Rat Periodontal Ligament Fibroblasts and Fetal Rat Pulmonary Cells Altered Gelatinase Expression upon Exposure to Cigarette Smoke Extract and Lipolysaccharide

By
Joumana Salame

A Thesis/Practicum 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

THE UNIVERSITY OF MANITOBA

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ABBREVIATIONS

aa: amino acids

APMA: 4-aminophenyl mercuric acetate

CSE: cigarette smoke extract

DG: day gestation

ECM: Extracellular matrix

EILISA: Enzyme Linked Immuno-Sorbent Assay

E/M: epithelial mesenchymal

HBSS: Hank's balanced salt solution

IH: immunohistochemistry

IU: Intra-uterine

LPS: Lipopolysaachride

MEM: minimum essential medium

MMP: matrix metalloproteinase

PDL: periodontal ligament

PAH: Polyaromatic hydrocarbons

TRE: Tumor promoter-responsive element

PEA3: Polyoma enhancer A binding protein-3 element

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ABSTRACT OF THE RESEARCH PROJECT

Background: Matrix metalloproteinases are a six group, 26 cell-membrane bound or secreted, inducible, Zinc-dependent proteolytic enzymes, which exhibit multi-domain structures and substrate specificities. They process or degrade a huge repertoire of extracellular molecules and thus play a pivotal role in a myriad of physiological processes, including many aspects of embryonic development, tissue morphogenesis and wound repair. MMPs aberrant and uncontrolled over expression results in pathological conditions, such as inflammatory diseases including periodontitis, gingivitis, chronic obstructive pulmonary disease (COPD), osteoarthritis and atherosclerosis. Furthermore they are considered major players during heart failure, fibrosis, tumor growth, metastasis and tissue invasion *(Nelson et al., 2000)*. On the other hand, tobacco smoking is a highly addictive social behavior and a major cause of morbidity and mortality (*Scott and Thliveris 2006*). It causes or exacerbates a multitude of pathological conditions and in both the adult and the fetus may have various detrimental effects. Tobacco smoke contains more than 4000 chemicals including a huge repertoire of addictive and/or toxic components including nicotine, benzene, cadmium, vinyl chloride, chromium and 2-naphthylamine, all of which are recognized carcinogens *(WHO)*. All of these agents have been implicated in a diverse array of pathological conditions. Nevertheless, the mechanisms by which tobacco smoke induces these pathological changes are not fully delineated. Several areas of particular relevance relate to smoke-induced connective tissue alterations and of course genetic changes. In the case of the former, smoke-induced oral cavity and skin related alterations in connective tissue have received some attention *(Liu et al., 2006; Morita, 2007)*. In both cases matrix metalloproteinases are implicated in

tissue degradation (*Yin et al 2000*) and reduced collagen synthesis. While such changes may not be life threatening, other smoke induced changes in organs such as the lungs (*Kim et al., 2004*) or heart *(Kuo et al., 2005)* carry with them potential for significant increases in mortality or morbidity. The direct exposure endured by the lungs to inhaled smoke, whether as primary smoke from the cigarette or as secondary from the environment, now generally termed passive smoking, may be associated with altered MMP gene in lung airway epithelium (*Lavigne and Eppihimer, 2005)* and is related to development of COPD or asthma (*Sampsonas et al., 2007).*

Hypothesis: We hypothesized that cigarette smoke may affect the viability and proliferation of the periodontal ligament fibroblasts and the fetal developing lung cells and may modulate specifically their gelatinases secretions. The rationale behind these model systems is that the fibroblasts of the ligament originate in part from the ectomesenchyme of the investing layer of the dental papilla. They are endowed with special properties that give the periodontium its resiliency, high turnover rate, rapid remodeling capacity and easy adaptability to the changing local conditions. All this necessitates MMPs continuous involvement to maintain its integrity and function. Furthermore, the oral cavity is the first site of exposure and contact with CSE. On the other hand, fetal lung development involves repetitive dichotomous branching of the duct system. This is dependent on tissue degradation and remodeling on a large scale and hence proteases activity (MMPs). Furthermore, this model system potentially can provide significant information on MMPs activation and function during the mid to late fetal period. It will also allow a direct comparison between the effects of smoking on fetal developing cells versus those of adult continually differentiating orally derived cells. Finally, it will enable us to relate periodontal diseases to systemic respiratory pathological conditions.

