Apoptosis and caspase-3 activity in isolated fetal rat lung cells, human A549 cells and rat periodontal ligament fibroblasts following exposure to cigarette smoke extract

By

Asra Ahmed

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE

Department of Oral Biology

University of Manitoba

Winnipeg

ABSTRACT

Lung development involves a complex phenomenon of continuous growth and remodeling to attain a mature stage for proper gas exchange. Exposure cigarette smoke (CS) during prenatal life is the leading cause of preventable premature death in the world. Moreover, it has been implicated in the pathogenesis of many pulmonary, oral and systemic diseases. The mechanisms by which pathological changes occur on exposure to first hand or second hand smoke are not clearly elucidated. The large surface area of the lungs increases the susceptibility of the lungs to toxic substances and pathogens. The oral cavity is the first site of exposure to CS and may be a possible source for the spread of toxins to other organs of the body. Chronic exposure of oral tissues to CS may be a predisposing factor for chronic destructive periodontitis, loss of tooth attachment, and necrotizing ulcerative gingivitis. Dose dependent *in-utero* exposure of CS either by active smoking or passive smoking is associated with developmental pulmonary toxicity and adverse respiratory outcomes. Apoptosis or programmed cell death is a methodical physiologic form of cell death which plays an important role during embryogenesis, regulation of the immune system and proper maintenance of homeostasis. Synchronization of cell proliferation, differentiation and apoptosis is the basis of organogenesis. Any defect in apoptotic processes during embryogenesis may lead to developmental abnormalities and damage to cells. Moreover, when the balance of cell death and cell proliferation is altered in adults it may lead to cancer development. The multi-step process of apoptosis involves complex biochemical events including activation of the caspase cascade. Caspases are considered as key mediators of apoptosis by

induction, transduction and amplification of intracellular signals leading to cell death. Caspases can be classified as initiator or effector caspases depending on their specific roles. Fourteen caspases have been identified so far. Caspases primarily exist in the cytosol as inactive forms or zymogens. Activation of caspase 3 is a key event during apoptosis. Caspase 3, is important for the downstream signaling for final execution of cell death. **Objective**: Since activation of caspase-3 is a main event in apoptosis, which is an important process for maintenance of homeostasis during embryogenesis and adult life, we explored the hypothesis that *in vitro* exposure of developing lung cells to cigarette smoke extract (CSE) may result in the alteration of normal homeostatic properties maintained by apoptosis through activation of caspase-3. Alongside we compared the responses of fetal lung cells with A549 cells and rat periodontal ligament (PDL) fibroblasts exposed to CSE in a dose dependent manner. **Methods**: Fetal rat lung fibroblasts and fetal rat lung type II alveolar epithelial (AE) cells were isolated from pregnant Sprague-Dawley rats on gestational day 21. Human A549 cell line and adult rat PDL fibroblast cells were sub-cultured in new born calf serum (NCS) media. Cells were exposed to different concentrations of CSE (5%, 10%, or 15%) (v/v) for sixty minutes and compared with unexposed controls. Detection of caspase-3 activity in cells when exposed to CSE was measured using a caspase-3 fluorometric assay. Effect of Z-VAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), an irreversible inhibitor of caspase-3 was detected using caspase-3 fluorometric assay. Effect of CSE on cell viability was measured using MTT formazan assay. Caspase-3 expression was detected by western blot analysis. Cellular localization of caspase-3 was determined by immunofluorescence using fluorescence microscopy. **Results**: Our results indicate that

isolated fetal rat lung fibroblasts and type II AE cells significantly ($p < 0.05$) elevated caspase-3 activity after exposure to 10% or 15 % (v/v) CSE *in vitro*, when compared to non-exposed controls. CSE significantly inhibited cell proliferation in the isolated fetal lung fibroblasts and type II AE cells at concentrations of 10% or 15 % (v/v). No significant differences were observed in the caspase-3 activity or cellular viability in A549 cells and rat PDL fibroblasts exposed to 5%, 10% or 15% (v/v) CSE. **Conclusion**: Activation of caspase-3, leading to eventual cell death due to apoptosis of fetal lung connective tissue and alveolar epithelial cells may be one of the reasons for the developmental pulmonary toxicity induced by CSE.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the encouragement and support of many people. First and foremost, I would like to thank my advisor Dr. James Elliot Scott, for his guidance, support and supervision. He gave me the freedom to explore and work independently which allowed me to enrich my ideas and understand the concepts in a better way. His excellence as a researcher and mentor has inspired me in many ways and added considerably to my graduate experience. I thank him for taking out his precious time to read this thesis and give critical comments. It has truly been a gratifying experience to work with such an exceptionally experienced scientist and a great person.

I would like to express my gratitude to Dr. James Thliveris and Dr. James Gilchrist for participating in my advisory and examining committee. I greatly appreciate Dr. Hugo Bergen for agreeing to be a part of my examining committee at a very short notice. My gratitude goes out to all the members of the Oral Biology staff and my colleagues for their friendly support and care which helped me stay focused during my graduate studies. I would like to thank Jasbir, Nisha, Bing and Claudia, for taking out time and helping me during technical difficulties. I gratefully appreciate Nichole Nayak for assisting me with my experiments when my hand was fractured.

My parents and my sisters deserve a special mention for their unconditional love and moral support. I wish to thank my children for giving me strength and energy in their own special way whenever I felt tired and discouraged. Lastly, and most importantly I would like to thank my husband, for his love, support and cooperation.

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ALP	Alkaline phosphatase
Apaf-1	Apoptotic protease activating factor-1
BSA	Bovine Serum Albumin
CARD	Caspase-activating recruitment domain
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSE	Cigarette smoke extract
DD	Death domain
DED	Death effector domain
DPPC	Dipalmitoylphosphatidylcholine
DISC	Death inducing signaling complex
ECM	Extracellular matrix
FADD	Fas-associated protein with death domain
FGF	Fibroblast growth factor
FPF	Fibroblast pneumocyte factor
GAGS	Glycosaminoglycans
HBSS	Hanks Balanced Salt Solution
LB	Lamellar bodies
LIC	Lipid interstitial cells
MEM	Minimal essential medium
NCS	Newborn calf serum
NLICs	Non-lipid interstitial cells

MAPK	Mitogen activated protein kinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide
NSCLC	Non-small cell lung carcinoma
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
SCLC	Small-cell lung carcinoma
SIDS	Sudden infant death syndrome
SP	Surfactant proteins
AE	Alveolar epithelial
TOP-I	Topoisomerase I
TNF	Tumor-necrosis factor
VGEF	Vascular endothelial growth factor
Z-VAD-fmk	Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

CHAPTER ONE

INTRODUCTION: PART ONE

RESPIRATORY SYSTEM

1.0 Respiratory System

The respiratory system is one of the most structurally complex and vital systems of the body. It can be broadly divided into two physiologic systems, the upper respiratory system (including the nose, nasopharynx, and oropharynx) and the lower respiratory system (including larynx, trachea, bronchi and lungs). Lungs are highly elastic, spongy organs with a large surface area of 118 ±11 m^2 of the human body (Coxson et al., 1999) and they function primarily for exchange of gases between the external and internal environment. Lungs are comprised of forty different types of cells and an irregular branching system. After repeated branching, the branching system ends as small, narrow terminal bronchioles, which further ends to form terminal air spaces known as the alveoli.

1.0.1. The Alveolus – The Gas Exchange Components of Lungs.

Alveoli are specialized respiratory units where exchange of oxygen and carbon dioxide from blood occurs. Alveoli are the major sites for gas exchange, which makes the alveoli susceptible to inhaled pathogens and toxic substances. Stereological studies have estimated that the number of alveoli in healthy adult lungs range from 274 to 790 million (Ochs et al., 2004). Moreover, a healthy human exhales and inhales more than a thousand liters of air each day. Alveoli are polyhedral, thin walled sacs, encircled by a rich network of capillaries which facilitate gas exchange. Capillaries are separated from the alveoli by

the presence of a thin walled blood air barrier formed by the fused basal lamina of type I alveolar epithelial (AE) cells and the endothelial cells of the capillaries. Alveolar structure is sustained by a mesh of collagenous and elastic fibers. Alveolar development and structural integrity of the lung is required for the proper functioning of the lung.

1.0.2. Lung Cells

There are 40 different types of lung cells. Among these the major cell types include fibroblasts, type I AE cells, type II AE cells and macrophages.

1.0.3. Lung Fibroblasts

1.0.3.1 Morphology of Lung Fibroblast

Fibroblasts are flat spindle-shaped elongated cells with long cytoplasmic processes and a large oval nucleus. The cytoplasm contains numerous rough endoplasmic reticulum, a well developed Golgi complex, and abundant mitochondria. Fibroblasts originate from the mesenchymal connective tissue. Fibroblasts are the dominant cell type found in the interstitial space in the walls of the alveoli. Fibroblasts account for 52% to 62% of the total interstitial cell population (Pinkerton et al., 1982). It has been well established that fibroblasts are heterogeneous depending on the phenotype and functions.

1.0.3.2. Functional Properties of Lung Fibroblast

The principal role of fibroblasts is to maintain the integrity of alveolar structure by the synthesis, secretion, maintenance, degradation and remodeling of extracellular

matrix (ECM) (McGowan and Torday, 1997). Furthermore, fibroblasts have been found to play an essential role in the immune mechanism of the body, through their responses to cytokines and through antigen presentation properties (Fries et al., 1994). Fibroblasts secrete key constituents of ECM including collagen, proteoglycans and fibronectin (Bradley et al., 1980) along with important biochemical mediators like growth factors and proteases.

Collagen is the major protein constituent of ECM. Primarily, type I collagen fibers (thick, cross-banded fibers) and type III collagen fibers (thin, randomly discrete) are secreted by lung fibroblasts. Type I collagen provides tensile strength and type III collagen contributes to the lung compliance. Fibroblasts are capable of synthesizing enzymes like collagenase, which can destroy the collagen it produces by breaking the peptide bonds in collagen (Pardo et al., 1992). Furthermore, fibroblasts are able to take up the degraded collagen fibers by phagocytic mechanisms to sustain the ECM.

Proteoglycans are large macromolecules which consist of a protein core with glycosaminoglycans (GAGS) attached in a brush like fashion. Major types of GAGS found in the alveolar wall are heparin sulfate, chondroitin sulfate and dermatan sulfate. GAGS act as molecular sponges as they are highly negatively charged molecules and hold water in the extracellular matrix providing rigidity and structural integrity to cells in the interstitial space. Fibronectin, is a glycoprotein involved in tissue repair and injury. Furthermore, fibronectin connects cells with collagen fibers in the ECM, facilitating cell movement. During lung development fibroblasts

contribute to the synthesis of pulmonary surfactant by secretion of fibroblast pneumonocyte factor (FPF) (Smith and Post, 1989). FPF is regulated by glucocorticoids and it accelerates lung maturation *in vivo* (Floros et al., 1985). Other functions of fibroblasts include protection of the alveolar structures by an antioxidant defense mechanism involving production of an enzyme superoxide dismutase. This enzyme helps prevent oxidative damage by conversion of superoxide radicals to hydrogen peroxide.

1.0.4. Two Types of Lung Fibroblasts

It is well documented that fibroblasts are not static cells, but rather are heterogeneous in nature (Fries et al., 1994; Smith et al., 1995). Fibroblasts differ in phenotype and function within and between tissues (Phipps et al., 1997). Lung fibroblasts differ from those found in the dermis depending on morphology, proliferative rates, ECM components and synthesis of cytokines (Fries et al., 1994). Two prominent subtypes of fibroblasts have been found in lungs, on the basis of presence or absence of lipid droplets called lipid interstitial cells (LICs) and non-lipid interstitial cells (NLICs) (Kaplan et al., 1985). During alveolarization, LICs and NLICs undergo cell division and are independent cells; they are not precursors of each other (McGowan and Torday, 1997). LICs are rich in lipid content and during lung development these cells are involved in synthesis of vital proteins of ECM, collagen and elastin. Furthermore, LIC contribute in the synthesis of surfactant as an accessory cell to the type II AE cells (McGowan and Torday, 1997). LICs decrease in number as the lung matures (Brody and Kaplan, 1983). LICs are also present in adults but are in fewer numbers when compared to fetal lungs. LICs contain

microfilaments and can be converted to a third subtype of fibroblast, the myofibroblast. LICs get transdifferentiated to myofibroblasts during lung development in cases of injury due to hyperoxia (Rehan and Torday, 2003).

1.0.5. Alveolar Epithelial Cells

The pulmonary alveolar epithelial wall covers ~99% of the total internal surface area of the lung (Wang et al., 2007) and it is lined by two main types of cells, type I AE cells and type II AE cells.

1.0.6. Type I Alveolar Epithelial Cells

Type I AE cells cover ~95% of the alveolar surface of peripheral lung. Type I AE cells are squamous epithelial cells and are also referred to as membranous pneumocytes. Type I AE cells are highly attenuated cells which facilitate exchange of oxygen and carbon dioxide, due to their tight intercellular junctions and their attachment to capillary endothelial cells by means of a thin basement membrane, as part of the blood-air barrier. Blood-air barrier has a mean thickness of only 0.6μm (Ochs, 2006). Type I AE cells are terminally differentiated cells (they do not possess the ability to divide). During lung injury, development and replacement of type I AE cells occurs by the de-differentiation of type II AE cells (Kauffman, 1980).

1.0.7. Type II Alveolar Epithelial Cells

1.0.7.1. Morphology of Type II Alveolar Epithelial Cells

Type II AE cells also referred to as granular pneumocytes, great alveolar cells, and type II pneumocytes, are multifunctional cells of the alveolar epithelium. The type II AE cells cover only ~5% of the total alveolar epithelial surface comprising 16% of the total lung cell population (Wang et al., 2007). They are cuboidal in shape interspersed between type I AE cells. Type II AE cells are characterized morphologically by the presence of large intracellular concentric membrane bound storage units of surfactant, known as lamellar bodies (LB). According to recent studies, in human lung, one type II AE cell contains 200 – 500 LB (Ochs, 2006). From type II AE cells LB are secreted into the alveoli at the air-liquid interface by means of exocytosis.

1.0.7.2. Functions of Type II Alveolar Epithelial Cells

It is well documented that the most important function of type II AE cells is synthesis, storage and secretion of surfactant. Furthermore, there is substantial evidence that type II AE cells are involved in the re-epithelialization of the alveolar wall after injury (Isakson et al., 2001). They also function in a defense role (Kannan et al., 2009) and transport sodium and fluid from the alveolar space into the interstitium to keep the alveolar space moderately free of fluid. Type II AE cells are thought to be progenitor cells of type I AE cells during development (Bishop, 2004) and injury (Borok and Crandall, 2009) of the lung.

INTRODUCTION: PART TWO

PULMONARY SURFACTANT

1.1. Pulmonary Surfactant

Surfactant is a surface-active material which prevents the collapse of the alveoli or atelectasis at the end of expiration by reducing surface tension. Surfactant is essential for normal biophysical and immunomodulatory functions of the lung. It is the first site of defense (Wright, 2003) against inhaled components of air such as cigarette smoke (CS). A shortage of pulmonary surfactant caused due to premature birth, lung injury or mutations in genes important for surfactant production causes respiratory failure. Insufficient amounts of surfactant leads to collapse of alveoli and is implicated in many pathological conditions like neonatal respiratory distress syndrome (RDS), caused due to a deficient amount of surfactant at birth. RDS is one of the leading causes of death in prematurely born infants.

1.1.1. Composition of Surfactant

Surfactant is a macromolecular complex composed of a mixture of phospholipids and proteins, with 90% lipid and 10% protein portions.

1.1.2. Surfactant Phospholipids

The surfactant phospholipids consist of 80% phosphatidylcholine, which is the most abundant component of surfactant, of which 40-50% occurs in a saturated form known as dipalmitoylphosphatidylcholine (DPPC). DPPC is primarily responsible for lowering

surface tension (Veldhuizen and Haagsman, 2000), although it exists in a gel state at body temperature. The rest of the phospholipids consist of phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine and other lipids. Cholesterol forms the majority of the natural lipids found in surfactant. PG is a glycerophospholipid, which is an indicator of the presence of surfactant. As the lungs mature levels of pulmonary surfactant increase. The rise in PG levels is used as an important marker of fetal pulmonary maturation. In surfactant from premature infants and rabbits with respiratory distress syndrome, levels of PG are absent from the surfactant (Bradford, 1976).

1.1.3. Surfactant Proteins

The protein portion of surfactant includes four major proteins, surfactant protein (SP)-A, SP-B, SP-C, and SP–D (Fehrenbach, 2001). SP-A is the most abundant of all the surfactant proteins. SP-A and SP-D are hydrophilic calcium-dependent proteins secreted by type II AE cells and bronchial epithelial non-ciliated Clara cells (Crouch, 2000). They have an important role in the body's innate host defense mechanism against microorganisms and pathogens. Furthermore, SP-A and SP-D are considered to have important roles in the recycling of surfactant lipids (Johansson and Curstedt, 1997). SP-B and SP-C, are low molecular weight proteins which are hydrophobic in nature (Korfhagen et al., 1998) and can easily associate with phospholipids of surfactant. SP-B and SP-C contribute to the formation, recycling and spreading of surfactant monolayer at air-liquid interface (Weaver and Conkright, 2001). SP-B is expressed in type II AE cells and non-ciliated Clara cells of the bronchial epithelium. Unlike SP-B, SP-A, and SP-D, SP-C is the only surfactant protein expressed exclusively in the lungs. SP-B is believed to

play an important role in tubular myelin formation (Ballard et al., 1995). Hereditary deficiency of SP-B during developmental stages of lungs can lead to RDS in preterm infants (Clements and Avery, 1998). SP-B gene knockout mice is lethal (Weaver and Conkright, 2001).

INTRODUCTION: PART THREE

LUNG DEVELOPMENT

1.2.0. Lung Development

Growth and maturation of the respiratory system occurs from proximal to peripheral segments. In humans development of the lung starts during the fifth week of gestation (Selman and Pardo, 2006). The developmental stages can be divided into 5 stages namely, embryonic, pseudoglandular, canalicular, saccular stage, and the alveolar period. Lung morphogenesis in humans is a very gradual and continuous process; there may be an overlap in the different stages of development. The information we have today regarding lung development is due to a vast number of studies done on rodents, especially mice. Development in humans and mice is similar in many aspects with two major exceptions, time frame of development and lobar formation. The development process in humans is much slower than in mice. Human lungs are divided into five lobes, two from the left bronchus and three from the right, whereas, mice have only one lobe from left primary bronchus and four lobes from right bronchus.

1.2.1. Embryonic Stage (4 - 6 weeks of gestation)

The human lung originates from the primordial foregut as a ventral diverticulum during the fourth week of embryonic life. The diverticulum elongates, grows caudally, and bifurcates into the left and right lung buds. The lung buds elongate in a posterio-ventral direction into the splanchnic mesenchyme, for further branching. The left lobe branches into two bronchi and the right lobe branches into three bronchi. Bronchi undergo further

dichotomous branching, in order to expand the major airways within each lobe of the lung. The embryonic stage is characterized by the formation of the lung buds, trachea, and primary bronchi. The primitive lung bud is lined by epithelium which is derived from the endoderm (Laudy and Wladimiroff, 2000).

1.2.2. Pseudoglandular Stage (6 - 16th week of gestation)

During this stage, developing lungs resemble a gland, therefore the name pseudoglandular. This stage involves repeated branching of the bronchi into the surrounding splanchnic mesenchyme until tracheobronchial tree formation takes place and is completed with the formation of conducting zone of respiratory system. After 16 weeks, growth of these branches takes place but further branching does not take place. Apoptosis, programmed cell death occurs all through embryonic development of lung, with its peak during pseudoglandular stage for proper branching and formation of tracheobronchal tree largely in mesenchymal cells. Further, another peak of apoptosis seems to occur during the saccular stage (Wongtrakool and Roman, 2008). Apoptosis is a form of cell death which is important for proper homeostasis and development during organogenesis. Towards the end of pseudoglandular stage, the process of cellular differentiation takes place; all the major elements of lungs are formed but respiration is not possible.

1.2.3. Canalicular Stage (16 - 28th week of gestation)

During this stage development of functional elements which assist in gas exchange takes place. The canalicular stage is marked by the growth of respiratory epithelium, formation

of respiratory bronchioles, formation of respiratory acini by alveolization, formation of a thin blood-air barrier and initiation of surfactant synthesis (McGowan and Torday, 1997). Moreover, there is decrease of mesenchyme and increase of vascular bed formation. Towards the 22 and 23 weeks of gestation epithelial differentiation from cuboidal type II AE cells to squamous type I AE cells takes place (Laudy and Wladimiroff, 2000). Concomitantly, the capillaries come in close approximation of type I AE cells forming the blood air barrier. Human fetuses born around 24 weeks can survive but the possibility of survival is low.

1.2.4. Saccular/Terminal Stage (28 - 36th week of gestation)

Extensive growth and maturation of the gas exchange surface of the lung takes place during this stage. Massive widening of airspaces causes an increase in the surface area for future exchange of gases. The widening of airspaces leads to thinning of the epithelium lining the alveoli or saccules which in turn leads to decrease in the mesenchymal tissue and thinning of interstitial space surrounding the saccules. Further cellular differentiation takes place with simultaneous development of pulmonary vasculature. Type II AE cells mature, formation of surfactant starts and elastic fibers are laid down in the interstitium for structural support. Adequate amount of surfactant is critical for the survival of a premature infant (Gortner, 1992).

1.2.5. Alveolarization- Postnatal Lung Development

Although alveolarization starts during fetal life, the process of alveolar formation continues on until 8 years after birth with its peak during the first two years (Laudy and

Wladimiroff, 2000). At birth only 50% of alveoli are present (Hislop et al., 1986). During postnatal development there is an increase in the number of alveoli but not the size of alveoli (Hyde et al., 2007). Alveoli are formed by sepation (subdivision) (Massaro and Massaro, 2000) during this stage, which results in smaller and more numerous alveoli with an eventual increase in the surface area for gas exchange. Moreover, during this stage, thinning of alveolar walls, increase in quantity of surfactant and further development of vasculature takes place until lung reaches its complete maturation. Some evidence suggests that exposure to cigarette smoke during developmental stages influences lung function and adversely affects respiratory health during childhood and persists into adulthood (Moshammer et al., 2006).

