choline kinase which in turn is converted to CDP-choline by choline-phosphate cytidylyltransferase [51]. DAG is converted to PC by cholinephosphotransferase. DSPC is formed through the deacylation-reacylation of phosphatidylcholine [51]. The significance of analyzing DSPC synthesis through [^{3}H]choline incorporation rather than any other substrates is in its ability to become incorporated directly into DSPC.

Research conducted on the effects of cigarette smoke or nicotine on surfactant-associated phospholipids show conflicting results. Phospholipid levels have been found to increase in 21 day old rat pups and the offspring of maternal rats demonstrate an increase in surfactant synthesis along with the accumulation of lamellar bodies after exposure to cigarette smoke [101, 103, 110]. However, recent studies show lower lung surfactant lipid contents for postnatal day 21 rats exposed to nicotine [111]. Similarly, lower yields of surfactant associated surface active phospholipids were found in bronchoalveolar lavage samples of cigarette smokers [135]. Our results show more specifically that [^{3}H]choline incorporation into DSPC increased after exposure of fetal rat lung alveolar type II cells to nicotine and/or cotinine which suggests an increase in surfactant-related phospholipid synthesis. The electron micrographs in our results further support these findings where intracellular lamellar body content increased in the fetal rat lung alveolar type II cells after exposure to nicotine or cotinine. The inhibition of glycolysis and glycogenolysis caused by nicotine may explain the apparent increase in surfactant synthesis [103]. Glucose and glycogen are important substrates in the formation of phosphatidic acid a precursor to DPPC. The loss of these substrates decreases the availability of DAG which is required for PC synthesis. To compensate for the reduced production of DAG an additional source is required. Studies have suggested that triacylglycerols serve as another source for DAG and that other fatty acids are utilized as precursors in surfactant synthesis [51, 101]. Although there is no direct evidence of what components are increased in the DSPC synthesis pathways, it is possible that the reduction of glucose and glycogen substrates results in the influx of

triacylglycerols and fatty acids which ultimately leads to an overproduction of surfactant related phospholipids.

Xue *et.al.*, suggest that the increase in surfactant after adult rat type II alveolar cells were exposed to cigarette smoke is due to a compensatory mechanism found within the type II alveolar cells [136]. In their study rat lung adult type II alveolar cell were isolated and cultured *in vitro* and cell attachment decreased after exposure to cigarette smoke. The decrease in type II cell numbers led to an increase in surfactant synthesis in the cells which contained the fewest cells per well [136]. This increase in surfactant synthesis suggests that the remaining type II alveolar cells up-regulate the synthesis of surfactant in order to compensate for inadequate surfactant levels within the lung. From our results we were able to show similar trends using nicotine or cotinine instead of cigarette smoke. The decrease in type II alveolar cells proliferation *in vitro* and the increase in surfactant production within the cells support the detrimental effects of cigarette smoke components and metabolites on fetal rat lung type II alveolar cells as well as the pulmonary system. The fact that isolated type II cells were used in both studies suggests further that nicotine, cotinine, or cigarette smoke have a direct effect on the type II alveolar cell and that there are no other cells such as type I alveolar cells or fibroblasts that may influence an up-regulation of surfactant in the type II alveolar cell.

Secretion of surfactant is a process that can occur through a constitutive or regulated pathway [61]. In the type II alveolar cells, studies have focused more on regulated secretion where a variety of stimuli can lead to the exocytosis of lamellar bodies from the cell [61]. Surfactant secretion can be regulated through physiological regulation and by three distinct signaling mechanisms [64]. Studies focusing on the

exposure of adult rat lung alveolar type II cells to cigarette smoke found a decrease in surfactant secretion [99, 123]. Wirtz *et. al.*, were the first to show an inhibition in phosphatidylcholine secretion for adult rat lung alveolar type II cells exposed to cigarette smoke. Studies on the exposure of 1 day old neonatal rat pups to maternal nicotine showed a significant increase of lamellar body content [110]. The decrease in surfactant secretion may be due to the suppression by cigarette smoke of protein phosphorylation which we have shown to be active in fetal adult lung [110, 137]. In the signal transduction pathways, protein phoshorylation is the last step prior to surfactant secretion and the suppression of protein phosphorylation may not only decrease the amount of surfactant being secreted from the cell, but may result also in the accumulation of lamellar bodies within the type II cells [64, 110]. Our studies suggest more specifically that nicotine or cotinine decrease DSPC secretion. The accumulation of lamellar bodies observed in the electron micrographs supports the previous findings. Exposure of fetal rat lung alveolar cells to nicotine or cotinine may increase DSPC synthesis however; the results suggest that both nicotine and cotinine disrupt the ability of the type II cell to secrete surfactant phospholipid.

The capillary surfactometer is a device that measures surfactant quality of a given sample. The Capillary Surfactometer simulates the size of the terminal airways. The technique involves the use of a glass capillary tube containing a narrow section in the middle portion. The sample is inserted inside the narrow section of the capillary tube and surfactant quality is based on the ability of a given sample to maintain patency (openness) once air pressure is applied [138]. The surfactant sample is regarded as good quality surfactant only if it can maintain patency within the glass capillary tube. The ability of a