Methods: Rat periodontal ligament (rPDL) fibroblasts and fetal developing rat cells were isolated following protocols established in our laboratory and exposed *in vitro* to increasing concentrations of cigarette smoke extract (CSE) prepared by a modification of Carp and Janoff's method (*Carp and Janoff, 1978)* using 2R1 research cigarettes from Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington Kentucky, USA. The conditioned media were collected and applied to 10% polyacrylamide-gels impregnated with MMP-specific substrate (Gelatin A, Sigma Aldrich), employing a highly sensitive quantitative technique, gel zymography. This reveals specific molecular masses as well as clear bands of substrate degradation corresponding to MMP activity. Immunosorbent Enzyme-Linked Assays (ELISA Kits, R&D Systems) were performed to further identify and quantitate total MMP-secretion. CSE-induced cellular and morphological changes were assessed by phase contrast microscopic imaging, while cellular proliferation and viability were examined by tryptan-blue viability assay, formazan and crystal violet assays.

Results: Our results showed that cigarette smoke extract (CSE) with or without LPS altered our cells of interest gelatinases secretion, viability and proliferation in a somewhat concentration-dependent manner.

Conclusion: Our studies suggest that CSE with or without LPS may alter the rPDL and fetal rat lung fibroblasts and type II alveolar pneumocytes viability and proliferation and may modulate their gelatinases secretion. All this may result in changes in the local conditions which may have a huge impact on the extracellular matrix formation, renewal and turnover rates, remodeling capacity and developmental interactions at the cellular level.

1. TISSUE DEVELOPMENT

1.1 Embryogenesis

Embryology is the study of the development, growth and differentiation of an embryo, from a single cell to a complex independent living organism. In animals, it commences when a sperm fertilizes an ovum giving rise to a *zygote*. The latter undergoes a series of cleavages or divisions resulting in the formation of a 12-32 cell, solid-ball called a *morula*. Cells of the morula will divide further to become a 128-cell structure called a *blatocyst,* which will be implanted in the uterus within the first two weeks of conception. This period is known as the *proliferative period* and is followed by a 3-8 week long *embryonic period*, during which cellular proliferation and development proceed resulting in the formation of the *gastula*, where a C-shaped, 4mm embryo becomes recognizable. Three distinct germ layers, the endoderm, mesoderm and ectoderm will form and induce organogenesis around this time. During the 15th day gestation a groove called the *primitive streak* develops on the dorsal aspect of the embryonic mass, with a knot called *Hansen's knot* from which the notochord develops. The mesoderm will spread between the overlying ectoderm and underlying endoderm, except in the prechordal and cloacal membranes *(Avery, 2002).* Distal to the notochord, the mesoderm induces thickening of the overlying ectoderm resulting in the formation of the neural plate, which will subsequently form the neural tube. This tube will become partly segmented in the region of the developing hindbrain giving rise to eight bulges, the *rhombomeres*. A specific group of cells called the *neural crest* cells, neuroectoderm or ectomesynchyme, will emerge in a very precise pattern from these rhombomeres, separate from the overlying ectoderm and migrate into the developing head region, where they populate mesodermal outpouchings called the *branchial arches*, which in turn, contribute to the development of most of the head and neck structures *(Noden, 1986-1991, Sperber et al., 1994; TenCate, 1997)*. Organogenesis commences around the 5th gestational week, where the endoderm will give rise to the digestive system, bladder and the lung. The skeleton, blood system and muscles will develop from the mesoderm, while the ectoderm will lead to the formation of the nervous system and the skin. So toward the end of the embryonic (organ differentiation) period the embryo's face and other body parts will be clearly identified and the third and last prenatal developmental period, the *fetal period*, where organ growth and maturation take place, will continue up until birth *(Avery, 2002)*.

1.2 Teeth development: Odontogenesis

Odontogenesis refers to the development of teeth. It is initiated between the sixth and eighth prenatal week, following the signaling of the migrated and orally residing neural crest cells to the overlying first arch oral epithelium to thicken and form horse shoe shaped structures called the primary epithelial bands or laminae. These bands will subsequently divide into anterior vestibular laminae and posterior dental ones. Cells of each dental lamina will proliferate giving rise to a tooth germ, which will undergo three stages of development, the bud, cap and bell stage. During the *bud stage* the dental lamina cells divide resulting in a spherical bud shaped epithelial (enamel) organ, which will be surrounded by mesenchymal cellular condensation. The enamel organ cells will divide further to attain a concave cap shaped structure. The surrounding and underlying mesenchymal cells will proliferate further and will give rise to the embryonic dental pulp or the *dental papilla*. Cells surrounding the papilla and lying outside the enamel organ will grow and divide to form the *dental sac* or *follicle*, which is the precursor of the supporting dental tissues. Proliferation of the cap shaped tooth germ will finally acquire an invaginated bell morphology with four distinct highly specialized cells, the outer and inner enamel epithelium, which encircle the stellate reticulum and stratum intermedium cells. Collectively these cells will contribute to the formation of the future tooth enamel and its underlying dentine. The dental papilla will give rise to the dental pulp, while the tooth supporting and surrounding structures will arise from the dental follicle. 52 tooth germs will be formed. 20 will give rise to the primary deciduous teeth and 32 to the permanent dentition. The latter develops lingual to their primary predecessors, except the three permanent molar laminae, which arise at a more posterior position in the dental arch *(Ten Cate, 1997; Avery, 2002).*