INTRODUCTION: PART FOUR

MATERNAL SMOKING AND DEVELOPMENTAL PULMONARY TOXICITY

1.3.0. Maternal Smoking and Developmental Pulmonary Toxicity

Exposure to tobacco smoke during prenatal life is the leading cause of preventable premature death in the world. Dose dependent exposure of tobacco smoke and second-hand smoke *in utero* has been associated with neonatal mortality, growth retardation (Roquer et al., 1995), low birth weight, sudden infant death syndrome (SIDS), preterm delivery and higher incidence of stillbirth (DiFranza et al., 2004). Cigarette smoking during pregnancy is a major source of prenatal exposure to harmful agents in tobacco smoke to the developing fetus. Despite the consequences, 30-40% of women smoke during pregnancy worldwide (Stocks and Dezateux, 2003). Exposure to the toxic substances in smoke is detrimental to the developing fetus (Table-1). Exposure to more than 4000 chemicals in tobacco smoke can be either due to direct exposure or by passive exposure to second-hand cigarette smoking. The fetus grows with an active trans-placental communication system with the mother. The placenta is a feto-maternal organ which is responsible for nutrient transfer and gas exchange between the mother and fetus. Prenatal exposure to CS is a concern because the immature lung is vulnerable to toxic substances in cigarette smoke. The immature lung is susceptible to toxic substances in CS primarily because of the fewer number of macrophages leading to a poor immune response (Naik et al., 2001) and also due to a decrease in surfactant quantity, which is considered as the first line of defense against pathogens. Furthermore, developmental

pulmonary toxicity due to CS exposure can lead to many morphological, biochemical and functional changes. Fetal pulmonary outcomes due to maternal smoking may lead to

Still births	(DiFranza et al., 2004).
Higher incidence of spontaneous abortions	(Haustein, 1999)
Low birth weight	(Dejmek et al., 2002)
Premature delivery	(Scott, 2004)
Increased lower respiratory tract illnesses in childhood	(Jedrychowski and Flak, 1996)
Sudden infant death syndrome	(Schoendorf and Kiely, 1992)
Fetal growth retardation	(Jacobson et al., 1994)
Placenta previa	(Handler et al., 1994)
Impairment of infant breathing	(Scott, 2004)
Early childhood bronchitis	(Yarnell and St Leger, 1979)
Increased prevalence of dental caries in childhood	(Tanaka et al., 2009)
Increased incidence of developing asthma in childhood	(Pattenden et al., 2006)
Impairs alveolarization during embryogenesis	(Bruin et al.)
Postnatal behavioral, psychiatric, and cognitive outcomes	(Cornelius and Day, 2009)
Impairment of pulmonary function	(Habek et al., 2002a)
Fetal hypoxia	(Bulterys et al., 1990)
Retardation hypoplasia	(Collins et al., 1985)

Table 1: Effects of fetal exposure to cigarette smoke through maternal smoking.

impairment of pulmonary function, reduction of fetal breathing movements, increased airway responsiveness and hypoxia (Habek et al., 2002a). Additionally, *in utero* exposure of rat pups to CS during lung development resulted in mesenchymal disorientation and

pulmonary cell apoptosis (Scott, 2004) leading to hypoplasia, with a significant decrease in alveolization, suggestive of a decrease in function of the surfactant system. It has been reported that maternal smoking of more than 20 cigarettes per day is associated with high risk for fetal hypoxia (Habek et al., 2002a). Studies in the past relate chronic fetal smoke exposure as a major predisposing factor for SIDS (Habek et al., 2002b). Studies also suggest that fetal growth and development is significantly reduced under hypoxic conditions (Gortner et al., 2005). Fetal respiration starts during the fifteenth week of gestation (Marchal and Droulle, 1988). At birth, the lungs must be sufficiently mature to quickly establish the vital physiological function of respiration. Epidemiologic and experimental studies confirm that smoking during pregnancy has detrimental long term effects during postnatal lung maturation and overall respiratory health with an increased predilection to development of asthma and bronchitis during childhood. These effects can be exacerbated by parental smoking during infancy (Jaakkola and Gissler, 2004). Many studies suggest that nicotine which is considered as the pharmacologically active ingredient in tobacco smoke may be responsible for the alterations of lung cells during development (Rehan et al., 2009). Nicotine has very low molecular weight and high lipid solubility enabling it to freely cross the placental barrier (Pastrakuljic et al., 1998) with minimal biotransformation to its metabolite, cotinine (Rehan et al., 2009). Previous studies have reported that immediately after five minutes of smoke exposure there was a decrease in the fetal breathing movements which persisted for 90 minutes after the start of smoking by the mother (Stocks and Dezateux, 2003).

1.3.1. Maternal Smoking and Routes of Fetal Exposure

Human fetal growth and development involves continuous growth and differentiation of organs and is highly influenced by the intrauterine environment (Strauss, 1997). The exposure of tobacco constituents during fetal life may compromise lung development. Although, the mechanisms by which tobacco causes adverse effects are not clearly elucidated it is widely accepted that inadequate blood flow to the fetus or utero-placental insufficiency may lead to compromised fetal growth. The outcome of fetal development depends on the mother for adequate supply of oxygen, nutrients and elimination of waste and toxic agents. Nutrients from the mother to the fetus are actively transported through the placenta. The placenta is the integration between mother and embryo. Chemicals with low molecular weight like nicotine can freely cross the placental barriers. Furthermore, cotinine, (a major metabolite of nicotine), cadmium (a heavy metal found in CS), and benzo-*a*-pyrene (a polyaromatic hydrocarbon in cigarette smoke) have been found in the follicular fluid of mothers who smoke (Talbot and Riveles, 2005). The adverse fetal effects of smoking during pregnancy may potentially be attributable to the exposure of the fetus to components of CS via two routes of exposure, through circulation and amniotic fluid. Carbon monoxide has higher affinity for fetal hemoglobin leading to increased content of carboxyhemoglobin in fetus than in the mother. Respiration during fetal life is considerably different and more complex when compared to the extra-uterine air-breathing pattern (Dawes, 1984). Observations of fetal breathing movements are possible through ultrasound fetal imaging. The fetus is submerged in amniotic fluid which is inhaled and exhaled by the fetus. In spite of increased clearance of nicotine during pregnancy, nicotine accumulates in the amniotic fluid, maternal milk, and fetal

tissues (Klein et al., 2004). Accumulation of nicotine is an important consideration for developmental toxicity in the fetus resulting in a disturbance of the normal molecular/cellular processes. Nicotine was detected in both maternal and fetal circulations, with fetal concentration equal to or higher than maternal concentrations of nicotine. It has been suggested that nicotine, a potent vasoconstrictor could be responsible for the decrease in fetal breathing movements (Stocks and Dezateux, 2003), due to decrease in blood flow, oxygen and nutrients. Reduction in smoking at any time during pregnancy resulted in an improvement in birth weight.

INTRODUCTION: PART FIVE

LUNG CANCER

1.4.0. Lung Cancer

Epidemiologic studies substantiate that cigarette smoking is the leading cause for lung cancer development. The risk involved in lung cancer development due to cigarette smoking depends on the number of cigarettes smoked and the duration of smoking. Despite increasing information and resources available to reduce tobacco use, lung cancer remains the leading cause of death worldwide. The lag period between smoking and progression to lung cancer is 20-30 years (Cristiano, 1997). Moreover, passive smoking, other environmental and occupational factors contribute to the pathogenesis of lung cancer. Extensive studies have been done, yet the mechanism of cancer development with exposure to tobacco smoke is still unclear. Tobacco smoke contains 55 carcinogens, of which 20 have been proven to induce lung cancer (Hecht, 1999). Carcinogens from tobacco smoke can enter the body either directly by smoking or indirectly by secondhand smoke. The intricate heterogeneity of normal lung cells gives rise to different types of cancers. Clinically, lung cancer can be classified into two distinct types, small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are subtypes of NSCLC. Adenocarcinoma is the most common type of lung cancer worldwide, accounting for 40% of all lung cancers. Adenocarcinoma is a slow growing tumor which originates from peripheral epithelial lung cells. Adenocarcinoma affects women (smokers and non-

smokers) more than men (Charloux et al., 1997), the reason may be due to higher susceptibility of women to carcinogens in cigarette smoke (Zang and Wynder, 1996).

1.4.1. Human A549 Cells

A549 cells are human lung adenocarcinoma cells, which are considered as a neoplastic transformation of type II AE cells (Lieber et al., 1976). A549 cells are flat morphologically and contain multi-lamellar bodies similar to type II AE cells. Two types of multi-lamellar bodies are found in A549 cells, one with loosely arranged concentric lamellae and a second type with tightly arranged lamellae (Lieber et al., 1976). Lamellar bodies are storage units of surfactant. Although A549 cells mimic type II AE cells with inclusion of lamellar bodies, they differ in the lipid composition. A549 cells have lipid composition similar to fibroblasts. Moreover, the phospholipid composition of disaturated phosphatidylcholine is much less in A549 cells compared to type II AE cells (Spragg and Li, 2000). The doubling time for A549 cells is 17.6 ± 0.5 hours (Sugimoto et al., 1990).

INTRODUCTION: PART SIX

PERIODONTAL LIGAMENT

1.5 Periodontal ligament

The alveolo-dental ligament, more commonly known as periodontal ligament (PDL), is a physically small but functionally important tissue which is primarily responsible for tooth support. It is a specialized thin, highly vascular, fibrous connective tissue which occupies the periodontal space between root of a tooth and the alveolar bone. The periodontium consist of tooth supporting structures including the alveolar bone proper, cementum and periodontal ligament. PDL forms a fibrous joint or gomphosis between the tooth and alveolar bone. It has been reported that the periodontal space is hour-glass shaped, with its widest portion at the cervical end of root and the narrowest portion in the middle third of the root near the fulcrum, the point at which the tooth moves. The width of PDL varies depending on age and functional state of tissue. Since, the PDL ligament is located in close proximity to the alveolar bone; it is always under high occlusal load of masticatory forces with a constant formation and remodeling of the alveolar bone (Lekic et al., 2001), as a functional response. The average thickness of PDL is 1.75 ± 0.24mm (Alpiste-Illueca, 2004). This thickness is constantly maintained throughout adult life, which is an important feature of PDL homeostasis (McCulloch and Melcher, 1983). PDL has been referred to as specialized connective tissue as it consists of soft connective tissue portion and a mineralized connective tissue portion (Lekic et al., 2001), it is well innervated, highly vascular with a high turnover rate (Beertsen et al., 1997). In health, PDL has a dense network of interconnecting collagen fibers which are oriented in the forms of

bundles (crestal, horizontal, oblique, apical, and interradicular) in different parts of the periodontal ligament space. Disease or damage to the PDL or any of the components of PDL results in the loss of fibrous attachment, leading to increased tooth mobility and eventually tooth loss. The average thickness of collagen fibers is thicker in adult humans than those in rats (Beertsen et al., 1997). Functionally, PDL is primarily responsible for resisting displacing forces on the tooth and anchorage of tooth to alveolar bone, allowing limited movement of the tooth within its socket. Additionally, PDL is supportive, sensory, homeostatic and nutritive in nature. Compelling evidence from clinical and epidemiological studies reported a strong association between habitual cigarette smoking and increased incidence of periodontal disease with a 2.7 times higher risk of developing periodontal disease than non-smokers (Calsina et al., 2002). The severity of periodontal disease associated with cigarette smoking depends on the number of cigarettes smoked and the time period of the habit (Bergstrom et al., 2000), (Calsina et al., 2002). Moreover, tobacco use in any from has an overwhelming effect on the oral cavity ranging from discoloration of oral mucosa to oral carcinomas. The biological mechanism behind cigarette smoke-induced destruction of the tooth supporting structures is not clear. Although, available studies unanimously agree that there is a two to six fold increase in the risk for development of periodontal disease in smokers compared to non-smokers (Kinane and Chestnutt, 2000). Furthermore, exposure to cigarette smoke in a dose dependent manner causes alveolar bone loss, pocket formation, attachment loss and eventually tooth loss (Johnson and Slach, 2001). CS has a negative impact on the normal healing process of the periodontium and causes a decrease in the immune response. Furthermore, the positive response to surgical, non-surgical and regenerative periodontal

therapy is reduced with an increased chance of recurrence of periodontitis in people who smoke (Tonetti, 1998). Tobacco smoke consists of many poorly defined components. Locally, smoking causes a decrease in gingival blood flow with a decrease in oxygen reaching gingival and a decrease in cellular number resulting in the overall decrease in tooth support. Nicotine, a major alkaloid in tobacco is known to cause vasoconstriction and it induces oxidative stress, which is speculated as contributing factors in the development of periodontitis. Reactive oxygen species (ROS) contributes to the activation of apoptosis (Wang et al., 2000). Previous molecular *in vitro* studies in human PDL fibroblasts suggest that nicotine was detected on the root surface of a periodontally involved tooth (Chang et al., 2002b). Salivary nicotine and cotinine levels are widely used as an indicator for total nicotine intake in the body (Rose et al., 1993). At the cellular level, nicotine along with other toxic substances in tobacco smoke inhibit attachment of periodontal ligament fibroblasts (James et al., 1999). Periodontal health is adversely affected by smoking, smoking cessation helps normalize the periodontal condition by increasing blood flow with an increase in gingival crevicular fluid content (Bergstrom, 2004).

1.5.1. Embryology of the Periodontal Ligament

Odontogenesis or tooth development involves a complex process where development of hard and soft tissues takes place and eventually teeth erupt in the oral cavity. During fetal life, development of primary teeth is initiated between six and eight weeks and permanent teeth development starts during the twentieth week. Microscopically, one of the earliest changes during tooth development includes distinction between the vestibular lamina and

the dental lamina. PDL is derived from the ecto-mesenchymal tissue of the dental follicle (inner layer) just after root development begins (Cho and Garant, 2000).

1.5.2. Cells of the Periodontal Ligament

Electron microscopic studies reveal that normal and healthy PDL is a highly cellular with fibroblasts as the predominant cell type. Other cell types in PDL include cementoblasts, osteoblasts, osteoclasts, odontoclasts, epithelial cell rests of Malassez, and defense cells including macrophages, mast cells and eosinophills. Cementoblasts line the root surface of the tooth and are responsible for secreting organic matrix of the cementum. Osteoblasts and osteoclasts line the surface of alveolar bone responsible for bone formation and resorption respectively.

1.5.3. Periodontal Ligament Fibroblasts

Fibroblasts are the dominant cell type found in the periodontium which originate from the ecto mesnchyme of the investing layer of the dental papilla (Beertsen et al., 1997). Morphologically, the fibroblasts are spindle-shape with a large nucleus and contain abundant cytoplasmic microtubules which contribute to the generation of eruptive forces during eruption of teeth. In addition, electron microscopic studies suggest the presence of large numbers of mitochondria, rough endoplasmic reticulum and Golgi complexes (Hassell, 1993). Fibroblasts are ubiquitous with significant functional properties. They have a major role in maintenance of the structural integrity of PDL during wound healing by synthesis of extracellular matrix. Additionally, PDL fibroblasts undergo rapid tissue remodeling and repair in response to injury (McCulloch, 1995). Fibroblasts construct a

connective tissue lattice which firmly anchors the tooth to the alveolar bone. The attachment fibers secreted by fibroblasts are composed of collagen (type I and III), which is responsible for the rapid turnover rate of the fibers or the regeneration of the PDL. Previous studies suggest that PDL fibroblasts secrete matrix metalloproteinases and collagenases which have the ability to degrade the extra cellular matrix (Hassell, 1993), thereby maintaining a dynamic equilibrium or homeostasis. Renewal process of PDL involves proliferation of PDL cells and stimulation of periodontal attachment of tooth to bone. PDL fibroblasts possess the ability of self renewal where the balance in the number of cell death and formation is maintained (McCulloch, 1995). Fibroblasts from different regions of the periodontium are heterogenous. Fibroblasts from gingival tissue differ from the fibroblasts found in the PDL. Differentiating factors in PDL include presence of glycogen pools, contractile microfilaments and distribution of GAGS, all these factors are absent in gingival fibroblasts (Phipps et al., 1997). GAGS are important components of connective tissue. PDL fibroblasts are different from other connective tissues of the body because of the alkaline phosphatase (ALP) activity (Beertsen et al., 1997). ALP is an enzyme which has an important role in the formation and calcification of hard tissues like cementum and alveolar bone. ALP enzyme activity and expression has widely been used in research as a marker for osteoblastic activity (Murakami et al., 2003).

PDL is constantly subjected to high amplitude of physical forces including forces of mastication. Fibroblasts play a pivotal role in maintaining homeostasis in the alveolar component and carry out an important function of synthesis and degradation of collagen fibers. Chronic exposure to CS may cause destructive processes of the periodontium. It

has been reported that smoking is a potential risk factor for loss of teeth, people who smoked 5 to 14 cigarettes per day were two times more likely to loose teeth compared to a non-smoker (Dietrich et al., 2007).

INTRODUCTION: PART SEVEN

APOPTOSIS

1.6. Apoptosis

Some common tobacco- induced diseases like interstitial pulmonary fibrosis (Drakopanagiotakis et al., 2008), acute respiratory distress syndrome (Li et al., 2004), chronic obstructive pulmonary disease (Hylkema et al., 2007), pulmonary emphysema (Kasahara et al., 2000), lung cancer (Hecht, 2002), periodontitis (Kibayashi et al., 2007), cardiovascular disease (Knight-Lozano et al., 2002), cerebrovascular diseases (Anbarasi et al., 2006) and peripheral vascular disease (Dieter et al., 2002) have been associated with pathological alterations due to cell injury and apoptosis. Additionally, chronic obstructive pulmonary disease (COPD) is the fourth leading cause of morbidity and mortality in North America (Yoshida and Tuder, 2007). The pathogenesis of COPD involves a number of pathogenic processes including abnormal inflammatory response to noxious particles and gases, variation in cell growth, apoptosis and abnormal response to cell repair (Macnee, 2007). During mild damage to cells, a normal cell repair process takes place whereas severe damage to cells may lead to unaltered cellular damage eventually contributing to disease progression (Kuwano, 2007). Furthermore, apoptosis has an important role in maintaining an inflammatory balance for host defense against pathogens and toxic substances (Krammer et al., 1994). Trans-placental exposure of tobacco to fetus may be responsible for restriction in growth and low birth weight (Dejmek et al., 2002), suggesting a decrease in cell proliferation. Furthermore, CS induced apoptosis in human umbilical venous endothelial cells (Wang et al., 2001) and

alveolar macrophages (Aoshiba et al., 2000). Apoptosis is a methodical physiologic form of cell death involving the elimination of cells. The apoptotic cells become activated by signals which converge to trigger a series of biochemical and morphological changes inside the cell leading to its death. Apoptosis is important for regulation of cell numbers during development and senescence. The term "apoptosis" was introduced by Kerr, Wyllie and Currie in 1972 (Degterev et al., 2003). Apoptosis is also known as programmed cell death as it involves genetically determined elimination of cells (Li et al., 2004). It is an irreversible, timely regulated form of cell death essential for proper maintenance of homeostasis, during embryogenesis and for functional regulation of immune system (Elmore, 2007). In contrast to apoptosis, necrosis is a passive, uncontrolled and energy independent form of cell death. Necrosis is characterized by rapid cytoplasmic swelling, plasma membrane rupture and organelle breakdown. The release of cytoplasmic contents in the surrounding tissues due to necrosis, triggers chemotactic signals resulting in an inflammatory response. Necrosis is a caspase-independent process and there is absence of nuclear degradation (Hampton and Orrenius, 1997). Autophagy is another form of programmed cell death involving lysosomal degradation of cells own organelles during starvation (Kuma et al., 2004).

Electron microscopic studies have identified that developing lungs undergo dramatic tissue growth and remodeling to achieve a mature architectural structure and apoptosis plays an important role in establishing mature lung structure. Any defect in apoptotic processes during embryogenesis may lead to developmental abnormalities and damage to cells due to metabolic stress (Haanen and Vermes, 1996). Furthermore increase in

apoptosis and DNA fragmentation was observed in human lung fibroblasts exposed to CSE extract for 3 hours (Carnevali et al., 2003).

1.6.1 Characteristic Morphologic Changes in Cells Undergoing Apoptosis

Histologically, the apoptotic cells appear as round or oval mass of cells detached from their surroundings with eosinophilic cytoplasm and hematoxylin-stained nuclear fragments due to karyorrhexis. Following karyorrhexis excessive plasma membrane blebbing occurs which leads to formation of membrane-bound vesicles called apoptotic bodies. Apoptotic bodies contain cytoplasm with or without nuclear fragments and tightly packed organelles. The remnant apoptotic bodies are recognized and sequestered by neighboring cells or macrophages, resulting in a decrease in cell volume (Matute-Bello and Martin, 2003). The process of apoptosis is not associated with any inflammatory response as the cellular contents of dying cells are not released in the surrounding interstitial tissues. Furthermore, apoptosis is a complex, multi-step process which involves biochemical events including activation of intracellular proteolytic caspase cascade, which is important in the regulation and execution of apoptotic cell death. The knowledge of importance of caspases in apoptosis has been possible because of studies on knockout animals deficient of particular caspases confirmed profound defect in apoptosis (Kohler et al., 2002). Moreover, studies involving use of inhibitors of caspases which effectively inhibit apoptosis also help understand the important role of caspases activation in apoptosis (Ekert et al., 1999). Consequently the measurement of caspase activity is considered as a good method to assess apoptotic process (Kumar and Dorstyn, 2009).

1.6.2. The Caspase Family

Caspases are proteases which belong to the family of cysteine-aspartic acid endo-peptidases. In general, proteases are enzymes which participate in the breaking down of peptide bonds by hydrolysis. The critical role of caspases in apoptosis was first observed in nematode worm Caenorhabditis elegans (Boatright and Salvesen, 2003). Apoptosis is an important biological process during embryogenesis, homeostasis and immune response (Ashkenazi and Dixit, 1998). Almost all cells contain caspases in their inactive forms. The activation of caspases leads to irreversible biochemical and morphological changes in apoptotic cells. Detection of active caspases may be used as a major indicator for apoptosis. There are 14 different types of mammalian caspases (Figure 2), 11 are human and 3 are murine in origin (Grutter, 2000). Caspases are primarily localized in the cytoplasm and are synthesized as inactive enzyme precursors or zymogens (Nicholson and Thornberry, 1997). Recent studies report the existence of caspases in the mitochondrial intra-membrane space (pro-caspase-2, -3,-8 and -9), endoplasmic reticulum (pro-caspase-12) nucleus and Golgi apparatus (pro-capaspase-2) (Chandra and Tang, 2003).

Caspases are activated from their inactive state by proteolytic processing at aspartic (Asp) residues (Fuentes-Prior and Salvesen, 2004). Caspases have a very high specificity for cleavage after an Asp-X residue (Thornberry and Lazebnik, 1998). The distinctive specificity of caspases for Asp is the main reason for the highly selective proteolytic events which take place in the cytoplasm of the cell (Garcia-Calvo et al., 1999). In addition, caspases are classified as cysteine aspartate proteases as cysteine is required for

catalytic mechanism and also because they cleave after an Asp residue (Shi, 2002). Once caspases are activated they can cleave other caspases. Previous studies made it clear that in addition to their important role in regulation of apoptosis, caspases are also implicated in pro-cytokine activation for inflammation (Zhang et al., 2005).