surfactant sample to maintain patency is based on the adsorption process. As pressure is exerted into the capillary tube, the surfactant sample is pushed out of the narrow portion and undergoes adsorption forming an air-liquid interface. At this point the surface tension is very low and this causes the capillary to remain open [139]. Research studies have yet to address the effects of cigarette smoke or nicotine surfactant function in fetal rat lung type II alveolar cells. Studies that have evaluated surfactant function were directed in other lung diseases. In these studies, surfactant function was found to be poor in chronic airway inflammation, asthma, and cystic fibrosis [113, 140, 141]. In our results, we were able to show that nicotine or cotinine were able to increase DSPC synthesis and lamellar body production in fetal rat lung type II alveolar cells. However we did not determine whether surfactant function was altered in any way. Therefore, the Capillary Surfactometer enabled us to test surfactant quality. Our results suggest a significant decrease in surfactant quality indicating that fetal rat lung type II alveolar cell exposure to nicotine or cotinine results in surfactant dysfunction. Although studies have yet to examine cigarette smoke or nicotine or cotinine effects on surfactant quality through the Capillary Surfactometer, there have been studies that identified a decrease in lung function after cigarette smoke exposure [94, 98, 101]. The increase in surfactant synthesis shown in our results may suggest a beneficial effect of nicotine or cotinine on surfactant metabolism however, the use of the Capillary Surfactometer demonstrates that surfactant quality and function were in fact poor. As a result, these results support further the notion that nicotine or cotinine is detrimental to surfactant metabolism.

Surfactant proteins play an important role in the adsorption, and secretion of pulmonary surfactant [30, 38]. Studies examining the effects of nicotine on surfactant

proteins have found variable results. Wuenscell *et.al.*, found that nicotine stimulated SP-A and SP-C mRNA gene expression [142]. Chen *et.al.*, however, found variable changes in fetal rat lung surfactant protein expression levels after maternal nicotine exposure [111]. Our results did not detect any significant changes in the cellular surfactant protein expression after exposure of fetal rat lung type II alveolar cells to nicotine or cotinine.

Total cellular protein synthesis was analyzed using [^{35}S]-methionine incorporation. [^{35}S]-methionine release by the fetal rat lung type II alveolar cells showed an interesting trend where an overall release was decreased suggesting a reduction in the release of protein by fetal rat lung alveolar type II cells. Treatment of fetal rat lung type II alveolar cells with phorbol ester resulted in a decrease in [^{35}S]-methionine release. Phorbol ester is an established secretagogue for surfactant phospholipids, and the decrease in [^{35}S]-methionine release suggests that protein synthesis, the majority of which would be surfactant-related, is controlled through a separate mechanism compared to the surfactant phospholipids. Thus the release of surfactant proteins and phospholipids may be independently regulated. This view is supported by some previous studies that SP-A in type II alveolar cells is secreted independently of lamellar bodies and that agonists that cause PC secretion do not necessarily affect SP-A secretion [43, 64]. Furthermore, the combination of phorbol ester and cotinine did not further decrease [^{35}S]-methionine release suggesting the two agents do not act synergistically via a common pathway.

Conclusion

Cigarette smoke is detrimental to the developing respiratory system causing a number of malfunctions within the lung. Maternal nicotine exposure of the fetus through maternal smoking is a problem that still occurs today. Nicotine absorption and transfer to the fetus from smoking mothers is rapid and thus gives rise to further complications to the newborn. Few studies have examined the effects of nicotine on fetal type II alveolar cells and in surfactant metabolism. Currently, studies have yet to analyze the effects of cotinine on fetal type II alveolar cells. Since fetal type II alveolar cells are the primary site for surfactant synthesis and secretion, the objective of this study was to determine what effect nicotine, a major component of cigarette smoke and its metabolite cotinine have on type II alveolar cells and on pulmonary surfactant metabolism. The results indicate that nicotine and cotinine have adverse effects on the fetal rat lung alveolar type II cell and pulmonary surfactant metabolism. In the fetal rat lung type II alveolar cells, nicotine or cotinine appeared to induce apoptosis where structural changes such as cytoplasmic blebbing and mitochondrial swelling occurred. Within the fetal rat lung alveolar type II cell the intracellular changes that occurred included an increase in mitochondrial activity, a decrease in DNA synthesis, and increase in DNA damage. This further demonstrated the detrimental effect of nicotine or cotinine on the type II cell. The changes that were shown within the fetal rat lung type II alveolar cell may be due to the inhibition of glycolysis and glycogenolysis caused by nicotine or cotinine. These pathways are important in providing ATP and glycogen to the type II alveolar cell for cell proliferation and other metabolic processes. DSPC synthesis appeared to increase and the electron micrographs illustrated an accumulation of lamellar bodies however,

secretion of DSPC decreased indicating a possible disruption to the secretion process of surfactant components. Furthermore the ability of surfactant to reduce surface tension may be reduced upon exposure of cells to nicotine or cotinine.

Future directions

Nicotine and cotinine induced changes to the fetal rat lung type II alveolar cells and altered the metabolism of pulmonary surfactant. The molecular mechanisms behind the structural changes to the type II alveolar cell and alteration of surfactant metabolism have yet to be determined. This study demonstrated that nicotine or cotinine induced apoptosis, however the biochemical pathways and receptors involved in this process have yet to be addressed. Whether or not nicotine or cotinine induce their adverse effect directly in the type II alveolar cell or via a receptor mediated process is an area of research that should be considered. The signaling pathways involved in the secretion of surfactant are complex and studies that can decipher how cigarette smoke or its major components inhibit secretion is a topic that would generate a lot of interest. The increased synthesis of DSPC and accumulation of lamellar bodies was established in the current and previous studies, however the substrates and enzymes involved in surfactant phospholipids synthesis is an important area that future studies needs to focus on. Although nicotine is a major component of cigarette smoke, there are thousands of other potentially toxic chemicals within cigarette smoke that are harmful to the type II alveolar cell and pulmonary surfactant system.

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