1.3 The periodontium

The periodontium comprises the tooth supporting and surrounding tissues including the alveolar bone proper, the periodontal ligament and cementum. It develops from the above-mentioned dental follicular cells during tooth eruption and root formation. In addition to its supporting and formative functions, the dental sac surrounds, stabilizes and protects the tooth germ during development. Furthermore, it innervates and nourishes it *(Avery, 2002).*

1.4 The periodontal ligament

The periodontal ligament is a unique, multifunctional, soft connective tissue structure that connects the tooth cementum to the investing and surrounding alveolar bone. It endows the teeth with a rapid, easy adaptability to the frequent insults they endure. Furthermore, its exceptionally high turnover rate and remodeling capacity maintains the tooth-bone attachment and enables micro-movements and adjustments to changes by the teeth *(Beertsen et al., 1997)*

1.5 Periodontal ligament composition

The periodontal ligament (PDL) comprises different cell populations embedded in a mostly collagenous matrix. The cellular compartment encompasses endothelial cells, epithelial rests of *Malassez*, cells associated with the sensory system, bone associated cells, cementoblasts and fibroblasts *(Lambrichts et al., 1992; Lang et al., 1995; Linden et al., 1995; Sims, 1995; Beertsen et al., 1997)*. On the other hand, the PDL matrix is formed of collagen fibers that are arranged in a specific fashion and bonded to the surrounding bone and cementum by unique collagenous fibers known as *Sharpey's* fibers. Interstitial collagen types I, III and type V, which is either buried or found within the other collagen types, *(Becker J et al., 1991; Birk et al., 1988)* give the periodontium its architecture *(Butler et al., 1975; Wang et al., 1980; Huang et al., 1991)*. Minimal amount of collagen XII can be found in the PDL matrix and it is suggested to play a role in PDL remodeling and regenerative capacity *(Karimbux et al., 1995)*. Moreover, *Fullmer, (1958)* described another form of apicocoronally oriented fibers called the oxytalan fibers, with which collagen VI, that is believed to mediate cell attachment, is associated.

1.6 Periodontal ligament fibroblasts

These cells predominate in the periodontium. They are oval in shape with abundant cytoplasmic microtubules that endow them with the unique contractile and migratory capacities *(Beertsen et al., 1974; Bellows et al., 1981; Pender et al., 1991).* Fibroblasts are aligned parallel to the ligament collagen fibers, which they secrete. They are attached by fibronexus plaques to the collagen fibers on which they exert traction forces and degrade, thus modifying their direction and position. Therefore, fibroblasts play crucial roles in the PDL formation, turnover and function *(Hynes et al., 1981; Singer, 1979; Garant et al., 1982).* Moreover, their enzymatic secretions, such as MMPs, maintain the PDL integrity by coordinating its synthesis and breakdown. Furthermore, PDL fibroblasts constitute an intricate system of communication, as they are connected by numerous types of adherence and gap junctions, which enable their direct interactions and hence coordinate group functioning *(Beertsen et al., 1980; Shore et al., 1981)*

1.7 The respiratory system and its development

One of the most complex systems in the body is the respiratory system. It is divided into an upper conducting tract, which extends between the nostrils and the conducting bronchioles and lower respiratory tissues. The latter comprise the respiratory bronchioles, alveolar ducts, alveolar sacs and the alveoli where gas exchange takes place. This vast surface presents the largest bodily area exposed to the environment and is estimated to be close in size to that of a tennis court *(Harding et al., 2004).*