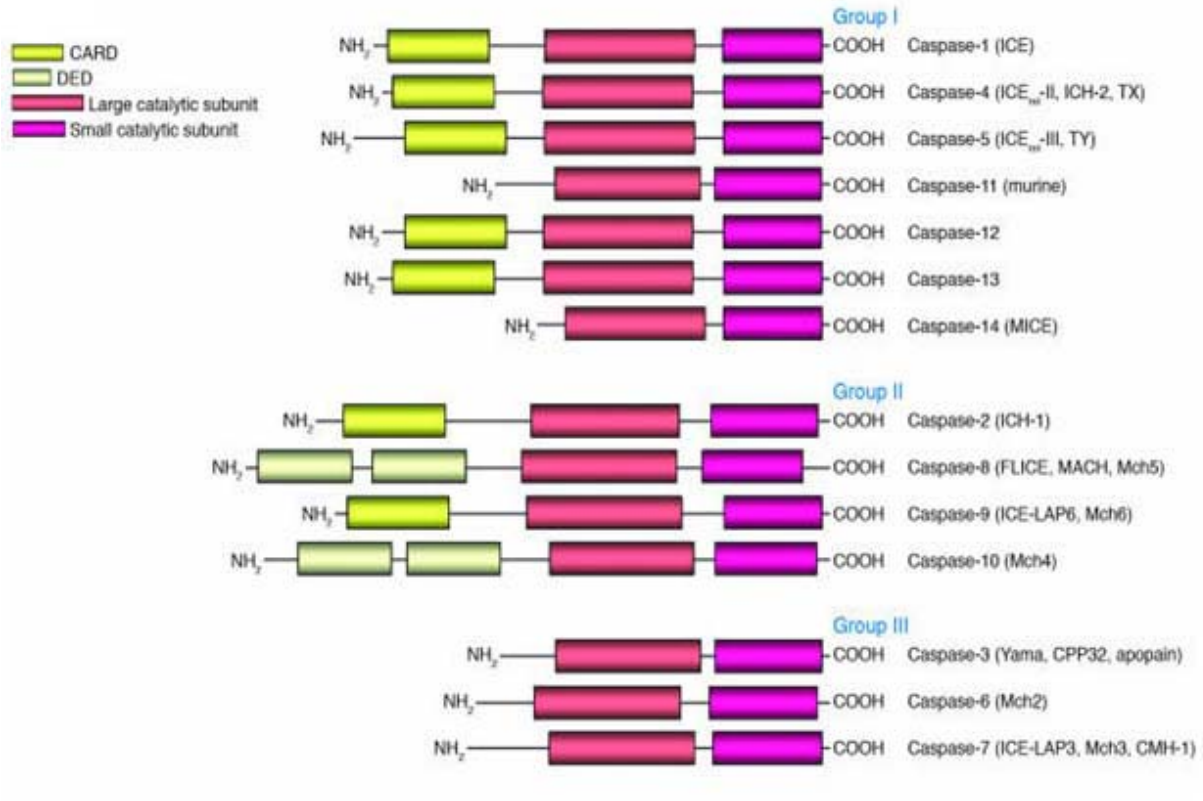


Figure 1: Schematic representation of the caspase family. There are 14 mammalian caspases which are broadly divided into three groups. Group I (caspase-1, -4, -5, -11, -12, -13, -14) are inflammatory caspases. Group II and group III are apoptotic caspases which are further subdivided as initiator (caspase-2, -8, -9, -10) effector caspases (caspase-3, -6 and -7). The colored portions represent different domains. (Lavrik et al., 2005)

1.6.3. Classification of Caspases

Caspases can be broadly classified into two groups (Figure 2) ; one which is thought to play a central role in apoptosis (caspases -2, -3, -6, -7, -8, -9, -10, and -12), which are further subdivided as initiator and effector/executioner caspases (Chowdhury et al., 2008). The second major group pf caspases are primarily involved in cytokine processing during inflammation (caspases -1, -4, and -5) (Fadeel et al., 2000). Caspases can also be classified into three sub-groups (group I, II, and III) based on the preference of a tetra-peptide sequence in the substrate (Kohler et al., 2002).

1.6.3.1. Initiator Caspases and Effector Caspases

Apoptotic caspases can be divided into initiator caspases and effector caspases (Figure 2), depending on the length of pro-domain and functional role in the initiation and execution of apoptosis. Initiator caspases (caspase -2, -8, -9 and -10) have large N-terminal pro-domains (>90 amino acids) and are thought to be involved in the initiation of the apoptotic process. By contrast, the effector caspases have shorter N-terminal pro-domains of 20-30 amino acids (caspase -3, -6, and -7) and are thought to amplify signals of the caspase cascade eventually leading to cell death (Shi, 2002).

The N- terminal pro-domains of initiator caspases include structural motifs which belong to the death domain superfamily comprising of proteins containing a death domain (DD), death effector domain (DED) and caspase-activating recruitment domain (CARD) (Lavrik et al., 2005). The mechanisms of activation of initiator caspases has been a point of research for a long time. But recent studies suggest that activation of initiator caspases

is by autolytic cleavage within a linker segment separating the large and small subunits due to an intrinsic proteolytic activity of the caspase zymogens (Salvesen and Riedl, 2008).

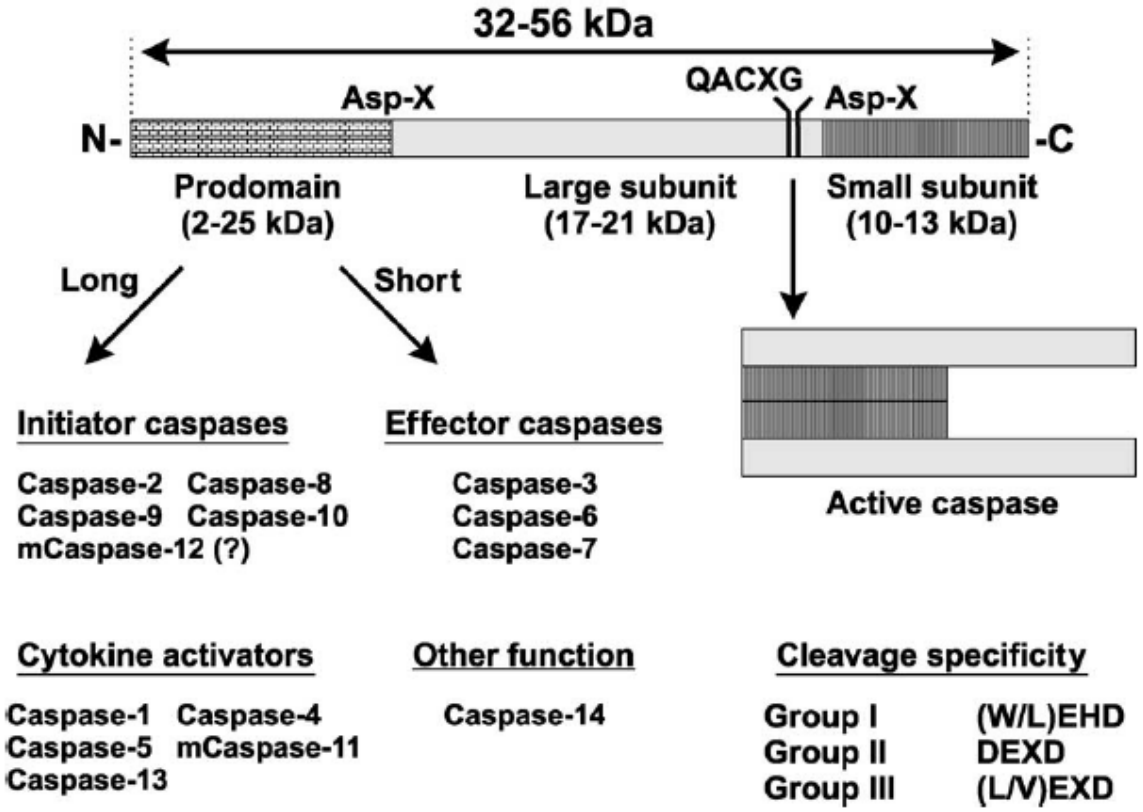


Figure 2: Structure and classification of caspases. Inactive precursor form of caspase zymogens is a single polypeptide chain made of 3 domains (32 kDa), amino-terminal pro-domain, a central large subunit (p20) (17 kDa) and a small subunit (p10) (12 kDa). The pro-domain is removed when caspases get activated. The active form of caspases is a tetramer with two large subunits and two small subunits. Apoptotic caspases can be divided into initiator and effector caspases depending on the length of the pro-domain. Caspases are also classified depending on the substrate specificity into three groups (group I, II and III) (Kohler et al., 2002).

Once activated the initiator caspases activate effector caspases (Degterev et al., 2003). The effector caspases lack the ability of self activation because of their small pro-domains. Activation of executioner caspases determines the fate of a cell. Activation of

initiator caspases does not always result in apoptosis, because members of anti-apoptotic family, Bcl-2 members can prevent the activation of effector caspases (Green and Kroemer, 1998).

Incidentally, caspase-2 has distinct properties compared to other initiator caspases (Degterev et al., 2003). Caspase 2 can act as both positive and negative regulators during cell death (Bergeron et al., 1998). Structurally, caspase 2 have long pro-domains, like initiator caspases and possess functional similarity to executioner caspases (Krumschnabel et al., 2009). Caspase -4, -5 and 13 share similarities to caspase -1 in cytokine activation but their exact role is unknown (Stennicke and Salvesen, 2000).

1.6.4. Caspase-3/ CPP 32/ YAMA/ Apopain

Caspase 3 also known as CPP32, YAMA or apopain is considered as one of the major executioners of apoptosis. It is the first of all the effector caspases to be activated for amplifying downstream apoptotic process. Caspase-3 can be activated through caspase-8 and caspase-9 by extrinsic or intrinsic signaling, respectively (Thornberry and Lazebnik, 1998). Suggesting, that apoptotic signal from either extrinsic or intrinsic pathways (two main apoptotic pathways) converge to the activation of caspase-3. Furthermore, activation of caspase-3 is a very rapid process in the apoptotic cell death process (Tyas et al., 2000) which may be associated with mitochondrial membrane permeabilization. Activated caspase-3 has been reported to contribute mainly to the characteristic morphologic changes in apoptotic cells including membrane blebbing, chromatin condensation and DNA fragmentation (Kohler et al., 2002). Knockout mice, lacking

caspase-3 gene die due to abnormal brain development (Kuida et al., 1996) and failed to show morphologic changes normally observed in cells dying due to apoptosis (Janicke et al., 1998).

1.6.4.1. Structure of Pro-Caspase-3

The understanding of the structure and substrate specificity of caspase helps in understanding the disease progression and in designing new therapies. Studies on caspase-3 demonstrated that the inactive precursor form of caspase zymogens is a single polypeptide chain made of 3 domains (32 kDa). The domains consist of a short amino-terminal pro-domain, followed by a central large subunit (p20) (17 kDa) and a small subunit (p10) (12 kDa) (Lavrik et al., 2005). All caspases have similar structure, caspase-1 and caspase-3 share high structural similarities (Nicholson and Thornberry, 1997) but, caspase-3 has a shorter pro-domain and the small linker sequence present in between the large and small sub-unit is absent in caspase-3 (Cohen, 1997). The catalytic diad of a cysteine group and histidine group is located on the large sub-unit.

Activation process involves a series of cleavage events. Caspases can be auto-activated or get activated by other caspases. The cleavage is mediated by a nucleophilic cysteine residue in the pent-peptide sequence of QACXG (Q-Glutamine, A-Alanine, C-Cystiene, X-unknown amino acid and G-Glysine) with an absolute requirement of aspartate at the P1 position (Thornberry and Lazebnik, 1998). The first cleavage site is between the pro-domain and large subunit, and the second cleavage site is between large and small subunit. On activation the pro-domains of pro-caspases are removed.

1.6.4.2. Structure of Active Caspase-3

It has been suggested that the active caspase-3 is a tetramer made of two large and two small sub-units (Crawford and Wells). Each tetramer has two active sites formed by both large and small sub-units which are located at opposite ends, and they function independently (Chang and Yang, 2000). The two hetero-dimers are closely associated with each other due to hydrophobic interactions between the p10 sub-units. Each heterodimer (formed by p20-p10 subunits) of the tetramer forms a single globular domain with six β-strands, five parallel and one anti parallel surrounded, by five α-helices (Donepudi and Grutter, 2002). In the active site cysteine, which is located in the p20 sub-unit is closely associated with imidazole ring of a histidine. Histidine attracts a hydrogen proton from cysteine leaving behind negatively charged sulfur ion, which in turn enhances the nucleophilic property of cysteine to attack the peptide bond.

1.6.5. Mechanisms of Caspase Regulation in Apoptosis

The mechanisms of apoptosis requires energy dependent cascade of molecular events. There are two main apoptotic pathways recognized in humans and mice, the extrinsic pathway and the intrinsic pathway (Fuentes-Prior and Salvesen, 2004) (Figure 3). Extrinsic pathway is also known as the death receptor pathway as it is initiated by extra-cellular signals which activates specific cell surface receptors (tumor-necrosis factor receptor, CD95 or Fas/Apo-1) (Tao et al., 2007). Activation of caspase-8 or -10 takes place via ligation of a trans-membrane death of tumor-necrosis factor (TNF). The TNF receptor family contains a death receptor proteins or death domains which will subsequently bind to adaptor proteins such as Fas-associated protein with death domain

(FADD). The adaptor proteins bind to pro-caspase-8 by homophilic interaction with the help of N-terminal DED. The death inducing signaling complex (DISC) is formed by the assembling of receptor, adaptor protein and pro-caspase-8. Caspase-8 (initiator caspase) gets activated within the DISC (Boatright and Salvesen, 2003). Once caspase-8 is activated it will activate effector caspases-3, -6 and -7 directly (Kumar, 2007) or by the cleavage of Bid, which is a pro-apoptotic member of the Bcl-2 family. Bcl-2 protein members may function as anti or pro-apoptotic regulators. Activation of Bid leads to the permeabilization of the mitochondrial membrane by Bax and Bak. The activation of Bax and Bak leads to the release of cytochrome-c.

Another major pathway of apoptosis is the intrinsic pathway or the mitochondrial pathway (Figure 3). This pathway involves non-receptor mediated intracellular signals initiated inside the cell in response to ionizing radiations, chemotherapeutic drugs (Boatright and Salvesen, 2003), stress and genotoxic damage. These signals act through p53 and other signals to activated BH-3 only proteins. BH-3 proteins belong to the pro-apoptotic members which inhibit the action of Bcl-2 members and promote cell death by the activation of Bax and Bac. With the activation of Bax and Bak the mitochondrion becomes selectively permeable and release of cytochrome c takes place. Cytochrome c binds to the apoptotic protease activating factor-1 (Apaf-1) to form apoptosome, which is a large multi-protein complex. It is the central component of intrinsic pathway because it recruits pro-caspase-9 and activates it via N-terminal CARD. According to electron microscopic studies apoptosome is shaped like a wheel with spikes and a central portion containing CARD which is responsible for activation of initiator caspase-9 (Boatright and

Salvesen, 2003). Active caspase-9 activates executioner caspases -3 and -7 which then further cleave other protein substrates and finally cell death takes place. Smac/DIABLO, are mitochondrial proteins which promote activation of caspases by inhibition of IAP (inhibitory apoptotic proteins).

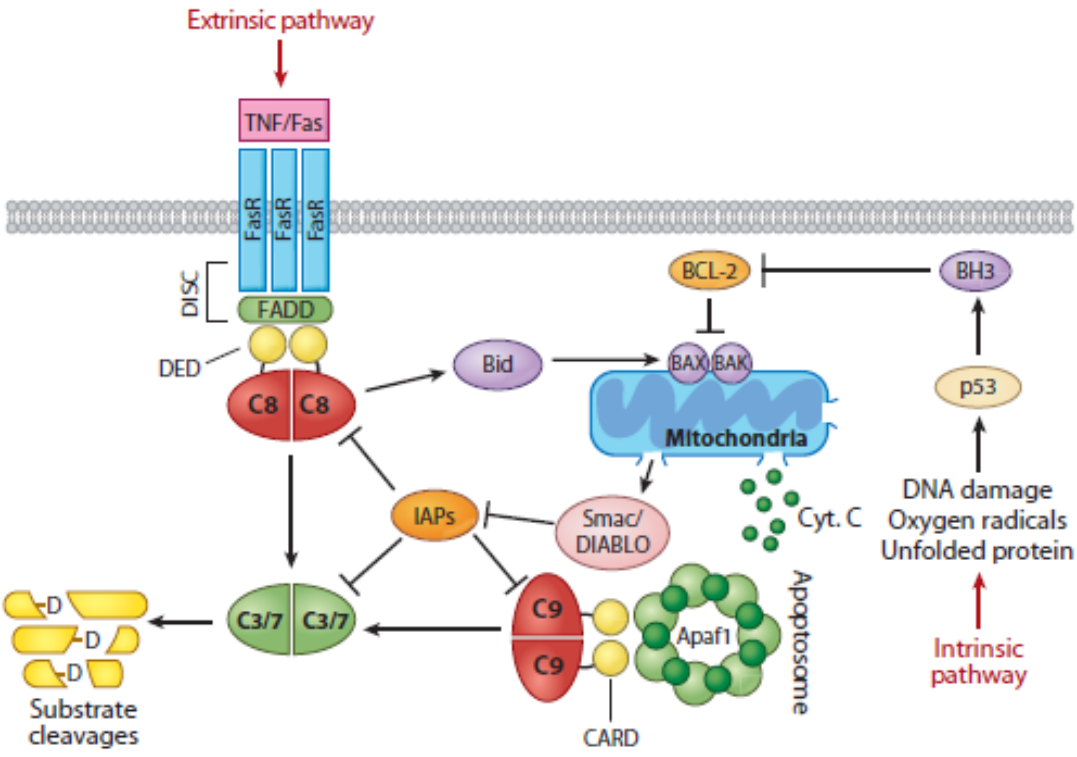


Figure 3: Mechanisms of caspase regulation in apoptosis. Two major pathways lead to apoptosis namely the extrinsic and intrinsic pathway. The extrinsic pathway is initiated by extra cellular signals leading to the activation of capase-8 which activates caspase-3 and -7. The intrinsic pathway includes release of cytochrome c leading to the formation of apoptosome and later activation of caspase-9 which activates caspase-3 and -7. IAPs are inhibitors of caspases (Crawford and Wells).

RATIONALE, OBJECTIVES AND EXPERIMENTAL APPROACHES

Rationale

Smoking during pregnancy is a major route of exposure to harmful chemicals in cigarette smoke to the developing fetus. The respiratory system has many different cell types and its development is complex. Cigarette smoke contains a plethora of ingredients which have been linked with pathological changes in various systems of the body including the oral cavity and the respiratory system. Smoke exposure during fetal development may lead to disturbance in basic cellular processes including proliferation, viability and cell death all of which are important for normal fetal development. The oral cavity, the first site of exposure to cigarette smoke as a result of smoking has been associated with periodontitis with eventual tooth loss due to loss of attachment. It is well documented that apoptosis is an important process during development and shaping of the organs. Any disruption or disturbance of apoptosis during the different stages of lung development may lead to decrease or increase in cell numbers. Cigarette smoke exposure to the fetus has been associated with respiratory distress syndrome characterized by the developmental insufficiency of surfactant during lung development. The insufficiency in surfactant may be due to damage to the surfactant producing cells. Type II AE cells are specialized cells which play a critical role in the production of surfactant that helps prevent the collapse of the alveolar spaces on expiration. Moreover, fibroblasts are the predominant cell type of the mesenchyme responsible for the production of extracellular matix in both the lungs and the PDL. Taking into account all the above factors this

present *in vitro* study was based on the rationale that cigarette smoke may lead to activation of caspase-3. Activation of caspase is an important event in the process of apoptosis.

Objectives

Hypothesis 1: The main hypothesis of the present in vitro study that cigarette smoke extract induces apoptosis through the activation of caspase-3 in smoke exposed fetal rat lung fibroblasts and type II AE cells.

Hypothesis 2: The second hypothesis is to compare responses of human A549 cells and rat periodontal ligament cells with fetal lung cells when exposed to CSE.

Experimental approaches

In order to investigate the influence of CSE on fetal lung cell apoptosis by the activation of caspase-3 and compare the same with human A549 cells and rat periodontal ligament cells the experimental approach was to:

a. Isolate fetal rat lung cells on the 21st day of gestation.
b. Culture human A549 cells and rat PDL fibroblasts.
c. Examine cells using phase contrast microscopy.
d. Prepare CSE and treat cells with increasing concentrations of CSE for 60 minutes.
e. Sub-cellular localization of caspase-3 by immunofluorescence.

f. Measure and analyze the caspase-3 activity in smoke exposed cells using fluorometric assay.

g. Study the effect of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), an irreversible inhibitor of caspase-3.

h. Determine whether CSE alters cell viability using MTT formazan assay.

i. Confirm the expression of caspase-3 activation by Western Blot.

j. Study the effect of camptothecin, an apoptosis inducing agent using caspase-3 fluorometric assay.

k. Analyze statistical differences between controls and cells exposed to CSE using *post hoc* Duncan's Multiple Range Test.

CHAPTER TWO

MATERIALS AND METHODS

2.0. Materials

The following materials were purchased from the following companies. Pregnant Sprague-Dawley rats were purchased on the 21st day of gestation from Animal Care Facility Services, University of Manitoba, Winnipeg Canada. Sodium pentobarbital (Euthanyl) Bimedia-MTC from Animal Health Inc. Cambridge, Ontario Canada. Hanks Balanced Salt Solution (10x), Minimal essential medium (MEM) Newborn Calf Serum (NCS), antibiotics/antimycotic and fungizone were purchased from Gibco, Ontario Canada. Trypsin from Sigma-Aldrich (Oakville, Ontario). Kodak films, 96 well black walled clear bottom plates, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-*fmk*), four well glass Chamber Slide System and Tissue culture flasks were purchased from Thermo Fisher Scientific (Toronto, ON). ECL-plus from GE Healthcare (NJ, USA). MTT based cell proliferation assay kits, and Prolong Antifade Gold reagent were purchased from Invitrogen (Burlington, ON, Canada). Human A549 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A). Research cigarettes from University of Kentucky (Kentucky Research Center). Caspase 3 fluorometric assay kits and camptothecin were purchased from BioVision, (MountainView, CA). Bovine Serum Albumin (BSA), Mini-PROTEAN precast gels (12%) and electrophoresis apparatus was purchased from BIO-RAD (Mississauga, ON). Rabbit polyclonal (sc-7148) primary antibody, goat anti-rabbit IgG-HRP secondary

antibody and donkey anti-rabbit IgG-FITC (sc-2090) were purchased from Santa Cruz Biotechnology, CA, USA.

2.1. Isolation and Culture of Fetal Rat Lung Fibroblast

Pregnant Sprague-Dawley rats purchased from Central Animal Services, University of Manitoba were used to isolate fetal lung fibroblasts and fetal lung type II alveolar cells. Rats were euthanized with an intraperitoneal injection of 1ml Euthanyl (240mg/ml sodium pentobarbital) on gestational day 21 (day 23 is term gestation). Fetuses were removed by hysterotomy, decapitated and placed in cold, sterile Hanks Balanced Salt Solution (HBSS, Gibco, ON Canada). Lungs were dissected from fetuses by making an incision in the mid-sternal region, and minced using a Sorval tissue chopper (Sorval Instruments, Newton, CT) in a laminar flow hood. The minced lung tissue was dissociated by incubating with trypsin-EDTA (0.05%) in HBSS at 37^0C for 45 minutes in a water-jacketed trypsinization flask which was placed on a magnetic stirrer. Minimal essential medium (MEM) (Gibco, ON Canada) containing 10% of newborn calf serum (NCS), antibiotics/antimycotic (1%) and fungizone (1%) (Gibco, ON Canada) was added to stop further enzymatic disaggregation. The dissociated cells were filtered through three layers of 150μm Nitex gauze to remove tissue fragments and centrifuged for 10 min at 1000 rpm at 4^0C. The cell pellet was resuspended in 10ml of MEM/NCS and cells were plated in five 75cm^2 tissue culture flasks in a humidified incubator (95% air/ 5% CO_2) and allowed to adhere for 1hour. Fibroblasts have the ability to attach faster when compared to type II AE cells (Hastings et al., 2005). After this period media from each flask containing nonadherent cells (including type II AE cells and red blood cells) was

collected in an autoclaved beaker. Fresh media (10% NCS) was added to the flasks which had attached fibroblasts. Media was changed after 24 hours for the first time and then at 48 hours thereafter. The cell monolayers were cultured for 3-4 days till they reached 80% confluence and sub-cultured in a ratio of 1:3.

2.2. Culture of Type II Alveolar Epithelial Cells

Fetal type II AE cells were separated by differential adherence (Batenburg et al., 1988). After one hour of incubation and adherence of fibroblasts, the media with unattached cells was collected. Cells were counted and re-plated at a density of 1.5 X 10^5 cells/flask. Type II cells were cultured in media supplemented with 10% carbon stripped serum (sNCS) in MEM and 1% antibiotics, antimycotic, fungizone and cultured in a humidified incubator (95% air/ 5% CO_2). Media was changed after 24 hrs to remove cellular debris and non-adherent cells. Cells were grown to 80- 90% confluence before use in other experiments. The cells reached confluence in 5-7 days. Type II AE cells were not sub-cultured like other cells. For the preparation of stripped serum, carbon (40-50 g) was added to 500ml of NCS and stirred overnight at 4^0C. The serum was centrifuged twice at 25000 rpm for 1 and a half hour at 4^0C in a Beckmans Ultracentrifuge C 8-70M using a SW 28 Rotor and filtered two to three times through Whatman filter paper followed by filtration through Millipore filter tubes in order to make it sterile.

2.3. Culture of Human A549 Cells

Human A549 cell line was purchased from ATCC and cultured in NCS media. These cells reached 80 -90 % confluence in 3- 4 days in culture. Frozen stocks of the cells were

thawed quickly. NCS media was added and the cells were centrifuged at 1000 rpm at 4^{o}C for 10min. The supernatant was aspirated and fresh media was added to the cell pallet and re-suspended in 25 cm^2 flasks. Media was changed at 24 hours for the first time and every 48 hours thereafter until they reached 70-80% confluence. The cells were sub-cultured in the ratio of 1:3, prior to trypsinization the cells were washed with HBSS and 2mls of 1x trypsin was added to 25cm^2 flasks. NCS media was added after the cells were detached and centrifuged at 1000 rpm for 10 min at 4^{o}C. The cell palette was resuspended in fresh media and plated in 75 cm^2 flasks.

2.4. Culture of Rat Periodontal Ligament Fibroblasts

Rat PDL fibroblasts were kindly supplied by Dr. C. Lekic which were isolated according to the method outlined by Lekic et al. (Lekic et al., 2005). Frozen stocks of the cells were thawed quickly. NCS media was added and the cells were centrifuged at 1000 rpm at 4^{o}C for 10min. The supernatant was aspirated and fresh media was added to the cell pallet and re-suspended in 25 cm^2 flasks. Media was changed at 24 hours for the first time and every 48 hours thereafter until they reached 70-80% confluence. The cells were sub-cultured in the ratio of 1:3, prior to trypsinization the cells were washed with HBSS and 2mls of 1x trypsin was added to 25cm^2 flasks. NCS media was added after the cells were detached and centrifuged at 1000 rpm for 10 min at 4^{o}C. The cell palette was resuspended in fresh media and plated in 75 cm^2 flasks.

2.5. Preparation of Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared according to method designed by Janoff and Carp (Janoff and Carp, 1977) (Figure-4). Unfiltered research cigarettes from University of Kentucky, each containing 2.45 mg nicotine/cigarette (Tabassian et al., 1988) were used. CS was drawn from each cigarette into a 50ml syringe for two seconds maintaining a gap of 20 seconds between each draw with the syringe and bubbled through 50ml of MEM at room temperature. This cycle was repeated till the end of the cigarette and then 50ml of fresh MEM was used for the next cigarette. The resulting smoke extracted MEM was considered to be 100% CSE. It was filtered using 0.22 um pore filters (Millipore) making it sterile and free from contaminants and stored at –80ºC. Further dilutions (5%, 10%, and 15%) (v/v) were made in serum free media containing antibiotics (1%) and fungizone (1%). Before treating cells with conditioned media, pH was adjusted to 7.2.

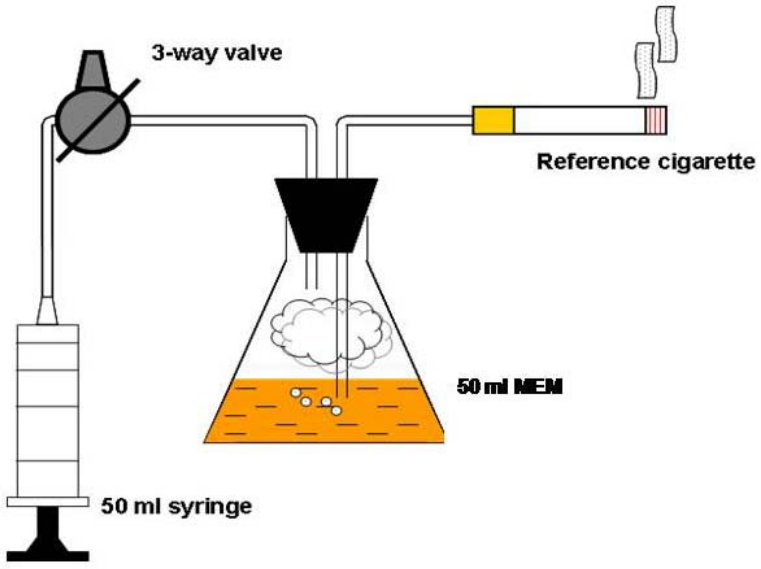


Figure 4: Apparatus for preparation of cigarette smoke extract medium: Smokometric concentron (Janoff and Carp, 1977). The 50 ml syringe was used to draw cigarette smoke and bubbled through 50 ml of minimum essential media.

2.6. Localization of caspase-3 by immunofluorescence

Sub-cellular localization of caspase-3 in cells exposed to CSE was observed using immunofluorescence microcopy. Cells were plated in four well glass chamber slides (Thermo Fisher Scientific, Toronto, ON) and left overnight in incubator at 37^{o}C for attachment on glass slide. The cells were exposed to 10% or 15% CSE in serum free media at 37^{o}C. After which the cells were washed with phosphate buffered saline (PBS) three times and fixed with cold methanol (-10º C) for 5 min followed by three washes of five min each with PBS, suction was used between each step to completely remove the reagents. The cells were blocked in 2% BSA / 1×PBS for one hour in a humidified chamber. The primary anti-body rabbit-polyclonal IgG, which recognizes active caspase-3 (Santa Cruz Biotechnology, CA, USA) was diluted (1:800 dilution) in blocking solution and added to the cells and incubated overnight at 4°C in a humidified chamber. Primary anti-body was not added to a few cells and these were considered as controls for comparison. After four washes with PBS, cells were incubated with secondary anti body, (FITC conjugated donkey anti-rabbit IgG) which recognizes rabbit IgG by immunofluorescence staining (Santa Cruz Biotechnology, CA, USA). The secondary antibody was diluted 1:80 in 2%BSA/1xPBS and cells were incubated in humidified chamber for one hour in the dark. All steps after this was done in the dark. After four washes of 5 min each with PBS the cells were stained with Hoescht 33342 (1:1000) for 15 seconds and washed four times with PBS. The slides were air-dried and mounted with cover slips using 40µl of Prolong Anti-fade Gold; the edges of the slides with cover slips were sealed with nail paint. The slides were later observed under an inverted fluorescence microscope (BX61 Olympus microscope) using ImagePro Software.

2.7. Caspase-3 Fluorometric Assay:

Caspase 3 activation in cells was determined using caspase 3 fluorometric assay kit, purchased from BioVision, (MountainView, CA, U.S.A). This is based on the detection of cleavage of DEVD-AFC substrate (AFC: 7-amino-4-trifluoromethyl coumarin) (figure 5). The DEVD-AFC substrate emits blue light (excitation at 404nm). When the substrate is cleaved by caspase-3, free AFC emits yellow-green fluorescence (detection at 505nm). The amount of fluorescence was quantified using a fluorometric plate reader at 505 nm. The amount of fluorescence is directly proportional to caspase-3 activity in cells.

After the monolayer of cells reached 70 to 80% confluence in 75 cm^2, the cells were washed with HBSS and trypsinized with 1Xtrypsin, centrifuged, counted and plated in 96 well black walled clear bottom plates (Thermo Fisher Scientific, Toronto, ON) at a density of 5 X 10^6 cells per well. The cells were left overnight in the incubator at 5% CO_2 at 37ºC and 100% humidity to adhere. Media was removed and the monolayer of cells was washed three times with HBSS and treated with different concentrations of CSE (5%, 10% or 15%) (v/v) diluted in serum free media, a few cells were not exposed to CSE which were considered as control cells and incubated for 60 min at 37ºC. The media containing CSE was removed and cells were washed with HBSS three times to ensure complete removal of traces of CSE. Caspase 3 activation in cells was determined following instructions of Caspase 3 fluorometric assay kit, purchased from BioVision, (MountainView, CA, U.S.A). The cells were lysed using the lysis buffer available with the kit (50ul per well) and incubated on ice for ten minutes, later incubated with 5ul of fluorogenic substrate 1mM DEVD-AFC in a reaction buffer (containing 10mM DTT) at

37ºC for two hours. The enzymatic activity was monitored using a fluorescence microplate reader with 400nm excitation and 505nm emission filter. Caspase 3 cleaves AFC substrate and releases a fluorogenic signal; this signal is directly proportional to the level of enzymatic activity of Caspase 3 in treated cells. Caspase 3 activity was calculated in treated samples and compared to untreated controls.

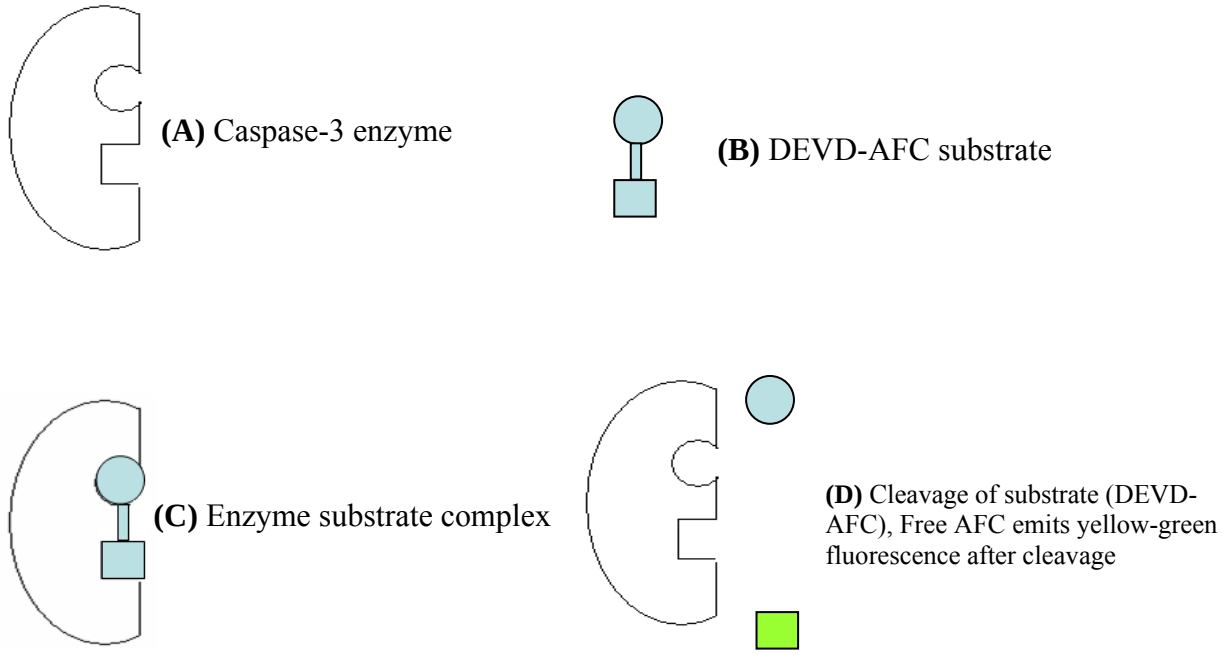


Figure 5: Schematic illustration of DEVD dependent detection of caspase-3 activity. Caspase-3 fluorometric assay kit (BioVision, Mountain View) is based on detection of cleavage of substrate DEVD-AFC by caspase-3. DEVD-AFC substrate emits blue light (400nm). After cleavage of the substrate by caspase-3, free AFC emits yellow-green fluorescence (505nm). The amount of fluorescence can be quantified using a fluorometric plate reader.

2.8. Effect of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk).

Z-VAD-fmk is a broad spectrum caspase inhibitor which was used in the present study to examine the involvement of caspases in cell death due to CS exposure. The cells were incubated with 80uM concentration of Z-VAD-fmk in serum free media at the time of exposure of cells to CSE for 60min. After which the cells were washed and the caspase-3 activity was measured using the fluorometric assay kit, purchased from BioVision, (MountainView, CA) as described above.

2.9. Determination of Cell Viability

The cells were isolated and cultured as described above. When the cells reached 70- 80% confluence they were trypsinized and plated in 96 well plates at a density of 5 X 10^6 cells/ well and kept overnight in the incubator at 5% CO_2 at 37ºC and 100% humidity to adhere. Media was removed, washed with HBSS twice and treated with different concentrations of CSE diluted in serum free media for 60 minutes with some untreated control cells. After incubation with different concentrations of CSE the cells were washed with HBSS three times to ensure complete removal of CSE. The cells were incubated with MTT solution for three hours according to the directions of the kit (Sigma Aldrich, St.Louis, MO, USA). The MTT based cell proliferation assay, is a calorimetric assay used to measure the ability of mitochondrial dehydrogenase of viable cells to reduce the key component, MTT or 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide, a yellow tetrazole to insoluble purple formazan crystals . Viable cells cleave the tetrazolium ring of MTT and the yellow water soluble dye is converted to insoluble

purple crystals of formazan. After three hours of incubation with MTT solution the crystals were dissolved in MTT solvent by pipetting three times in order to completely dissolve the crystals. The plates were read using a spectrophotometer at an absorbance of 540nm.

2.10. Whole Cell Lysate Preparation

Fetal lung fibroblasts, fetal type II AE cells and PDL cells were cultured as described above. The cells were washed with HBSS twice and incubated at 37^{o}C with CSE diluted (5%, 10% and 15%) (v/v) in serum free media for 60min and some cells were untreated and considered as controls. At the end of 60min, cells were washed three times with HBSS to ensure complete removal of any remnants of CSE. The cells were lysed by adding one ml of 2x RIPA buffer with protease inhibitor tablet [20mM Tris-HCL pH 7.6, 316mM NaCl, 2mM EDTA, 2% triton X100, 0.2% SDS, 2% sodium deoxycholate, 1mM PMSF, 1mM Na_3VO_4, 1 protease inhibitor tablet] on ice, after which the lysate was scraped and aliquots were stored at -80 until processing.

2.11. Measurement of Protein Concentration

After protein extraction the samples were quantified using Bradford protein determination method (Bradford, 1976). This method is based on Coomassie-blue dye which can bind to proteins. It exits in two forms and upon binding with proteins; the red dye changes its color to blue. The intensity of blue color was measured using a spectrophotometer at 590nm. BSA purchased from BIO-RAD (Mississauga, ON) was used as a standard with known concentration of proteins (two mg/ml). All protein extracts collected from cells exposed

to CSE (5%, 10% or 15%) was used for the assay and made in triplicates in increasing concentrations of proteins. The dye (BIO-RAD, Mississauga, ON), was diluted two-thirds in distilled water and added to each sample. The optical density of protein samples was read at 595nm using spectrophotometer. The weight of proteins was plotted as a graph against absorbance using Soft max pro software.

2.12. Sample Protein Preparation

Sample preparation was done by dilution of cell extracts in SDS reducing buffer [final concentration: 0.5M Tris-HCL, pH6.8, 20% Glycerol, 10% (w/v) SDS, 2-b-mercaptoethanol, 0.05% (w/v) bromophenol blue]. The samples were heated in water at 95^{o}C for five min in order to disrupt the disulphide bonds in the proteins. The samples were cooled before loading. 25ug of protein sample was loaded on gels. Pre-stained standards were used as indicator for relative molecular weight in Western Blots.

2.13. SDS-PAGE Analysis

The cells were grown to confluence, exposed to different concentrations of CSE, washed with HBSS and protein was extracted as described above. After determination of protein concentration 25ug of protein sample was loaded onto 12% sodium dodecyl sulfate-polyacrylamide precast gels (BIO-RAD, Mississauga, ON), electrophoresed at 180V at room temperature, to separate the proteins depending on their molecular mass. The gels with proteins were stained with Coomassie blue stain (45% methanol, 9% acetic acid, 0.2 % Coomassie blue) for forty five minutes, Coomassie blue stain is an anionic die which can bind to proteins non-specifically. Excess dye was removed with destaining solution

(15% methanol, 7.5% acetic acid in water). The gels were exposed to a final destain solution (10% methanol, 7.5% acetic acid, 3% glycerol). After staining and de-staining of the gels, proteins were detected as blue bands.

2.14. Western Blot Analysis of Caspase-3 Expression

The proteins which were electrophoresed and fractionated on gels were transferred onto nitrocellulose membranes by electrophoresis at 120V for one hour at 4^{o}C using transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol]. The membranes were placed in blocking buffer (10% skimmed milk in 1 X TBS- buffered saline containing 0.1% Tween 20) for two hours to reduce nonspecific binding of proteins. To detect the cleaved active form of Caspase 3, blots were incubated with Caspase 3 primary antibody (rabbit polyclonal raised against 1-277 amino acids) (Santa Cruz Biotechnology) (Santa Cruz, CA, USA), diluted 1:500 in blocking buffer and left overnight at 4^{o}C. This antibody recognizes both the pro (32kD) and active (20 and 17 kD) form of Caspase 3 (Santa Cruz, CA, USA). Blots were washed three times (15 min each wash) in TBS-T, and later incubated with goat anti rabbit IgG-HRP secondary antibody (Santa Cruz, CA, USA) at a dilution of 1: 1000 in blocking buffer for two hours at room temperature. The blots were washed three times in TBS-T. The primary anti-body binding sites were visualized using ECL-plus detection kits (GE Healthcare, NJ, USA), which was prepared according to the manufacturer's instructions and exposed on Kodak films. The densitometric analysis of the protein bands of interest was quantified using Quantiscan.

2.15. Effect of camptothecin on adherent cells

Camptothecin is an apoptosis inducing agent which acts by inhibiting cell proliferation. The cells were isolated and cultured as described above. When the cells reached 70- 80% confluence they were trypsinized and plated in 96 well plates at a density of 5 X 10^6 cells/ well and kept overnight in the incubator at 5% CO_2 at 37ºC and 100% humidity to adhere. Media was removed, washed with HBSS twice and treated with camptothecin (BioVision, MountainView, CA) at concentrations of 4uM, 8uM, 10uM or 15uM for 60 minutes in 37^{o}C incubator, cells not exposed to camptothecin were kept as controls. The media containing camptothecin was removed and cells were washed with HBSS three times to ensure complete removal of traces of camptothecin. Caspase 3 activation in cells was determined following instructions of Caspase 3 fluorometric assay kit as described above. Caspase 3 activity was calculated in treated samples and compared to untreated controls.

2.16. Statistical Analysis

Statistical differences between group means were carried out using *post hoc* Duncan's Multiple Range Test. A value of $p < 0.05$ was considered for statistically significant differences between the treated and untreated groups.

CHAPTER THREE

RESULTS

3.1 Phase contrast microscopy

Phase contrast microscopic images of all the cells used in the present study were taken after cells reached 70%-80% confluence. The fetal rat lung cells were isolated on the 21st day of gestation. Figure 6 shows the appearance of untreated isolated fetal rat lung fibroblasts after four days *in vitro* (40X magnification). The cells appear as spindle-shaped, elongated and closely associated to each other. Nuclei are centrally placed with very little free culture vessel space.

Figure 7 shows the appearance of untreated isolated fetal rat lung type II AE cells after six days *in vitro* (40X magnification). The cells appeared cuboidal in shape interspersed between fibroblast cells. The cells appear granular with numerous cytoplasmic inclusions and cover culture vessel space completely.

Figure 8 shows the appearance of A549 cells after three days *in vitro* (40X magnification). The cells appeared to lie in groups and were generally cobble-stone in shape. Nuclei were distinguishable and the cells appeared to contain numerous cytoplasmic inclusions. Very little free culture vessel space was present. The cells appeared to abut one and other.

Figure 9 shows the appearance of rat PDL fibroblasts after seven days *in vitro* at 40X magnification. The cells appear as spindle-shaped, elongated and closely associated with each other. Nuclei were distinguishable and the cells appeared to contain some cytoplasmic inclusions. Very little free culture vessel space was present.