1.7.1 *Embryonic lung development*

Embryonic lung development starts around the 3rd week intrauterine (IU) life, when the embryonic foregut endoderm proliferates forming a primordium pouch that invades the adjacent mesoderm. It grows in a ventral and caudal direction giving rise to the primitive trachea. By the end of the 4th week, this diverticulum divides into two sacs, the future lung buds *(Nelson, 1985)*. These buds will grow in a tree-like dichotomous branching fashion, known as *branching morphogenesis*. Endodermal epithelial-cellular interactions with the surrounding mesenchymal cells, through several extracellular matrix components and growth factors control and regulate early lung development and its dichotomous branching *(Spooner et al., 1970; Harding et al., 2004)*. Around the 4th week, the larynx starts to take form *(O' Rahilly et al., 1973)*, two tiny saccules, on the left side and three on the right corresponding to the future lung lobes and future lobar bronchi appear. Between the sixth and eighth week IU, lobar bronchi will undergo segmentation giving rise to the first (48 day gestation) and second generation (51 day gestation) of subsegmental bronchi *(Nelson, 1985; Mc Donald, 1997)*. Subsequently, the lung enters the first stage of its *fetal*-prenatal development, which is mainly divided into several stages: the pseudoglandular, canalicular, saccular and the alveolarization stage, which is further subdivided into three phases *(Hodson, 1977; Nelson, 1985)*.

1.7.2 *Fetal lung development*

1.7.2.1 *The pseudoglandular phase* starts around the 52nd day of gestation and terminates during the 16th week IU. The bronchopulmonary segmentation that started during the fetal-period continues resulting in the formation of most of the pulmonary conducting airways. So toward the end of this phase the bronchiolar tree ends in structures known as the terminal bronchi, which in turn give rise to 3-4 generation of respiratory bronchioles. Segmentation of the latter will terminate in the presumptive alveolar ducts. The airway duct epithelia start to differentiate, except in their most distal aspect, where they maintain their nonspecific, tall columnar epithelial morphology, until the following canalicular phase *(Burri, 1997).* During this period the lung assumes the appearance of a gland and hence the name pseudoglandular *(Hodson, 1977; Nelson, 1985).*

1.7.2.2 *The canalicular phase* commences approximately after the 10 week long pseudoglandular period. In human, this lasts around 16-24 weeks *(Burri, 1997).* The extensive multiplication of capillaries that takes place during this stage, results in canalization of the lung parenchyma and hence the name "*canalicular*" *(Mc Donald, 1997).* This phase is marked by the appearance of acini, an increase in vascularization, partial differentiation of the alveolar epithelium and a decrease in lung connective tissue. Flattening out of the glycogen-rich, cuboidal tubule-lining epithelial cells with rearrangement of the junctional complexes creates a thinner air-blood barrier in preparation for a saccular phase. This signals the naissance of the lamellar body rich, surfactant manufacturers, type II- alveolar pneumocytes and type I epithelial cells *(Harding et al., 2004; Mc Donald, 1997).*

1.7.2.3 The *saccular phase* is the third morphological differentiation period, which spans about 24 weeks in human, during which the peripheral airways form terminal clusters of widened airway spaces called saccules *(McDonald, 1997).* These structures will develop, lengthen and branch *(Nelson, 1985; Burri, 1997) and* the vascular network will continue to expand. The interstitium between these airspaces thins during this phase resulting in a steady increase in the spaces available for gas exchange in the distal part of the lung *(Nelson, 1985; Burri, 1991; McGowan et al., 1997; Del Riccio et al., 2004).*

1.7.2.4 *Alveolarization* starts before birth around the 36 week IU and is likely to terminate between the 18th-24th month posnatally. During this phase, secondary septa start protruding from the walls (primary septa) of the terminal sacs, resulting in the formation of the alveolar ducts, which in turn give rise to the alveolar sacs and alveoli *(Harding, 2004).* Septation in humans starts *in utero* and ends between 2-5 years of age, while in the rat it occurs during the first three postnatal weeks *(Burri, 1991).* Thinning and lengthening of these septa mark the commencement of the second phase of alveolarization, where a decrease in the mesenchymal component, an increase in the lung volume and remodeling of the capillaries are recognizable. The third phase of alveolarization is the growth stage, which is marked by a continual increase in lung volume at the expense of its septa, thus increasing further the air exchange surface. Mammalian lungs continue to grow postnatally. Usually their growth ends prior to the termination of the growth of long bones *(Harding, 2004. Burri, 1991).*