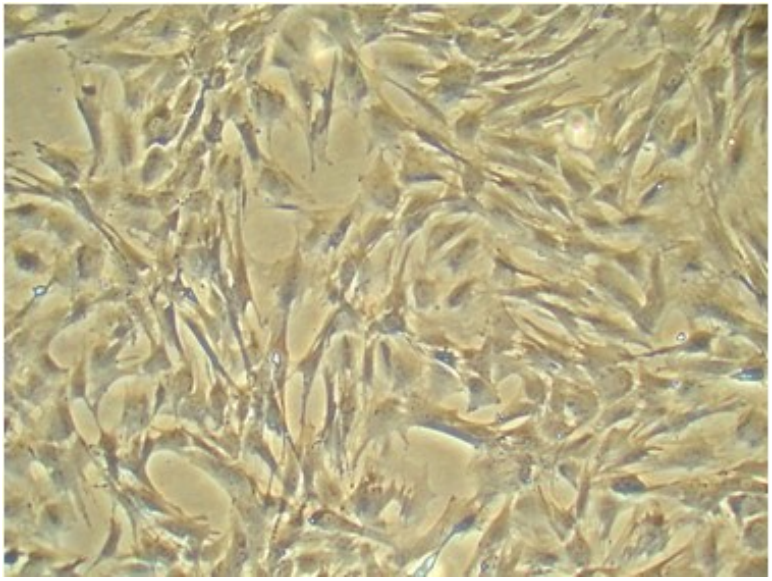


Figure 6: Phase contrast microscopic image of isolated fetal rat lung fibroblasts. Sub-confluent monolayer of isolated fetal rat lung fibroblasts under culture conditions after four days *in vitro*. Phase contrast microscopy; 40X objective.

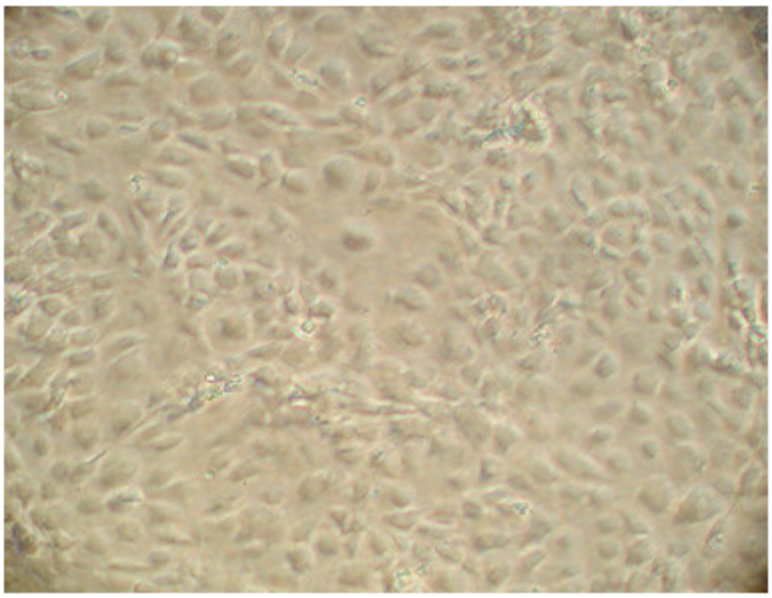


Figure 7: Phase contrast microscopic image of isolated fetal rat lung type II alveolar epithelial cells. Sub-confluent monolayer of isolated fetal rat lung type II alveolar epithelial cells under culture conditions after six days *in vitro*. Phase contrast microscopy; 40X objective.

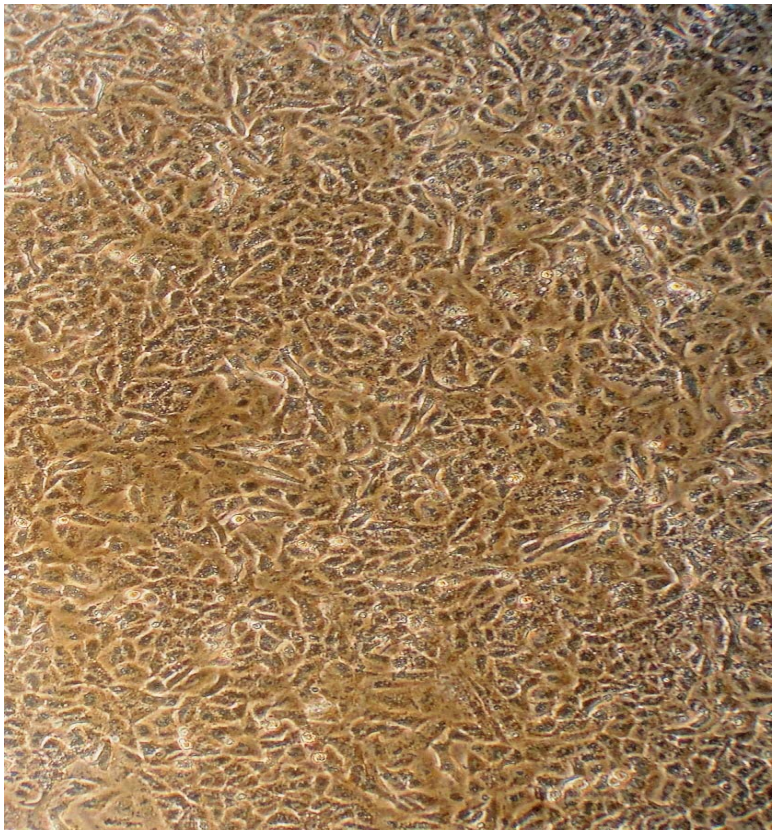


Figure 8: Phase contrast microscopic image of human A549 cells. Phase contrast microscopic image of monolayer of human A549 cells *in vitro*, three days after sub-culture. Phase contrast microscopy; 40X objective.

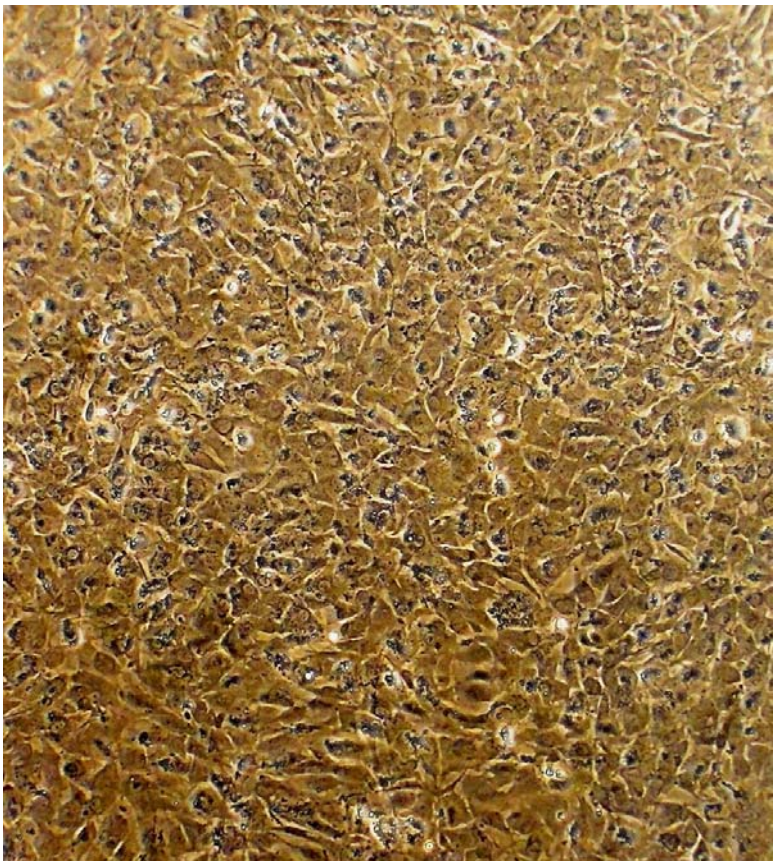


Figure 9: Phase contrast microscopic image of rat periodontal ligament fibroblasts. Phase contrast microscopic image of monolayer of rat periodontal ligament fibroblast *in vitro*, seven days after sub-culture. Phase contrast microscopy; 40X objective.

3.2 Localization of caspase-3 by immunofluorescence

It is generally accepted that caspase-3 exists as inactive zymogens in many cells and gets activated only during apoptosis (Degterev et al., 2003). The sub-cellular localization of caspase-3 expressed in all cell types used in the present study after exposure to CSE was determined by immunofluorescence using fluorescence microscopy. The controls consisted of samples in which the primary antibody against caspase-3 was omitted.

Immunofluorescence was clearly observed in the cytoplasm of isolated fetal rat lung fibroblasts (figure 10), human A549 cells (figure 12) and rat PDL fibroblast (figure 13) after exposure to CSE. The controls did not show any significant fluorescent staining. In fetal rat lung type II AE cells (figure 11) two distinct forms of reactions were present. The first were definite fluorescence which filled the majority of the cytoplasm but did not extend to the plasma membrane. The second were a more localized fluorescence which appeared to be constricted to specific round cytoplasmic inclusions.

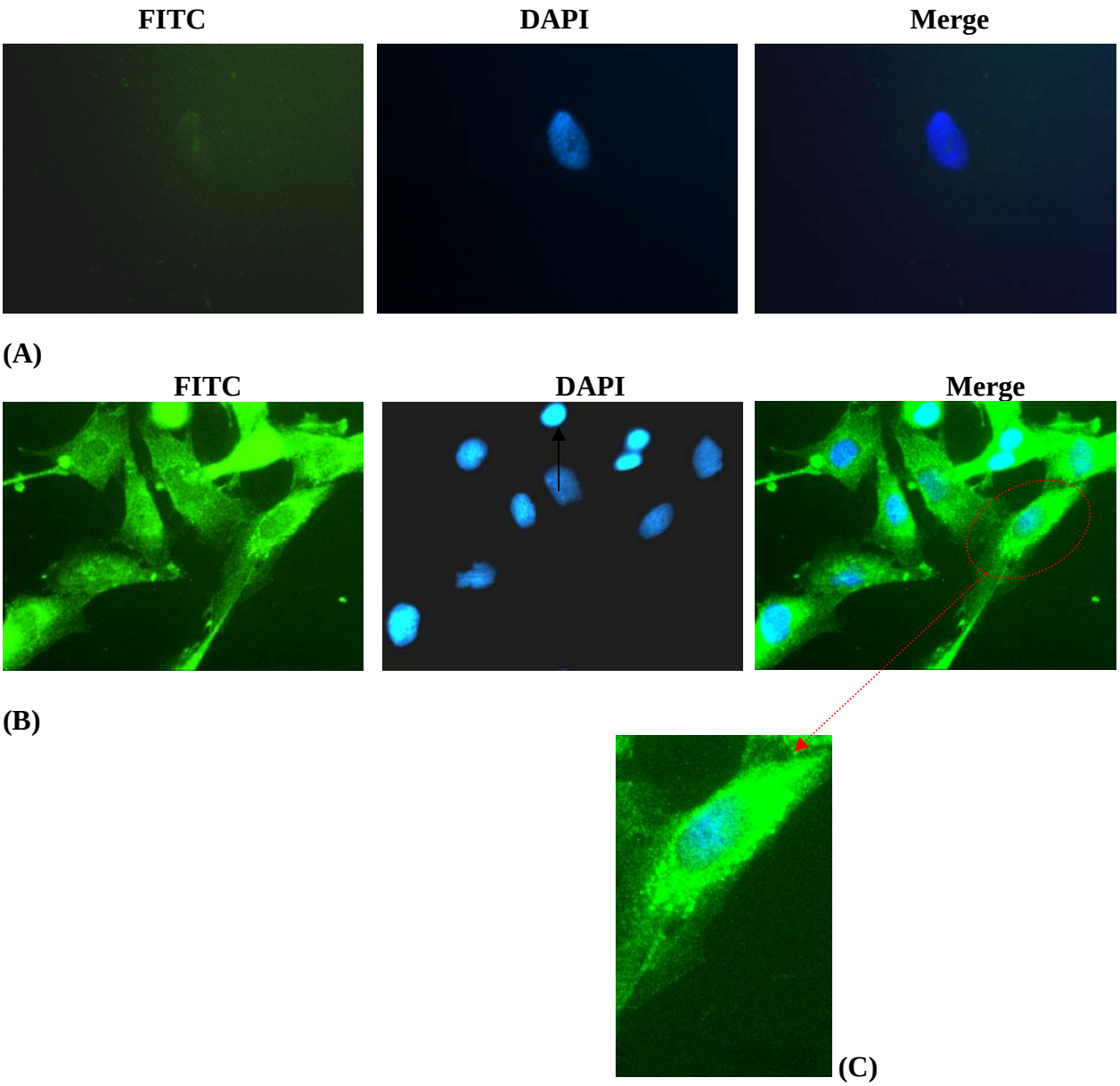


Figure 10: Immunofluorescence staining for detection of caspase 3 expression in isolated fetal rat lung fibroblasts. Isolated fetal rat lung fibroblasts were exposed to CSE (15% v/v) for three hours. Immunofluorescence was performed using caspase 3 rabbit polyclonal IGg antibody. Caspase 3 was visualized using donkey anti-rabbit IGg-FITC (green fluorescence) and counter stained with Hoescht 33342 (nuclear staining - blue). Image **(A)** shows controls (without primary anti-body). Image **(B)** shows expression of caspase 3 (green) primarily localized in the cytoplasm. **(C)** Shows enlarged image of single cell.

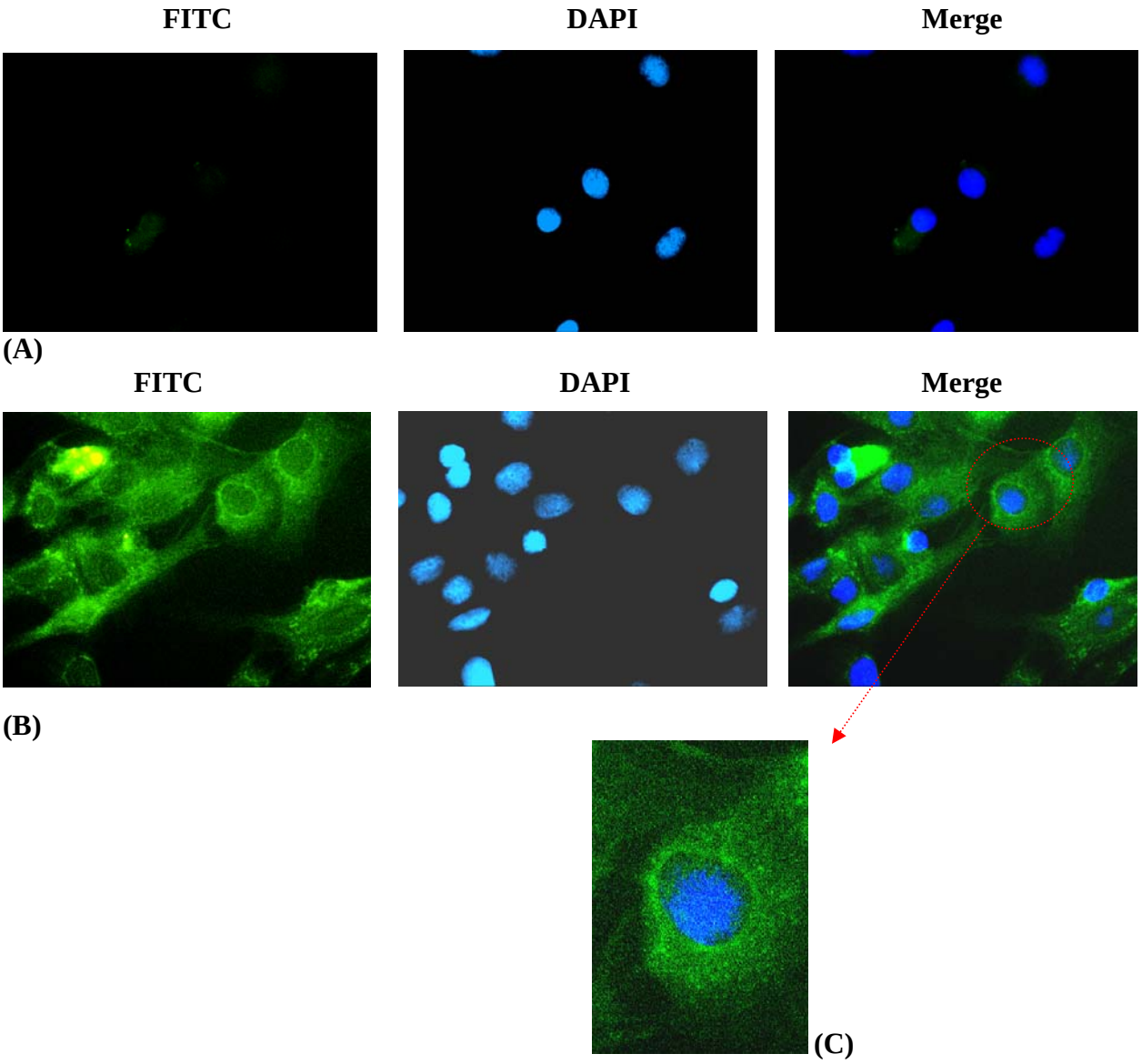


Figure 11: Immunofluorescence staining for detection of caspase 3 expression in isolated fetal rat lung type II AE cells. Isolated fetal rat lung type II AE cells were exposed to CSE (15% v/v) for three hours. Immunofluorescence was performed using caspase 3 rabbit polyclonal IGg antibody. Caspase 3 was visualized using donkey anti-rabbit IGg-FITC (green fluorescence) and counter stained with Hoescht 33342 (nuclear staining - blue). Image **(A)** shows controls (without primary anti-body). Image **(B)** shows expression of caspase 3 (green) primarily localized in the cytoplasm. **(C)** Shows enlarged image of single cell.

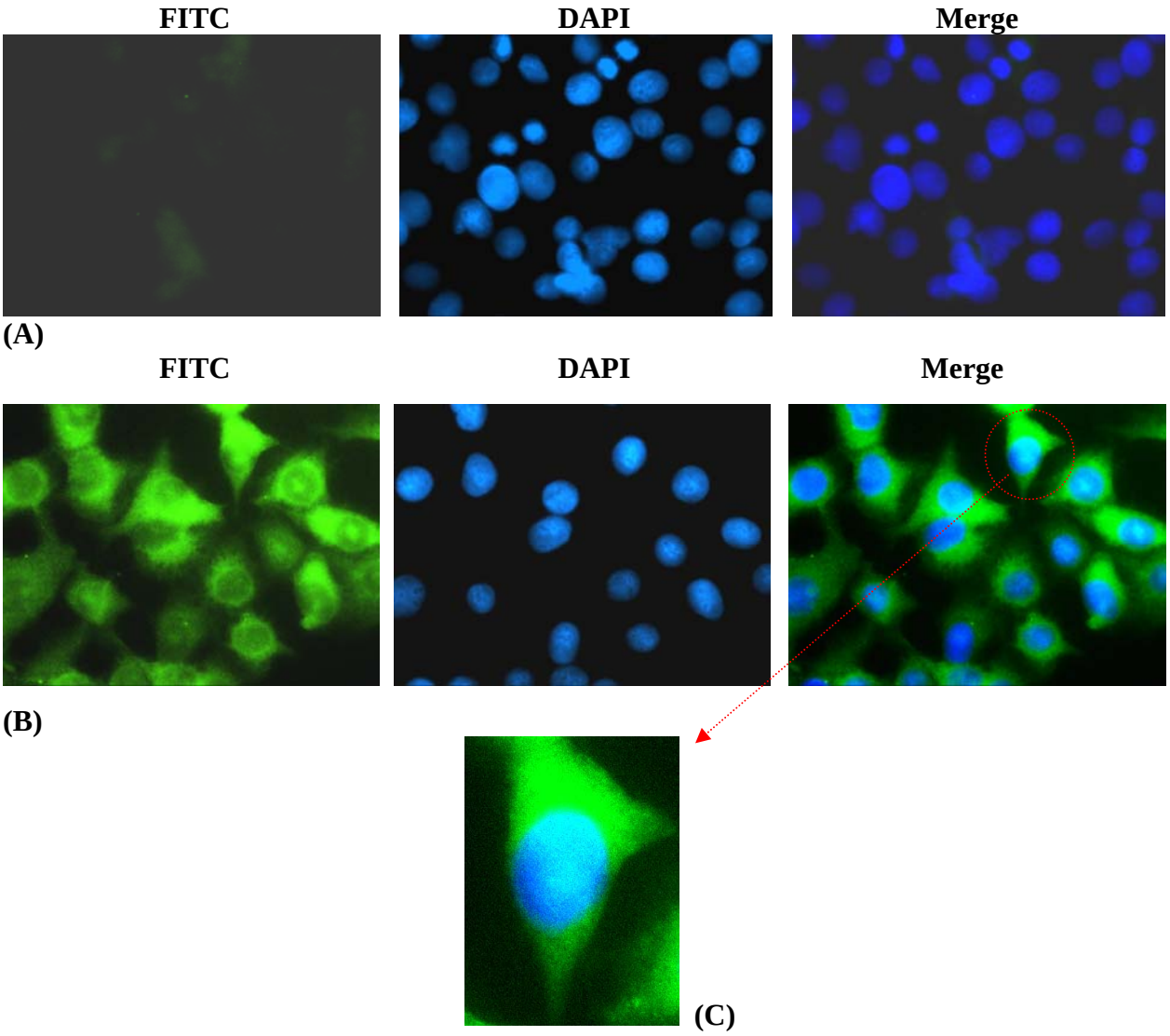


Figure 12: Immunofluorescence staining for detection of caspase 3 expression in human A549 cells. Human A549 cells were exposed to CSE (15% v/v) for three hours. Immunofluorescence was performed using caspase 3 rabbit polyclonal IGg antibody. Caspase 3 was visualized using donkey anti-rabbit IGg-FITC (green fluorescence) and counter stained with Hoescht 33342 (nuclear staining - blue). Image **(A)** shows controls (without primary anti-body). Image **(B)** shows expression of caspase 3 (green) primarily localized in the cytoplasm. **(C)** Shows enlarged image of single cell.