1.8 The cellular population of the lung

The lung is the main organ of the respiratory system. It performs versatile respiratory and nonrespiratory functions. Nonrespiratory functions include filtration of small blood clots, protection of the heart by flanking it and acting as a shock absorber and altering the arterial blood concentration of biologically active substances and drugs used in medicine. Whereas the respiratory functions encompass conduction, filtration and warming of the inhaled air and above all exchange of gases. These functions are carried out by an intricate population of more than 40 specialized cell types. The respiratory system extending between the nose and the bronchi is lined with a pseudostratified, ciliated columnar epithelium. Simple, ciliated columnar to simple cuboidal epithelial cells line the bronchioles, while the alveolar ducts and the alveoli are covered by a simple squamous epithelium composed of type I and type II alveolar cells. Type I alveolar cells cover about 95% of the alveolar surface. They are terminally differentiated cells, which are unable to divide. They form a thin, blood-gas barrier through which gas exchange occurs. Type II alveolar cells are cuboidal in shape with short microvilli-covered apical surfaces. They are considered the stem cells which are capable of differentiating into both type I and type II alveolar cells. They secrete the pulmonary surfactant, which decreases the surface tension at the alveolar surface, allowing the alveoli to expand during inspiration, and preventing their collapse at expiration. Dispersed within this lining epithelium are olfactory cells and goblet mucous-secreting cells. Furthermore, within the lining of bronchioles special mitotically active cells can be found called *Clara* cells. These cells divide and differentiate to form both ciliated and nonciliated epithelial cells. They secrete glycosaminoglycans, a major component of the extracellular matrix and metabolize airborne toxins via the smooth endoplasmic reticulum cytochrome P-450 enzymes. Surrounding and supporting the lung is the pulmonary interstitium, which contains a rich mesenchymal cell population including myofibroblasts, undifferentiated fibroblast-like cells and interstitial fibroblasts. The latter are the most prevalent cell type, accounting for 52-62% of the lung parenchyma population *(Pinkerton et al., 1982)*. These spindle shaped cells with oval nuclei *(Ross et al., 1968; Khun, 1978)* play crucial roles in maintaining the integrity of the interstitial connective tissue by secreting connective constituents, such as collagen fibers, fibronectins and proteoglycans. Furthermore, their interaction with the overlying pneumocytes are critically important in modulating fetal lung development.

2. MATRIX METALLOPROTEINASES

2.1 Overview of Matrix metalloproteinases

Matrix metalloproteinases (MMPs), also known as matrixins, are zinc-dependent neutral endopeptidases, belonging to a large family of proteases known as metzincin. The metzincins, descendants of the metalloproteinase superfamily, are characterized by a conserved methionine-turn located beneath the zinc in the active site and a (HEBXHXBGBXHZ) zinc-binding motif. In this motif B is a hydrophobic residue, X is a variable residue, and Z is a family-specific amino acid, which is serine in all the metzincins' derivatives, the serralysins, astacins and ADAMs/adamalysins However, few MMPs reflect a similar structure with regards to the serine residue (*Stöcker et al., 1995*).

MMPs constitute a multigene family of over 25 secreted and cell surface vertebrate proteolytic enzymes, 22 of which are fully characterized at the protein level in humans (*Visse et al., 2003*). They process or degrade a huge repertoire of pericellular substrates and structural extracellular matrix proteins, including interstitial and basement membrane collagens, proteoglycans, fibronectins and laminins. In addition, they have been shown to degrade clotting factors, chemotactic molecules, proteinases, proteinase inhibitors, latent growth factors, growth factor-binding proteins, cell-cell adhesion molecules and cell surface receptors. The degradation of the extracellular matrices alters the stromal environment, modifies cellular differentiation and interaction with their immediate surroundings, thereby permitting multi-cellular organisms to develop, grow and function normally. This may impact on the composition of the cell matrix and paracrine cellular signals. Therefore, MMPs control basic processes such as cellular differentiation, morphogenesis, migration and death in anchorage-dependent cells and influence diverse physiologic and pathologic processes. Moreover, aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases and cancers may be MMP-dependent conditions. This necessitates and dictates a requirement for a tight regulating and controlling enzymatic activity, to avoid deleterious over-expression and consequently hyperfunction. MMPs, like other proteins, are regulated at various levels including gene transcription, mRNA processing, exporting, translation, stability and degradation. Protein secretion, localization, catabolism and clearance levels are also regulated. These molecules are synthesized as zymogens, inactive latent enzymes, which need activation to carry out their degrading function (*Harper et al., 1971*). Their intracellular as well as extracellular activations are complex, unique and highly regulated processes. Furthermore, posttranscriptional control is achieved by endogenous inhibiting proteins, by such molecules as α-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) (*Bauer et al., 1975*). Four TIMPs have been shown to bind MMPs in a 1:1 ratio regulating their proteolytic activity. Disruption of this MMPs/TIMPs balance may result in serious pathological conditions like tumor growth, tumor metastasis and invasion, pulmonary emphysema, fibrosis, heart failure, osteoarthritis and atherosclerosis.

2.2 Historical background

The discovery of a B. *fragilis* metal-containing enzyme suggested that MMPs existed even before bacteria, which have existed for more than 3.5 billion years *(Franco et al., 1997*). Similarly MMPs with a high degree of domain homology, have been identified in various, long-time existing species, such as plants, invertebrates, as well as vertebrates.

However, the first discovery that MMPs display collagenolytic-activity was in 1962, by the pioneers in the MMP field, Jerome Gross and Charles Lapiere, who demonstrated the production of a diffusible collagenolytic factor operating at neutral pH and 27°C on undenatured collagen, in living anuran tadpole tail tissues in culture. The tadpole was selected for their study, because of the rapid dissolution of large tissue masses during natural or hormone-induced metamorphosis. This they detected and measured by an increasing area of lyses around the explant and by the hydroxyproline or ^{14}C content of degraded radioactive collagen (*Gross & Lapiere, 1962*). Following their report, there has been a rapid, burgeoning development of this field, where more than 1200 MMP- related articles are published every year.

2.3 Nomenclature

Vertebrate MMPs have been named according to their presumed substrate, or classified by sequential, mainly chronological numbers, which run from MMP-1 to MMP-28, missing MMP-4, 5 and 6.To date, there are 25 well-characterized MMPs, 22 of which are of human origin, while the remaining three have been cloned from xenopus and chicken MMPs; those latter ones have not yet been fully sequenced (*Klaus, 2005).*

2.4 Classification and substrate specificity

MMPs are grouped mainly according to their function, substrate specificity, sequence similarity and domain organization into:

2.4.1 *Collagenases*

MMP-1 (collagenase-1, interstitial collagenase), 57 kDa.

MMP-8 (collagenase-2, neutrophil collagenase), 75 kDa.

MMP-13 (collagenase-3).

MMP-18 (collagenase-4, xenopus collagenase).

A common feature of these enzymes is their ability to cleave interstitial collagen types I, II and III at a specific site three fourths of the sequence away from the N-terminus. They can also digest a number of other ECM and non-ECM molecules. In breast cell carcinoma, MMP-1 activates protease-activated receptor-1(PAR-1), by cleaving the same Arg-Ser bond as thrombins, promoting cellular growth and invasion *(Nagase et al., 2006*).

2.4.2 *Gelatinases*

MMP-2 (Gelatinase A, type IV collagenase), 72 kDa.

MMP-9 (Gelatinase B), 92 kDa.

Both enzymes digest gelatins and cleave laminins, aggregans and collagen type IV, Vand XI *(Nagase et al 2006*), but are mainly effective against type IV collagen (*Klaus, 2005*). MMP-2 digests type I, II and III collagens (*Nagase et al., 2006*) and is mainly secreted from skin fibroblasts and human melanoma cells. On the other hand, keratinocytes and polymorphonuclear leukocytes produce MMP-9.

2.4.3 *Stromelysins*

MMP-3 (Stromelysin-1), 55-60 kDa.

MMP-10 (Stromelysin-2), 55-60 kDa.

MMP-11 is stromelysin-3, but is usually grouped with other MMPs, because its substrate specificity diverges from that of MMP-3 (*Visse et al., 2003*) and its propeptide-domain.

Furin recognition motif is responsible for its intracellular activation *(Nagase et al., 2006)*. MMP-3 activates a number of pro-MMPs, such as MMP-1(*Visse et al., 2003*) and has a higher proteolytic efficiency than MMP-10, in general. Stromelysins act with broad specificity on noncollagen matrix molecules, laminins, fibronectins and elastins *(Klaus, 2005)*.

2.4.4 *Matrilysins*

MMP-7 (matrilysin-1) is the smallest of the MMPs. It is synthesized in epithelial cells and secreted apically (Nagase et al., 2006).

MMP-26 (matrilysin-2).

Both MMPs are also called endometases. They degrade ECM components. MMP-7 processes cell surface molecules, such as: pro-α- defensin, fas ligand, pro- tumor necrosis factor α and E-cadherin *(Visse et al., 2003)*. MMP-26 is expressed in normal endometrial cells and in some carcinomas and is largely stored intracellularly (*Nagase et al., 2006).*

2.4.5 *Membrane- type MMPs* (MT-MMPs)

MT-MMPs are divided into two subgroups:

2.4.5.1 -Type-I transmembrane proteins:

MMP-14 (MT1-MMP).

MMP-15 (MT2-MMP).

MMP-16 (MT3-MMP).

MMP-24 (MT5-MMP).

2.4.5.2 -Glycosylphosphatidylinositol(GPI) anchored proteins:

MMP-17 (MT4-MMP).

MMP-25 (MT6-MMP).