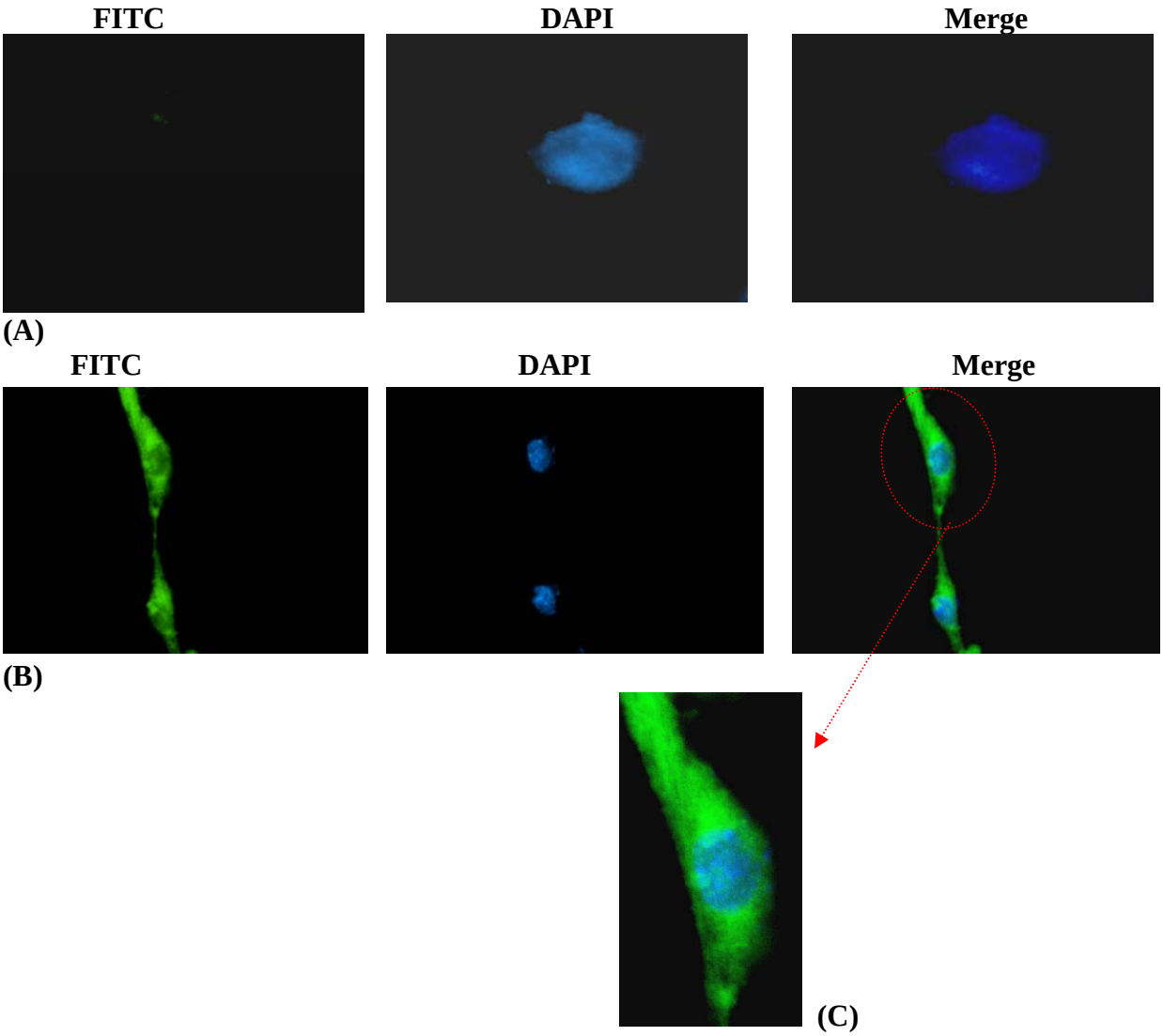


Figure 13: Immunofluorescence staining for detection of caspase 3 expression in rat PDL cells. Rat PDL cells were exposed to CSE (15% v/v) for three hours. Immunofluorescence was performed using caspase 3 rabbit polyclonal IGg antibody. Caspase 3 was visualized using donkey anti-rabbit IGg-FITC (green fluorescence) and counter stained with Hoescht 33342 (nuclear staining - blue). Image **(A)** shows controls (without primary anti-body). Image **(B)** shows expression of caspase 3 (green) primarily localized in the cytoplasm. Image **(C)** Shows enlarged image of single cell.

3.3 Detection of caspase-3 activity in adherent cells exposed to cigarette smoke extract (CSE)

The main executioner caspase to be activated in the process of apoptosis is caspase-3. A fluorometric assay was used to further analyze caspase-3 activity in isolated fetal rat lung fibroblasts, fetal rat type II AE cells, A549 cells and PDL fibroblasts exposed to CSE (5%, 10% or 15%) (v/v) for 60 minutes. This is based on the detection of cleavage of DEVD-AFC substrate (AFC: 7-amino-4-trifluoromethyl coumarin). The DEVD-AFC substrate rapidly penetrates into the cell which is later cleaved at aspartate residue to release a fluorescent compound, AFC (Tao et al., 2007). The DEVD-AFC substrate emits blue light (excitation at 404nm). When the substrate (DEVD-AFC) is cleaved by caspase-3, free AFC emits yellow-green fluorescence (detection at 505nm). The amount of fluorescence was quantified using a fluorometric plate reader at 505 nm. The amount of fluorescence is directly proportional to caspase-3 activity in cells.

Figure 14 shows caspase-3 activity in isolated fetal rat lung fibroblasts exposed to CSE (5%, 10% or 15%) (v/v) for 60 minutes. The enzyme activity in samples not exposed to CSE produced readings of approximately 13 arbitrary fluorescent units. Exposure to CSE at concentrations of 10% and15% (v/v) produced significantly elevated activity ($p < 0.05$) of caspase-3 compared to the non-exposed cells. No significant differences were observed in the caspase-3 activity in cells exposed to 5% CSE when compared to the non-exposed cells.

Figure 15 shows caspase-3 activity in isolated fetal rat lung type II AE cells exposed to CSE (5%, 10% or 15%) (v/v) for 60 minutes. The enzyme activity in samples not exposed to CSE produced readings of approximately 7 arbitrary fluorescent units. Exposure to CSE at concentrations of 10% and15% (v/v) produced significantly elevated activity ($p < 0.05$) of caspase-3 compared to the non-exposed cells. No significant differences were observed in the caspase-3 activity in cells exposed to 5% CSE compared to the non-exposed cells.

Figure 16 shows caspase-3 activity in human A549 cells exposed to different concentrations of CSE (1%, 5%, 10%, 15% or 20%) (v/v) for different periods of time (three, six and eighteen hours). There was a significant increase in caspase-3 activity in samples exposed to 20% CSE (v/v) for three hours compared to the non exposed control samples. No significant differences were observed in caspase-3 activity in cells in all other samples compared to the associated controls.

Figure 17 shows caspase-3 activity in rat periodontal ligament fibroblasts exposed to CSE (5%, 10% or 15%) (v/v) for 60 minutes. The enzyme activity in samples not exposed to CSE produced readings of approximately 50 arbitrary fluorescent units. No significant differences were observed in the caspase-3 activity in cells exposed to 5%, 10% or 15% (v/v) CSE compared to the non-exposed cells.

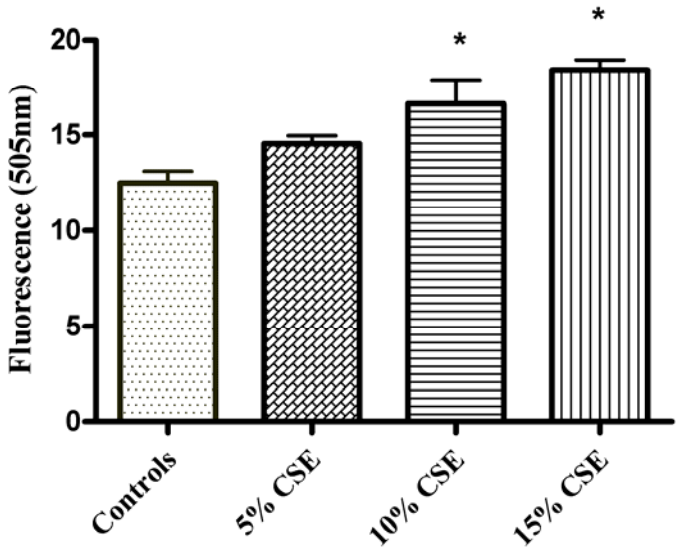


Figure 14: Effect of CSE on caspase 3 activity in fetal rat lung fibroblasts. Fluorometric assay to assess the activity of caspase-3 in fetal rat lung fibroblasts exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes in 37^{o}C incubator. Cells not exposed to CSE were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

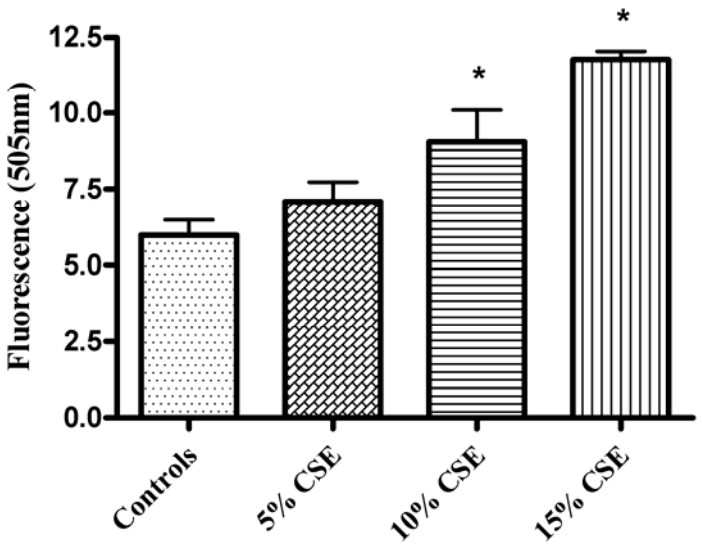


Figure 15: Effect of CSE on caspase 3 activity in isolated fetal rat lung type II alveolar epithelial cells. Fluorometric assay to assess the activity of caspase-3 in fetal rat lung type II AE cells exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes in 37^{o}C incubator. Cells not exposed to CSE were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

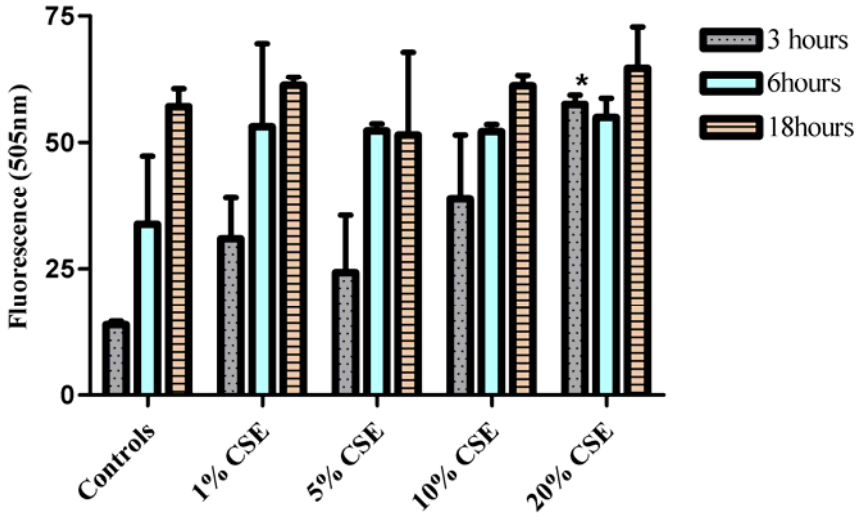


Figure 16: Effect of CSE on caspase 3 activity in human A549 cells. Fluorometric assay to assess the activity of caspase-3 in fetal rat lung type II AE cells exposed to different concentrations of CSE (1%, 5%, 10% or 20%) (v/v) for different periods of time (three, six and eighteen hours) in 37^{o}C incubator. Cells not exposed to CSE were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

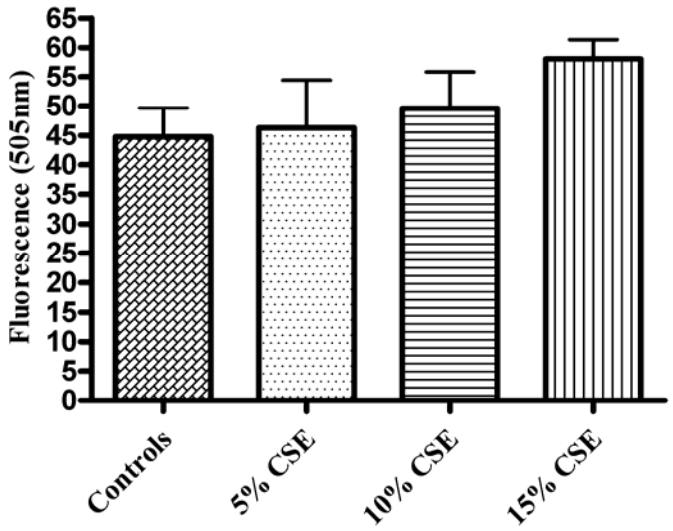


Figure 17: Effect of CSE on caspase 3 activity in rat periodontal ligament fibroblasts. Fluorometric assay to assess the activity of caspase-3 in rat periodontal ligament fibroblasts exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes in 37^{o}C incubator. Cells not exposed to CSE were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

3.4 Effect of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), an irreversible inhibitor of caspases

Figure 18 shows effects of Z-VAD-fmk on caspase-3 activity in isolated fetal rat lung fibroblasts exposed to different concentrations of CSE (5%, 10% and 15%) and Z-VAD-fmk (80uM concentration) for 60 minutes. Z-VAD-fmk is a membrane permeable irreversible inhibitor of caspases and blocks apoptosis. The caspase-3 activity in CS exposed cells with and without the Z-VAD inhibitor was measured using the fluorometric assay kit at 400nm excitation and 505nm emission. In all samples Z-VAD-fmk inhibited caspase-3 activity.

Figure 19 shows effects of Z-VAD-fmk on caspase-3 activity in isolated fetal rat lung type II AE cells exposed to different concentrations of CSE (5%, 10% and 15%) and Z-VAD-fmk (80uM concentration) for 60 minutes. The caspase-3 activity in CSE exposed cells with and without the Z-VAD inhibitor was measured using the fluorometric assay kit at 400nm excitation and 505nm emission. CSE significantly elevated caspase-3 activity at concentrations of 10% and 15% (v/v). In all samples Z-VAD-fmk inhibited caspase-3 activity.

Figure 20 shows effects of Z-VAD-fmk on caspase-3 activity in human A549 cells exposed to different concentrations of CSE (5%, 10% and 15%) and Z-VAD-fmk (80uM concentration) for 60 minutes. The caspase-3 activity in CSE exposed cells with and without the Z-VAD inhibitor was measured using the fluorometric assay kit at 400nm excitation and 505nm emission. No significant differences were observed in caspase-3 activity in cells exposed to CSE at all concentrations compared to the controls, which were not exposed to CSE. Caspase-3 activity was not inhibited in these cells.

Figure 21 shows effects of Z-VAD-fmk on caspase-3 activity in rat PDL fibroblasts exposed to different concentrations of CSE (5%, 10% and 15%) and Z-VAD-fmk (80uM concentration) for 60 minutes. The caspase-3 activity in CSE exposed cells with and without the Z-VAD inhibitor was measured using the fluorometric assay kit at 400nm excitation and 505nm emission. No significant differences were observed in the caspase-3 activity in cells exposed to 5%, 10% or 15% (v/v) CSE compared to the non-exposed cells. In all samples Z-VAD-fmk inhibited caspase-3 activity.

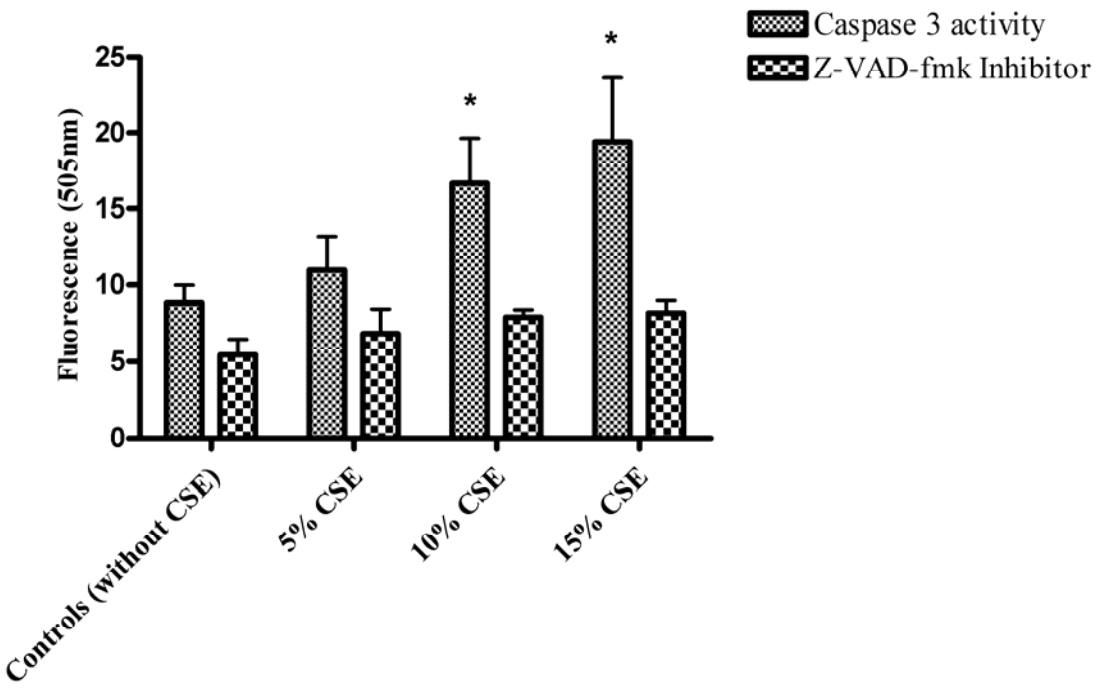


Figure 18: Effect of Z-VAD-fmk and caspase 3 activity in isolated fetal rat lung fibroblast cells exposed to CSE. Caspase 3 activity and effect of Z-VAD-fmk was measured using caspase 3 fluorometric assay in isolated fetal rat lung fibroblast cells exposed to 5%, 10% or 15% (v/v) CSE for 60 minutes. Each bar represents the mean of ± SEM of three experiments of 16 samples each. (*) indicates significantly ($p<0.05$) different from the corresponding controls.

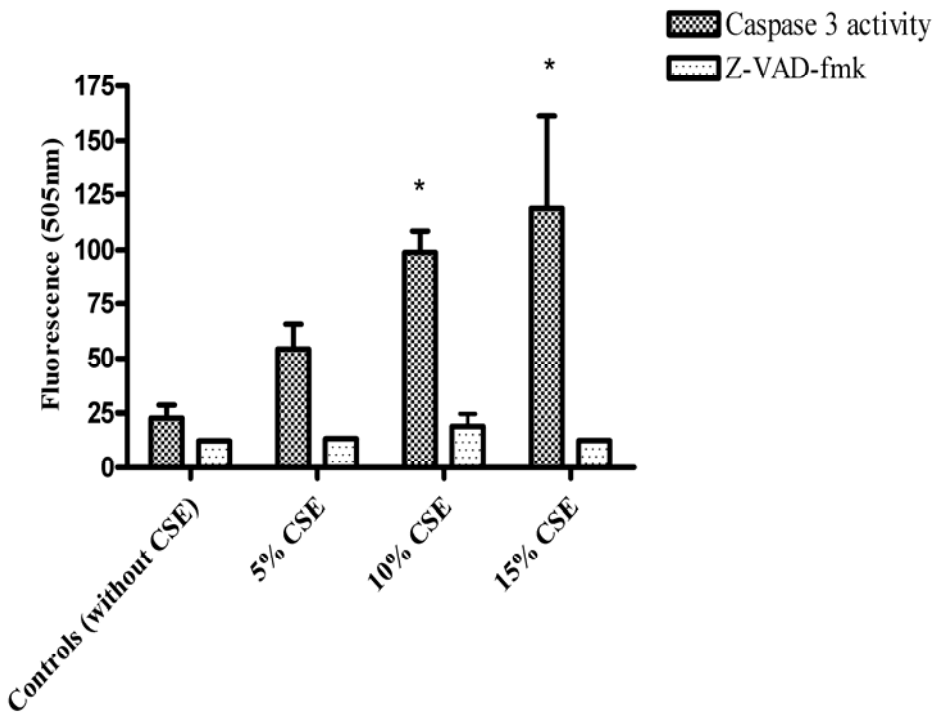


Figure 19: Effect of Z-VAD-fmk and caspase 3 activity in isolated fetal rat lung type II AE cells exposed to CSE. Caspase 3 activity and effect of Z-VAD-fmk was measured using caspase 3 fluorometric assay in fetal rat lung type II cells exposed to 5%, 10% or 15% (v/v) CSE for 60 minutes. Each bar represents the mean of ± SEM of three experiments of 16 samples each. (*) indicates significantly ($p<0.05$) different from the corresponding controls.

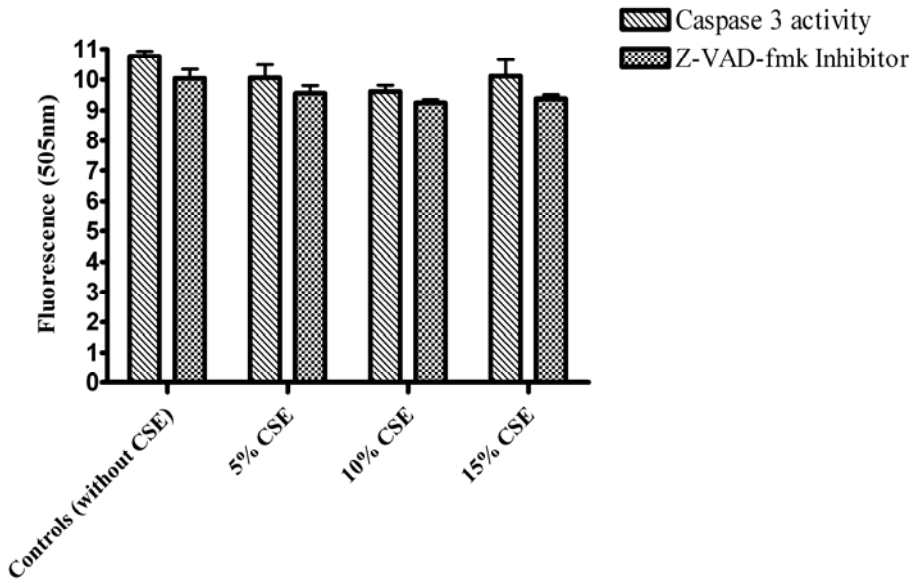


Figure 20: Effect of Z-VAD-fmk and caspase-3 activity in human A549 cells exposed to CSE. Caspase 3 activity and effect of Z-VAD-fmk was measured using caspase 3 fluorometric assay in human A549 cells exposed to 5%, 10% or 15% (v/v) CSE for 60 minutes. Each bar represents the mean of ± SEM of three experiments of 16 samples each. There were no significant ($p<0.05$) differences noted.

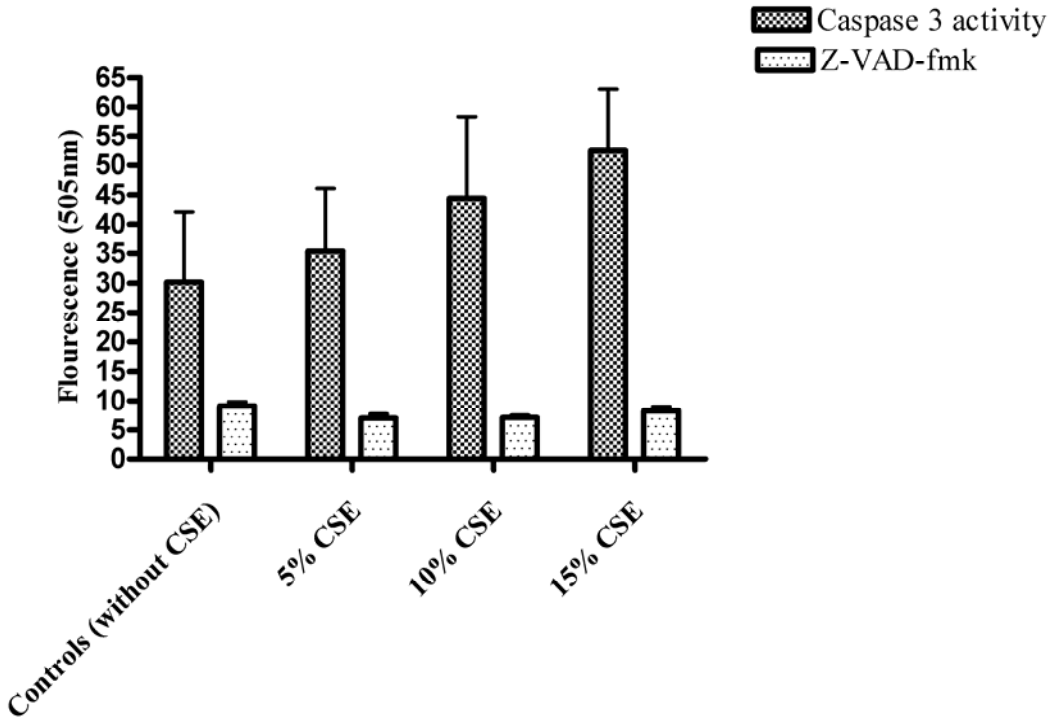


Figure 21: Effect of Z-VAD-fmk and caspase 3 activity in rat PDL fibroblasts exposed to CSE. Caspase 3 activity and effect of Z-VAD-fmk was measured using caspase 3 fluorometric assay in rat PDL fibroblast cells exposed to 5%, 10% or 15% (v/v) CSE for 60 minutes. Each bar represents the mean of ± SEM of three experiments of 16 samples each. No significant differences were noted.

3.5 Effect of CSE on cell viability

The effects of CSE on cellular viability in cells exposed to different concentrations of CSE (5%, 10% and 15%) for 60 minutes were measured using MTT formazan assay. The formazan assay measures cellular mitochondrial dehydrogenase activity within a cell and is based on the conversion of mitochondrial-dependent MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow water soluble dye which is readily taken up by viable cells and reduced to purple formazan crystals (Denizot and Lang, 1986). The intensity of purple color was quantified using a spectrophotometer at a wavelength of 540 nm. The intensity of purple color in the solution results in an increase in absorbance level, indicative of the number of living cells.

Figure 22 shows effects of CSE on viability of isolated fetal rat lung fibroblasts. The cells were treated with CSE at different concentrations of CSE (5%, 10% and 15%) in serum free media for 60 minutes. There was a significant ($p < 0.05$) decrease in the mitochondrial activity of cells exposed to 10% or 15% CSE compared to the cells non exposed control cells.

Figure 23 shows effects of CSE on viability of isolated fetal rat lung type II AE cells. The cells were treated with CSE at different concentrations of CSE (5%, 10% and 15%) in serum free media for 60 minutes. There was a significant ($p < 0.05$) decrease in the mitochondrial activity of cells exposed to 10% or 15% CSE compared to the cells not exposed to CSE or to cells exposed to 5% CSE.

Figure 24 shows effects of CSE on viability of human A549 cells. The cells were treated with CSE at different concentrations of CSE (5%, 10% and 15%) in serum free media for 60 minutes. There was no significant ($p<0.05$) decrease or increase in the mitochondrial activity of cells exposed to 5%, 10% or 15% CSE compared to the cells not exposed to CSE.

Figure 25 shows effects of CSE on viability of isolated rat PDL fibroblasts. The cells were treated with CSE at different concentrations of CSE (5%, 10% and 15%) in serum free media for 60 minutes. No significant decrease or increase in the mitochondrial activity of cells exposed to 5%, 10% or 15% CSE was observed compared to the cells not exposed to CSE.

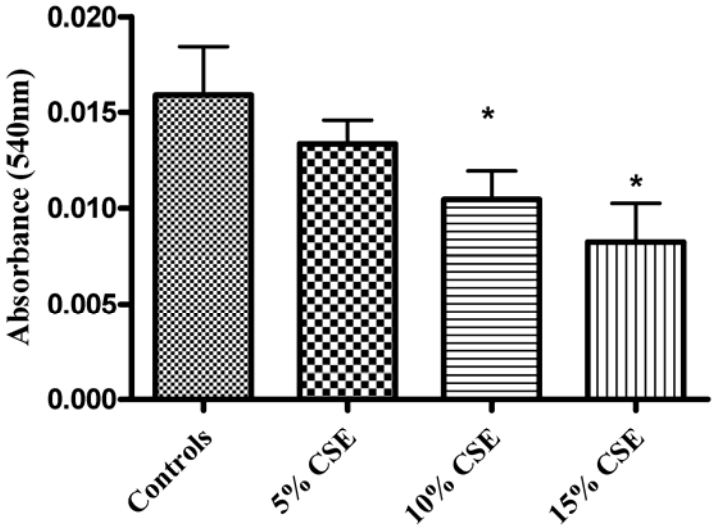


Figure 22: Effect of CSE on cell viability in isolated fetal rat lung fibroblasts. MTT activity was measured in fetal rat lung fibroblasts exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes. Cells not exposed to CSE were considered as controls. Level of absorbance was measured at 540nm. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

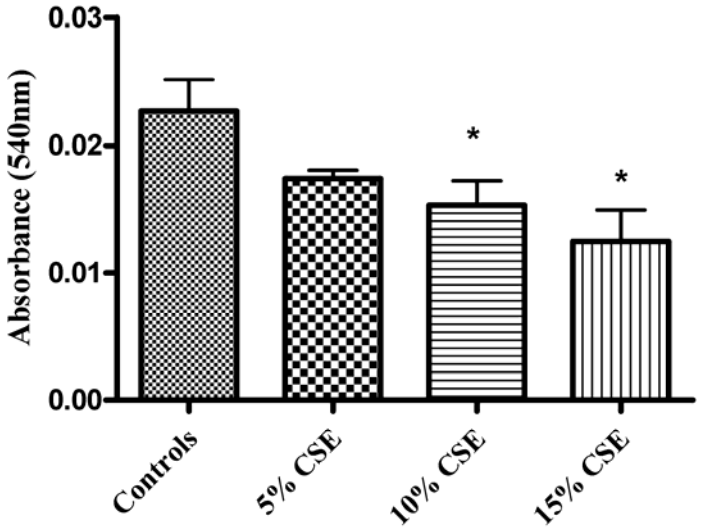


Figure 23: Effect of CSE on cell viability in isolated fetal rat lung type II AE cells. MTT activity was measured in fetal rat lung type II AE cells exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes. Cells not exposed to CSE were considered as controls. Level of absorbance was measured at 540nm. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

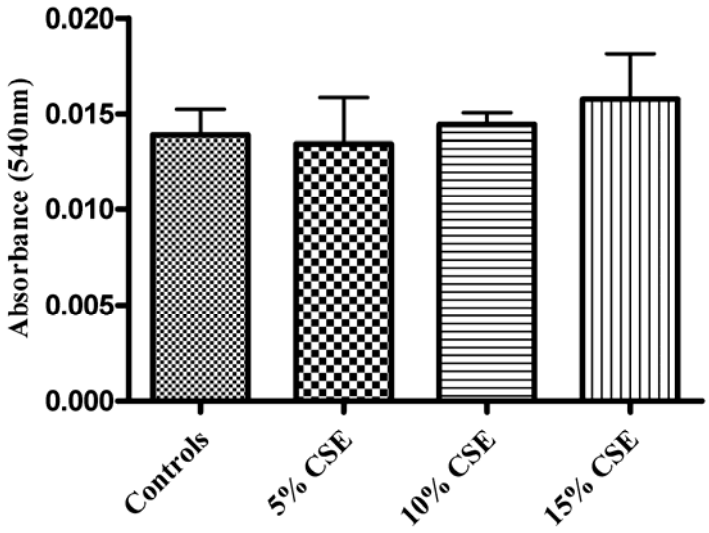


Figure 24: Effect of CSE on cell viability in human A549 cells. MTT activity was measured in human A549 cells exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes. Cells not exposed to CSE were considered as controls. Level of absorbance was measured at 540nm. Each bar represents the mean ± SEM of three experiments of 16 samples in each. No significant ($p<0.05$) differences were noted.

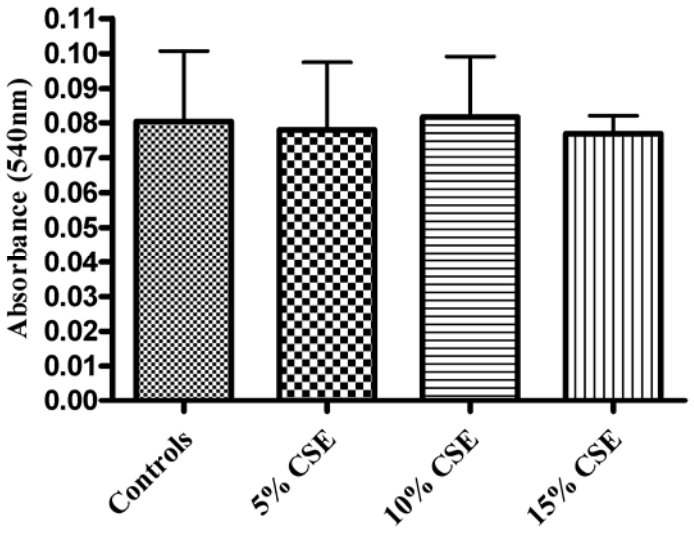


Figure 25: Effect of CSE on cell viability in rat PDL fibroblasts. MTT activity was measured in fetal rat PDL fibroblasts exposed to different concentrations of CSE (5%, 10% or 15%) (v/v) for 60 minutes. Cells not exposed to CSE were considered as controls. Level of absorbance was measured at 540nm. Each bar represents the mean ± SEM of three experiments of 16 samples in each. No significant ($p<0.05$) differences were noted.

3.6 Detection of caspase-3 activity in cells exposed to camptothecin

The caspase-3 activity was investigated in all cell types used in the present study when treated with different concentrations of camptothecin (4uM, 8uM, 10uM or 15uM) for 60 min. Camptothecin executes nuclear topoisomerase I mediated apoptosis, a process known to result in mitochondrial dysfunction leading to release of cytochrome c and the opening of mitochondrial permeability transition pores (Pommier, 2006). After 60 min of incubation the caspase activity was measured using a fluorometric assay kit. This is based on the detection of cleavage of DEVD-AFC substrate (AFC: 7-amino-4-trifluoromethyl coumarin). The DEVD-AFC substrate emits blue light (404nm). When the substrate is cleaved by caspase-3, free AFC emits yellow-green fluorescence (detection at 505nm). The amount of fluorescence was quantified using a fluorometric plate reader at 505 nm. The amount of fluorescence is directly proportional to the caspase-3 activity in cells.

Figure 26 shows caspase-3 activity in isolated fetal rat lung fibroblasts exposed to camptothecin, (4uM, 8uM, 10uM or 15uM) for 60 minutes. The enzyme activity in samples not exposed to camptothecin produce a reading of approximately 40 relative fluorescent units. Exposure to camptothecin at all concentration levels produced significantly elevated activity ($p<0.05$) of caspase-3.

Figure 27 shows enzymatic activity of caspase-3 in isolated fetal rat lung type II AE cells exposed to camptothecin (4uM, 8uM, 10uM or 15uM) for 60 minutes. The caspase-3 activity in control fetal rat lung type II AE cells was observed to be double than the control cells of fetal rat lung fibroblasts. The enzyme activity of caspase-3 in cells

exposed to camptothecin at all concentration levels was significantly elevated ($p<0.05$), in contrast to the cells not exposed to camptothecin.

Figure 28 illustrates caspase-3 activity in human lung A549 cells exposed to different concentrations of camptothecin (4uM, 8uM, 10uM or 15uM) for 60 minutes. The enzymatic activity in cells exposed to 10uM and 15uM displayed a significant decrease ($p<0.05$) in caspase-3 activity in contrast to those not exposed to camptothecin. Moreover, no significant differences in caspase-3 activity were noted in cells exposed to 4uM concentration of camptothecin.

Figure 29 shows caspase-3 activity in rat PDL fibroblasts exposed to camptothecin (4uM, 8uM, 10uM, and 15uM) for 60 minutes. Caspase-3 activity in rat PDL fibroblasts exposed to camptothecin at concentration of 8uM, 10uM and 15uM was significantly increased ($p<0.05$) when compared to those cells not exposed to camptothecin. No significant differences in caspase-3 activity were observed in cells exposed to 4uM concentration of camptothecin.

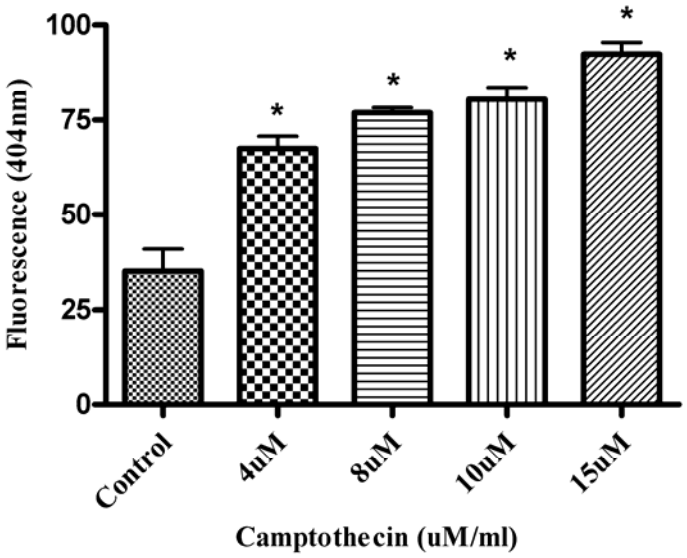


Figure 26: Effect of camptothecin on caspase 3 activity in isolated fetal rat lung fibroblasts. Fluorometric assay to assess the activity of caspase-3 in fetal rat lung fibroblasts treated with camptothecin at different doses (4uM, 8uM, 10uM and 15uM) for 60 minutes in 37^{o}C incubator. Cells not exposed to camptothecin were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

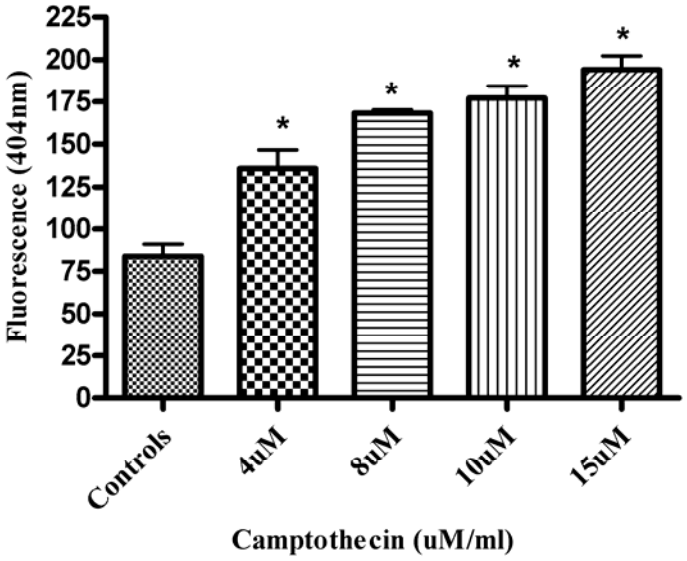


Figure 27: Effect of camptothecin on caspase 3 activity in isolated fetal rat lung type II alveolar epithelial cells. Fluorometric assay to assess the activity of caspase-3 fetal rat in lung type II alveolar epithelial cells treated with camptothecin at different doses (4uM, 8uM, 10uM and 15uM) for 60 minutes in 37^{o}C incubator. Cells not exposed to camptothecin were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

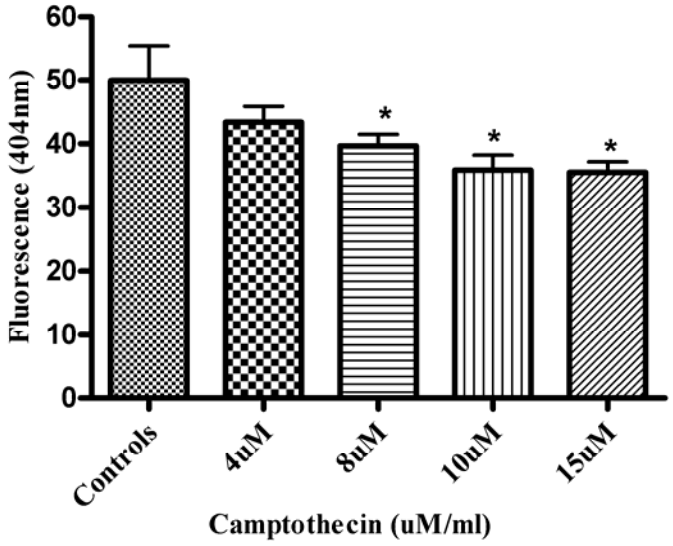


Figure 28: Effect of camptothecin on caspase 3 activity in human lung A549 cells. Fluorometric assay to assess the activity of caspase-3 in human lung A549 cells treated with camptothecin at different doses (4uM, 8uM, 10uM and 15uM) for 60 minutes in 37^{o}C incubator. Cells not exposed to camptothecin were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

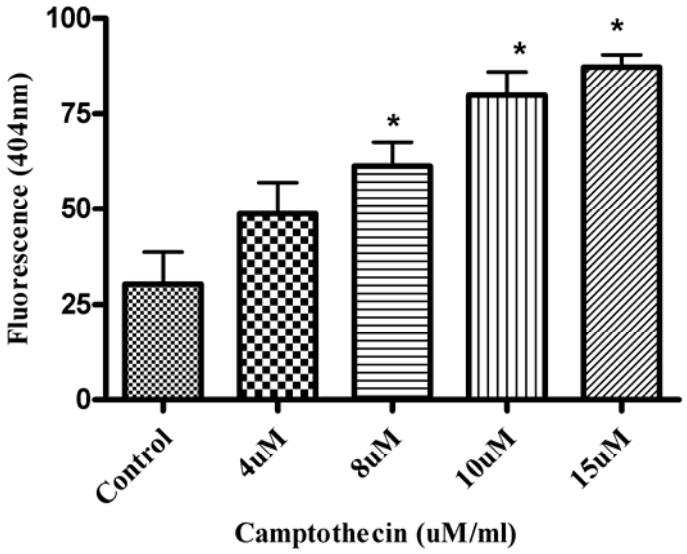


Figure 29: Effect of camptothecin on caspase 3 activity in rat periodontal ligament fibroblasts. Fluorometric assay to assess the activity of caspase-3 in rat periodontal ligament fibroblasts treated with camptothecin at different doses (4uM, 8uM, 10uM and 15uM) for 60 minutes in 37^{o}C incubator. Cells not exposed to camptothecin were considered as controls. Each bar represents the mean ± SEM of three experiments of 16 samples in each. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

3.7 Detection of caspase 3 activity by Western blot

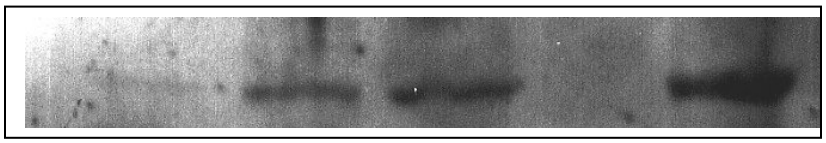
The expression of caspase-3 in isolated fetal rat lung fibroblasts, fetal rat type II AE cells and adult rat PDL fibroblasts was analyzed by SDS-PAGE and Western Blotting. Lysates of cells not exposed to CSE were considered as controls. The results of all cell types used in the present study showed that an antibody specific for detecting active form of caspase-3 bond to the protein band with relative molecular mass of 17 kDa, which is the accepted molecular mass of active caspase-3 (Kilic et al., 2002). The band intensity was measured by densitometric analysis.

Figure 30 shows the densitometric analysis of caspase-3 expression in fetal rat lung fibroblasts. On a protein level caspase-3 expression was enhanced in fetal rat lung fibroblasts exposed to CSE when compared to the non exposed controls. The activity was significantly increased ($p<0.05$) in the samples exposed to 10% or 15% CSE.

Figure 31 shows the densitometric analysis of caspase-3 expression in fetal rat lung type II AE cells. On a protein level caspase-3 expression was enhanced in fetal rat lung type II AE cells exposed to CSE when compared to the non exposed controls. The activity was significantly increased ($p<0.05$) in the samples exposed to 10% or 15% CSE.

Figure 32 shows densitometric analysis of caspase-3 expression in PDL fibroblasts, no significant differences were observed in the intensity of the bands exposed to CSE.

(A)



Controls 5% CSE 10% CSE 15% CSE

(B)

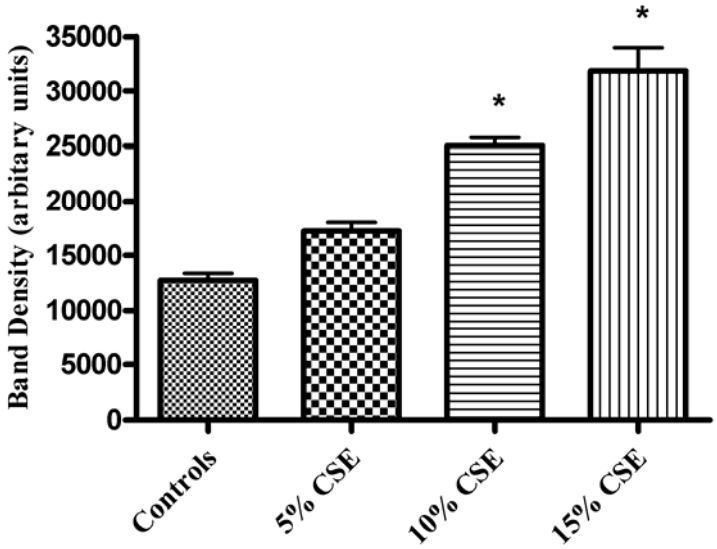


Figure 30: Western blot analysis of caspase-3 from lysates of isolated fetal rat lung fibroblasts exposed to different concentrations of CSE. Fetal rat lung fibroblasts were isolated on the 21st day of gestation and grown to 70 -80 % confluence in an incubator at 37^{o}C. The cells were exposed to different concentrations of CSE (5%, 10% and 15%) (v/v) in serum free media for 60 min keeping controls which were not exposed to CSE. **(A)** Lane 1: cell lysates not exposed to CSE. Lane 2: cell lysates exposed to 5% CSE. Lane 3: Cell lysates exposed to 10% CSE. Lane 4: Cell lysates exposed to 15% CSE. **(B)** Densitometric analysis of band intensity shows each bar represents the mean ± SEM of three experiments. (*) indicates significantly different from the corresponding controls ($p<0.05$).