Membrane-type MMPs can digest a number of ECM molecules and all are capable of activating proMMP-2, except MT4-MMP. MT1-MMP has a collagenolytic activity on type I, II and III collagens and plays an important role in angiogenesis (*Sounni et al., 2002).* MT5-MMP is brain specific and mainly expressed in the cerebellum. MT6-MMP is expressed almost exclusively in peripheral blood leukocytes and in anaplastic astrocytomas and glioblastomas *(Visse et al., 2003).*

2.4.6 *Other MMPs*

Seven MMPs are not classified in the above categories.

2.4.6.1 *MMP-12, Metalloelastase, is* expressed mainly in macrophages and is essential for macrophage migration (*Nagase et al., 2006).*

2.4.6.2 *MMP-19* digests many ECM molecules including components of the basement membrane. It is found in the lymphocytes and plasma cells of patients suffering from rheumatoid arthritis. MMP-19 expression is increased in systemic lupus erythematosis an inflammatory, multisystemic, autoimmune disease of the connective tissue, which affects the kidneys, spleen, and various other organs. It is characterized by fever, skin lesions, joint pain or arthritis, and anemia *(Nagase et al., 2006).*

2.4.6.3 *MMP-20, Enamelysin* is localized within the newly formed tooth enamel. It digests its main ECM low-molecular-weight protein, amelogenin. This protein regulates the initiation and growth of hydroxyapatite crystals during the mineralization of enamel. It also helps in organizing enamel rods during tooth development and aids in the development of cementum, by directing cells that form cementum to the teeth root-surfaces. Mutation of the cleavage site of the pro-MMP-20 molecule leads to *amelogenisis imperfect,* a genetic disorder of tooth development. This condition causes teeth to be unusually small, discolored, pitted or grooved, and prone to rapid wear and breakage. Other dental abnormalities are also possible, and the defects vary among affected individuals. These problems can affect both primary (baby) and permanent teeth *(Visse et al., 2003).*

2.4.6.4 *MMP-22* has been cloned from chicken fibroblasts. A human homologue is identified, but its function is still not known *(Visse et al., 2003).*

2.4.6.5 *MMP-23* is mainly expressed in the reproductive system. It is suggested to be a type-II membrane protein, with the transmembrane domain in the N-terminus part of the propeptide. It lacks the cysteine switch motif in the prodomain, as well as the hemopexin domain. MMP-23 has an immunoglobulin-like domain and a furin recognition motif in its propeptide, so it is cleaved by proconvertases in the Golgi apparatus and released as an active enzyme into the extracellular spaces *(Visse et al., 2003).*

2.4.6.6 *MMP-28, Epilysin,* is the most recently discovered MMP, expressed mainly in keratinocytes and may have a role in tissue homeostasis and wound repair *(Visse et al., 2003).*

2.4.6.7 *MMP-4, 5, 6 (the missing enzymes)*

These enzymes were named directly after their discovery, yet it was determined subsequently, that they were the already existing enzymes *(Woessner and Nagase, 2000).*

2.5 Gene structure

MMP-genes are highly conserved. They exhibit remarkable similarity among species, where comparable exons are of similar sizes with closely conserved exon-intron junctions. Collagenases, stromelysins 1 and 2 and the human macrophage metalloelastase (HME) genes, each contains 10 exons and 9 introns in 8-12 kbp of DNA *(Belaaouaj et al., 1995; Das et al., 2003).* On the other hand, MMP-7 (matrelysin-1) lacks exons 7 to 10, which encode the hemopexin-like domain, as well as most of the hinge region encoding exon-6. Gelatinases A and B genes are 26-27 kbp respectively, and contain three additional exons, each of which encodes a fibronectin-like collagen binding domain *(Huhtala et al., 1991).*

2.6 Chromosomal location

MMP-1:11q22-q23
MMP-2: 16q13
MMP-3:11q23
MMP-7: 11q21-q22
MMP-8: 11q21-q22
MMP-9 : 20q11.2-q13.1

MMP-10: 11q22.3-q23
MMP-11: 22q11.2
MMP-12: 11q22.2-q22.3
MMP-13 : 11q22.3
MMP-14, MT1-MMP: 14q11-q12
MMP-15, MT2-MMP: 15q13-q21
MMP-16, MT3-MMP: 8q21
MMP-17, MT4-MMP: 12q24.33
MMP-18: not found in humans
MMP-19: 12q14
MMP-20, Enamelysin: 11q22.3
MMP-23:1p36.3
MMP-24, MT5-MMP: 20q11.2
MMP-25, MT6-MMP: 16p13.3
MMP-26: 11p15
MMP-27: 11q24
MMP-28: 17q21.1
(Nagase et al., 2006).