(A)

Controls **5% CSE** **10% CSE** **15% CSE**

(B)

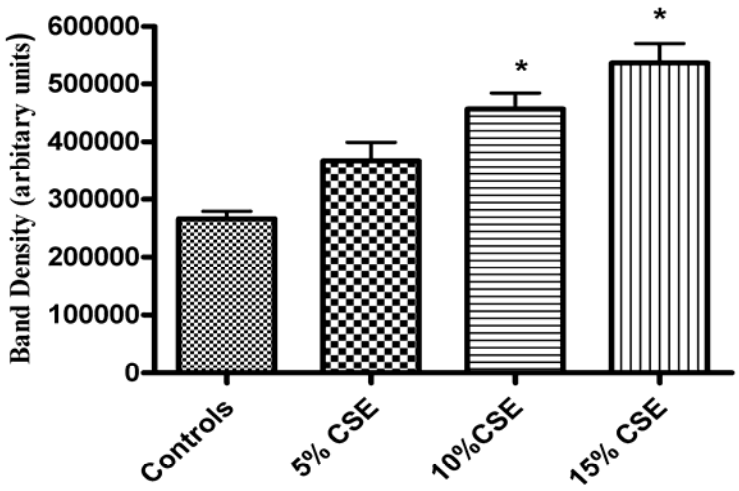
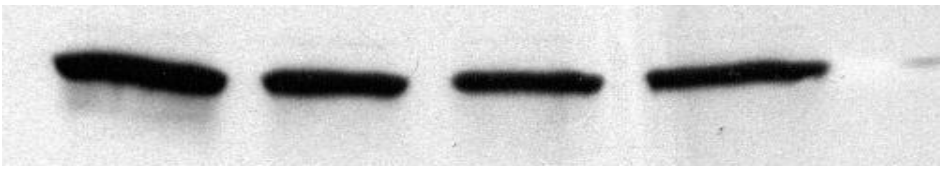


Figure 31: Effect of CSE on caspase-3 expression in fetal rat lung type II AE cells
(A) Representative Western bolt showing caspase-3 expression in fetal rat lung type II AE cells treated with 5%, 10% and 15% (v/v) CSE for 60 min. Cells not exposed to CSE were considered as controls. **(B)** Densitometric analysis of band intensity shows each bar representing the mean ± SEM of three experiments. (*) indicates ($p<0.05$) significantly different from the corresponding controls.

(A)



Controls **5% CSE** **10% CSE** **15% CSE**

(B)

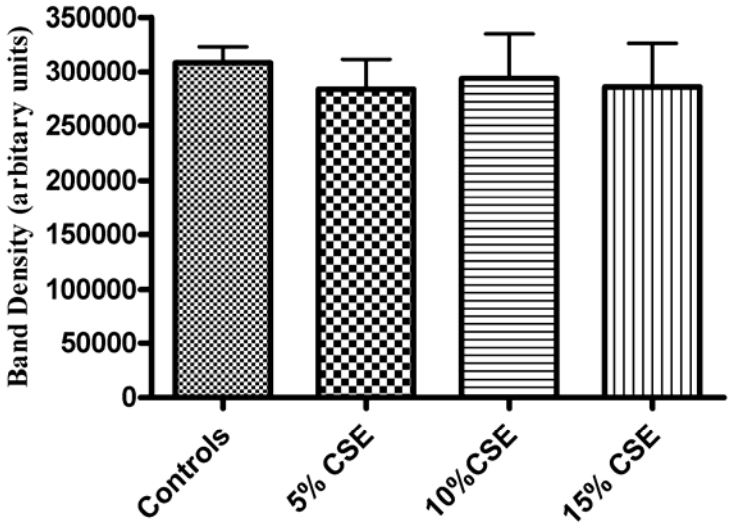


Figure 32: Western blot analysis of caspase-3 from lysates of rat Periodontal ligament fibroblasts exposed to different concentrations of CSE. Rat PDL fibroblasts cultured and grown to 70 -80 % confluence in an incubator at 37^{o}C. The cells were exposed to different concentrations of CSE (5%,10% and 15%) (v/v) in serum free media for 60 min keeping controls which were not exposed to CSE. **(A)** Lane 1: cell lysates not exposed to CSE. Lane 2: cell lysates exposed to 5% CSE. Lane 3: Cell lysates exposed to 10% CSE. Lane 4: Cell lysates exposed to 15% CSE. **(B)** Densitometric analysis of band intensity shows each bar represents the mean ± SEM of three experiments. No significant ($p<0.05$) differences were noted.

CHAPTER FOUR

DISCUSSION

CS contains more than 4000 chemicals and has been implicated in the pathogenesis of many pulmonary, oral and systemic diseases. Given that the lungs have a large surface area, the respiratory system is drastically affected by the insults of tobacco smoke. CS is identified as a major risk factor for lung cancer and chronic obstructive pulmonary disease (COPD) (Iribarren et al., 1999). By the year 2030, COPD is expected to be the fourth leading cause of death worldwide (Mathers and Loncar, 2006). Furthermore, cigarette smoking is recognized as the leading cause of preventable death (Mokdad et al., 2004). Approximately, 22 million women smoke in the United States and the prevalence of deaths due to lung cancer is more than deaths due to breast cancer (Patel, 2005). Smoking during pregnancy has a negative impact on the developing fetus, possibly due to fetal hypoxia (Cnattingius and Nordstrom, 1996). Maternal smoking is a risk factor for spontaneous abortion, intrauterine growth retardation and sudden infant death syndrome (Raza et al., 1999). Successful transition of intrauterine to extrauterine gas exchange states is one of the most critical challenges faced by the newborn. The lungs have to be sufficiently mature to face these challenges. Fetal lung development involves complex biochemical processes resulting in dramatic changes particularly as term gestation approaches and these changes continue even after birth (Zeltner and Burri, 1987). In humans, lung development starts during the fifth week of fetal life (Selman and Pardo, 2006). Morphogenesis of the lung involves a series of events and disruption during the stages may lead to congenital malformation (Perelman et al., 1981). Moreover, fetal

growth and development is influenced by intrauterine environment and governed by physical, environmental, genetic and hormonal factors (Joshi and Kotecha, 2007). This environment is carefully regulated and several factors play an important role including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) (Joshi and Kotecha, 2007). These hormones contribute to the type II alveolar cell differentiation, production and secretion of surfactant proteins (Joshi and Kotecha, 2007). Moreover, alterations in the intra-uterine environment due to exposure to cigarette smoke may affect lung function (Rona et al., 1993). It may result in decrease in alveolarization (Stocks and Dezateux, 2003), with a decrease in the formation of the pulmonary surfactant. The mechanisms underlying the pulmonary effects due to CSE exposure are not clearly elucidated. It is clear that lungs have to be sufficiently mature at birth in order to acquire normal gas exchange of oxygen and carbon dioxide for survival in the extra-uterine environment. The immature lung is susceptible to toxic substances in CS primarily because of the fewer number of macrophages leading to a poor immune response (Naik et al., 2001) and also due to a decrease in surfactant quantity, which is considered as the first line of defense against pathogens (Wright, 2003).

Many researchers agree that apoptosis plays an important role during all stages of lung development (pre and post-natal) and repair of lungs during injury (Li et al., 2004). Moreover, CS absorption starts in the oral cavity and many studies have provided convincing evidence that CS exposure is a risk factor for oral pathological conditions ranging form staining of teeth, discoloration of the gingival to degradation of tooth supporting structures (Chang et al., 2002b). The PDL is a unique soft connective tissue

which forms a fibrous joint, primarily responsible for anchorage of tooth to alveolar bone. Degradation of this connection between the tooth and bone may lead to loss of attachment, increased tooth mobility eventually leading to tooth loss (Krall et al., 1999). PDL fibroblasts are dominant cell types of the periodontium, responsible for the high turn over rate, rapid remodeling capacity during injury and repair. This necessitates continuous involvement of the apoptotic process for maintenance of PDL integrity. Furthermore, the presence of chronic periodontal conditions in a regular smoker may be a potential risk factor for spread of infection leading to the development of systemic diseases (Vellappally et al., 2007). Activation of caspase 3 is the key event in the downstream execution process of apoptosis. In the present study, we primarily examined the influence of CSE on apoptosis by activation of caspase 3 in primary lung cells. For comparison, we examined the influence of CSE on apoptosis by activation of caspase3 in human A549 cells and PDL fibroblasts.

Our animal model for the study consisted of randomly bred timed-pregnant Sprague Dawley rats. Fetal rat lung fibroblasts and type II AE cells were isolated on the 21st day of gestation. The term of gestation for these rats is 22 to 23 days (Shirley, 1984). It has been documented that smoking during the third trimester of pregnancy is a potential risk factor for a considerable retardation of fetal growth (Lieberman et al., 1994). Additionally, isolation of fetal lung cells during the late fetal period may help to clarify the role of apoptosis in the late fetal period by exposure to CS. Fetal lung type II AE cells produce surfactant during the later stages of lung development (Burri, 1984).

Phase contrast microscopy

Our present study included isolated fetal rat lung fibroblasts and type II AE cells cultured during the late gestational period or saccular stage of lung development. During this stage, expansion of the airspaces takes place with maturation of type II AE cells and thinning of interstitial spaces. Apoptosis plays a vital role in all the stages of normal lung development for proper maintenance of homeostasis (Del Riccio et al., 2004). Type II AE cells produces pulmonary surfactant which is a complex mixture of phospholipids and proteins. The pulmonary surfactant is a surface active material essential for lowering surface tension. The pulmonary surfactant plays an important role in defense against microorganisms and toxic substances in cigarette smoke. In the normal process of fetal development, oxygen is supplied to the fetus through the placenta and immediately after birth lungs become inflated and a normal respiratory pattern of breathing starts. Pulmonary surfactant is very critical for the adaptation of fetal respiration from a prenatal aqueous environment to neonatal air-breathing state after birth (Daniels and Orgeig, 2003).

In the present study, morphologically as observed through phase-contrast microscopy the fetal lung fibroblasts and PDL fibroblasts appeared to be similar, both the cells showed typical fibroblast-like appearance of being spindle shaped, elongated with a centrally placed nucleus. Nevertheless the proliferation rates of these cells differed, the fetal lung fibroblasts had faster rate of proliferation when compared to the adult PDL fibroblasts. Fibroblasts are mesenchymal cells which are responsible for many important functions during development and during adult life including secretion of ECM and in wound

healing process. There is evidence that fibroblasts cultured from different parts of the body have distinct phenotypes (Chang et al., 2002a). Moreover, fetal lung fibroblasts and PDL fibroblasts differ in functions. Fetal lung fibroblasts contribute significantly during developmental stages of lung as they play a vital role in the transition from saccular stage to alveolar stage. Moreover, during fetal lung development, epithelial-mesenchymal interactions are important for proper morphogenesis and differentiation of epithelial cells.

The A549 cells and type II AE cells differed immensely were considerably different in appearances. The type II AE cells presented a cobble-stone appearance and in this regards were similar to the A549 cells although the later were generally smaller in circumference. Furthermore, A549 cells and type II AE cells behave differently in culture, the fetal type II AE cells lose their unique phenotype after one or two weeks in culture.

Detection of caspase 3 activity in adherent cells exposed to cigarette smoke extract (CSE)

CS can be divided into two phases, the tar phase/particulate phase and gas phase, both the phases are rich sources of free radicals (Pryor, 1997). The tar phase contains free radicals which are stable and are retained on the filter when a cigarette is smoked. In contrast, the gas phase smoke contains less stable free radicals, which are more reactive and can penetrate through the filter (Ambrose and Barua, 2004). The gas phase can be further divided into mainstream smoke and side stream smoke. The mainstream smoke is directly inhaled through the cigarette and contains 8% tar and 92% gaseous components (Ambrose and Barua, 2004). Side stream smoke is the smoke emitted from the burning

end of the cigarette. Environmental smoke is a combination of side stream and exhaled mainstream smoke. Most of the components of cigarette smoke have been implicated in carcinogenesis (Pryor, 1997). The respiratory system is affected by ingredients in CSE either due to exposure to chemicals through direct inhalation or by absorption through systemic circulation (Lee et al., 2000). The exposure of CSE to fetus during gestation and lactation is mainly by systemic circulation transferred by means of maternal circulation during gestation and through mother's milk to the newborn.

In the present study CSE was prepared as described in materials and methods section using unfiltered research cigarettes, which includes both phases of the smoke components. Water soluble components remain in the media during exposure to cells. The solution considered as 100% CSE, which was further diluted for all assays. Since previous studies by other investigators in our lab have found these high levels of CSE to be cytotoxic. Apoptosis or programmed cell death is a normal and essential biological process for development, maintenance of homeostasis and host defense (Chang and Yang, 2000). The highly regulated process of apoptosis can be triggered by extra-cellular or intra-cellular signals resulting in cell death with the absence of inflammation. Moreover, apoptosis plays an important role during lung development and during postnatal adaptation of lung after birth for proper gas exchange (Kresch et al., 1998). Considerable research in the past supports a role of apoptosis in remodeling of lung tissue after lung injury (Martin et al., 2005). The deregulation of apoptosis may lead to development of lung disease. Increased apoptosis in the epithelial lung cells may lead to inadequate re-epithelialisation (Drakopanagiotakis et al., 2008). Similarly, PDL

fibroblasts are important for the maintenance of periodontal health and also contribute in the periodontal regeneration during repair process. The localization of caspase-3 in all cell types used in the present study was confirmed by immunofluorescent staining. Activation of caspase 3 is a prominent feature of an irreversible process of cell death, apoptosis. Both the major pathways of apoptosis (extrinsic and intrinsic pathways) are mediated by caspases. Caspase 3 is responsible for the characteristic morphological changes in apoptotic cells (Bressenot et al., 2009). Although caspase-3 is the central caspase in the caspase cascade that mediates the execution of apoptotic process of cell death (Kumar, 2007), little is known about the ability of tobacco generated smoke, either main stream or side stream smoke to activate caspase-3 in fetal lung cells, A549 cells or PDL fibroblasts. Previous studies reported cytototoxic effects of CSE on human lung fibroblasts (Nakamura et al., 1995) and human type II AE cells (Hoshino et al., 2001).

Our present study suggests that CSE induces apoptosis by activation of caspase-3 in developing fetal rat lung fibroblasts and type II AE cells *in vitro* in a dose-dependent manner. For comparison we used human A549 cells and rat PDL fibroblasts. Interestingly, these latter two cell types differed remarkably in their response to CSE compared to the fetal lung cells. It is speculated that the difference in the response of A549 cells could be because it is derived from a tumor cell line. Fibroblasts from different regions in the body may resemble in morphology, but these cells behave differently as they are heterogeneous in nature.

The observations of the present study suggest that the two cell types (fetal lung fibroblasts and PDL fibroblasts) used in the study differ from each other *in vitro*. Studies in the past have shown that fibroblasts of the periodontium show phenotypic differences from the gingival fibroblasts (Somerman et al., 1988). PDL fibroblasts originate from ecto-mesenchyme (McCulloch et al., 2000), specifically the investing layer of the dental follicle and the gingival fibroblasts originate from the follicular cells or the neighboring cells from the embryonic ridge mucosa (Lekic et al., 1997). It is speculated that the difference in the periodontal and gingival fibroblasts could be due the difference in the doubling times of these two cells (Somerman et al., 1988) and the difference in the origin of the cells (Lekic et al., 1997). Similarly, we speculate that the difference in response of fetal lung fibroblasts and PDL fibroblasts when treated with CSE *in vitro* could be due to the difference in the origin and functions of these two cell types. PDL fibroblasts are ecto-mesenchymal in origin (McCulloch et al., 2000) were as lung fibroblasts are mesenchymal in origin.

An increase in apoptosis or reduction of fetal rat lung fibroblast numbers through activation of caspase-3 when exposed to CSE is suggestive of a decrease in rate of alveolarization because these cells play an important role in transition from saccular stage to alveolar stage, with a resulting consequence of decrease in the total respiratory surface area. It has been suggested that there is a possibility that exposure of the fetus to CS during the phases of rapid cell development may predispose to the development of lung cancer (Maritz, 2009). Specific mechanisms underlying these changes are still not completely understood. It is likely that cellular changes due to exposure to tobacco smoke

may be attributed to inflammation, oxidative stress and mitochondrial damage (Yang et al., 2007). It has been estimated in the past that each puff of cigarette smoke contains 10^{14} free radicals. These radicals can induce oxidative stress and injury resulting in DNA damage. The ROS may induce cellular reactions via mitogen activated protein kinase (MAPK) pathways (Rahman, 2003).

Effect of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), an irreversible inhibitor of caspases

Previous studies have shown that Z-VAD-fmk is a broad spectrum caspase inhibitor which is widely used in the studies for evaluating the involvement of apoptosis in biological processes. Caspase inhibitors act by covalent binding to the cysteine of the active site (Kohler et al., 2002). Z-VAD-fmk is an irreversible inhibitor of caspases and has been reported to prevent the activation of caspase-3 during apoptosis (Hirsch et al., 1997). Z-VAD-fmk is considered as the key compound for studies based on apoptosis (Van Noorden, 2001). In the present study Z-VAD-fmk effectively inhibited caspase-3 activity in isolated fetal rat lung cells and rat PDL cells when treated with different concentrations of CSE. The caspase-3 activity was not inhibited in A549 cells. Z-VAD-fmk is a reliable inhibitor for caspase activity because caspases have stringent substrate specificity for aspartic acid in the P1 position. Previous studies have reported that in hepatic cells Z-VAD-fmk inhibitor resulted in complete inhibition of pro-caspase 3 activation (Chandler et al., 1998).

Effect of CSE on Cell Viability

In the present *in vitro* study, CSE exposure had differing effects depending on the cell type. In fetal lung fibroblasts and fetal lung type II AE cells CSE appeared to have an anti-proliferative effect. The analysis of the MTT formazan activity in these cells suggested that CSE depressed caspase activity in fetal lung fibroblasts and type II AE cells. A similar response in terms of MTT formazan activity was noted in malignant oral keratinocytes exposed to desferrioxamine (Lee et al., 2006). Interestingly, the activity of MTT in human A549 and adult rat PDL fibroblasts differed greatly after exposure to CSE. Again this difference may be associated with the origin of these cells, the maturity of these cells in the metabolic activity of these cells.

Detection of caspase-3 activity in adherent cells exposed to camptothecin

Camptothecin is an alkaloid which inhibits the DNA enzyme topoisomerase I (TOP I) (Oberlies and Kroll, 2004). TOP I is present in almost all somatic cells (Slichenmyer et al., 1993) and it is one of the key enzymes for DNA replication and consequent cell proliferation. It was isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca, Happy tree). Camptothecin has been widely used for cancer therapy as it is a strong inducer of DNA strand breaks in mammalian cells (Kunimoto et al., 1987), (Hsiang and Liu, 1988) leading primarily to cell death due to apoptosis. Some cancer cell types are resistant to camptothecin (Urasaki et al., 2001). According to the results shown in the present study camptothecin effectively increased caspase-3 activity suggestive of an increase in apoptosis in fetal rat lung fibroblasts, fetal rat lung type II AE cells and PDL fibroblasts. However results in A549 cells exposed to camptothecin were different

were capase-3 activity was decreased suggesting that there was a decrease in apoptosis induced by camptothecin. Our findings are consistent with other studies as A549 cells (human lung cancer cell line) were shown to be cross-resistant to camptothecin induced apoptosis (Sugimoto et al., 1990).

Detection of Caspase-3 Activity by Western Blot

Caspase-3 is synthesized as inactive 32kDa zymogens. Caspases have high specificity for cleavage after an aspartate residue resulting in fragments of a large sub-unit and small sub-unit. The importance of the role of caspases in apoptosis is well recognized (Chowdhury et al., 2008). Studies in the past confirmed that caspase knockout mice had profound defects in apoptosis resulting in developmental defects (Zheng and Flavell, 2000). More than a dozen caspases have been found but it is well documented that activation caspase-3 in a major event in apoptosis. Diverse cell types including hepatocytes, thymocytes and mouse embryonic fibroblast from caspase -3 knockout mice showed defective morphological and biochemical changes including defects in the characteristic morphological changes (Zheng et al., 1999) which are normally observed in apoptosis. Additionally caspase-3 deficient mice were perinatally lethal, meaning that these mice die before three weeks after birth (Zheng and Flavell, 2000). We analyzed the expression of active caspase-3 in all cell types exposed to CSE through western blotting. Our results suggest that caspase-3 expression was all cell types used in the present study respond to CSE induced apoptosis in a different way. Fetal rat lung fibroblasts and fetal rat lung type II AE cells show higher level of active caspase-3 expression when exposed to CSE depending on the dose. While adult rat PDL fibroblasts did not show drastic

changes when compared to the controls. The contrasting differences in the behavior of cells which was observed in the present study suggests that all cells do not respond to the effects of CSE in a similar way, probably due to the difference in the origin of the cells.

Conclusions

The focus of the present study was to characterize the apoptotic responses of developing fetal rat lung cells in response to cigarette smoke. The lung is a heterogeneous organ with a large number of different cell types. Exposure to CS in fetal life is detrimental and is evidently associated with pre-term delivery, spontaneous abortions, still birth, reduced lung function, decreased body weight, early neonatal mortality and an increased risk of development of asthma in childhood. All these effects may potentially be attributable to the exposure of the fetus to components of CS via two routes of exposure, through the circulation and amniotic fluid. Furthermore, smoking has a negative impact on the oral cavity and may be responsible for periodontitis, oral cancer, leukoplakia and high caries incidence (Vellappally et al., 2007).

The detection of apoptotic cells is important in the investigation of the role of apoptosis in cell proliferation and differentiation. Our present *in vitro* study suggests that exposure of fetal rat lung cells to CS may trigger apoptotic signals leading to cell death by activation of caspase-3 consequently alter cellular viability and proliferation in a dose dependent manner. We subsequently found that exposure of A549 cells and rat PDL fibroblasts to CS did not show a significant change in the activation of caspase-3. Although the present *in vitro* study gives an insight of the changes in cellular functions,

clearly further studies are required to elucidate the trigger factors inducing apoptosis in smoke exposed tissues. However, the identification of the critical role of caspases in the regulation of apoptosis has been beneficial in understanding the ways by which apoptosis contributes during tissue injury and repair.

Future Studies

The adverse effects of maternal smoking on respiratory health during childhood are well recognized, its potential impact on early lung development is less clear. Our observations from the present study raise a number of key questions such as, what is the magnitude of active maternal smoking during pregnancy on the development of lung? Another question which can be addressed here is why and how does CS cause apoptosis? What are the possible mechanisms and what may be the trigger factors which lead to caspase activation in smoke exposed cells? Future studies are clearly needed to evaluate the effects of smoke exposure in periodontal ligament cells.

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