2.7 General structural features of MMPs, Figure 1

2.7.1 *N-terminal signal sequence*

MMPs are synthesized with a hydrophobic, 18-30 amino acid residue signal peptide, which begins with a Met. Removal of this sequence, upon insertion of the MMP into the endoplasmic reticulum, yields a latent proenzyme *(Klaus, 2005; Woessner and Nagase, 2000).*

2.7.2 The *prodomain*

This domain extends from the N-terminus created after removal of the signal-peptide to the beginning of the catalytic domain, typically spanning 80 residues. It has a highly conserved cysteine switch sequence PRCGVPD, positioned directly opposite to the zinc atom of the catalytic domain and bound to it by a sulfhydryl group (–SH) of Cys73, in a manner that covers the active site *(Springman et al., 1990).* The purpose of this domain is to maintain latency of the enzyme until an activation signal recieved *(Das et al., 2003).*

The C-terminal segment that connects the prodomain to the catalytic one, is not sharply defined and is susceptible to autoproteolytic cleavage or cleavage by proconvertases, such as furin and sheddases *(Klaus, 2005)*. Upon activation, this domain is cleaved in several steps *(Das et al., 2003)*. The propeptide is not necessary for protein folding. This is based on the ease of refolding after removal of the SDS denaturing effect during zymography *(Woessner and Nagase, 2000)*.

2.7.3 *Catalytic domain*

This domain is a C-terminal in matrilysins, but is followed by a hemopexin domain in all other MMPs. It is linked to the prodomain and consists approximately of 175 residues. Its C-terminal contains two Zn atoms, a structural and a catalytic one and two or three calcium atoms, which are located approximately 12Å from the catalytic Zn. The catalytic zinc is bound to the highly conserved sequence, HELGHXXGXXH, by three His residues and is essential for the proteolytic activity of MMPs *(Massova, 1998)*. The Zn atoms together with the calcium ions are required for the stability and expression of the enzymatic activity of matrixins *(Nagase et al., 1999)*. This domain also contains a conserved, eight residue long, Met-turn after the zinc binding motif. This turn forms a base that supports the structure around the catalytic zinc atom *(Nagase et al., 2006)*. The crystal structure of this domain shows a shallow active-site cleft notched into the front surface separating the smaller 'lower subdomain' from the larger 'upper subdomain'. This cleft extends horizontally across the molecule and binds a peptide substrate from left to right *(Klaus, 2005)*.

2.7.4 *MMP Specific domains*

2.7.4.1 *Fibronectin repeats*

These are three tandem repeats, each of 58 residues. They are related to fibronectin type-II domain and contribute about 20 kDa of the mass of the molecule. They occur in gelatinase A and B, shortly before the catalytic domain Zn-binding motif *(Woessner and Nagase, 2000)*. They also assist these enzymes in binding to gelatins and collagens *(Das et al., 2003).*

2.7.4.2 *Hinge region*

This region is 15 to 65 residue, variable-length linker between the catalytic and the hemopexin domains. It contributes to the stability of the enzyme, due to its highly conserved proline residues, which are in direct contact with the catalytic and the hemopexin domains. On the other hand, this domain aids in the enzymatic degradation of complex substrates, such as fibrillar collagen by collagenases *(Klaus, 2005).* This region may adopt a conformation similar to that of collagen, which could then interact with the collagen triple helix to form a sort of proline zipper that may destabilize the helix and allow the strand to enter the active pocket of the enzyme (*Woessner and Nagase 2000).*

2.7.4.3 *Hemopexin domain*

This domain got its name due to the sequence similarity to hemopexin (a plasma heme-binding and heme-transport protein). It is found in all MMPs, except MMP-7, 26 and 23 and is involved in substrate recognition and specificity. In collagenases, it enables the enzyme to process the collagen triple-helix.

2.7.4.4 A *transmembrane domain*

This domain extends after the hemopexin domain in MT-MMPs. It is a highly hydrophobic, 74-residue domain in MT4-MMP *(Woessner and Nagase, 2000).*

2.7.4.5 *Type IV collagen insert*

This is a proline-rich residue found only in gelatinase B, between the active center and the hemopexin domain. It is similar to the α-chain of type IV collagen. The exact role of this insert is still not elucidated *(Woessner and Nagase, 2000).*

2.7.4.6 *Furin susceptible insert*

MMP-11,-23,-28 and MT-MMPs have an 11-residue furin-recognition site that extends between the propeptide and the catalytic domain. It has an ARNRQKR sequence *(Woessner and Nagase, 2000)*, which is susceptible to cleavage by furin-like convertases resulting in intracellular enzyme activation *(Das et al., 2003).*

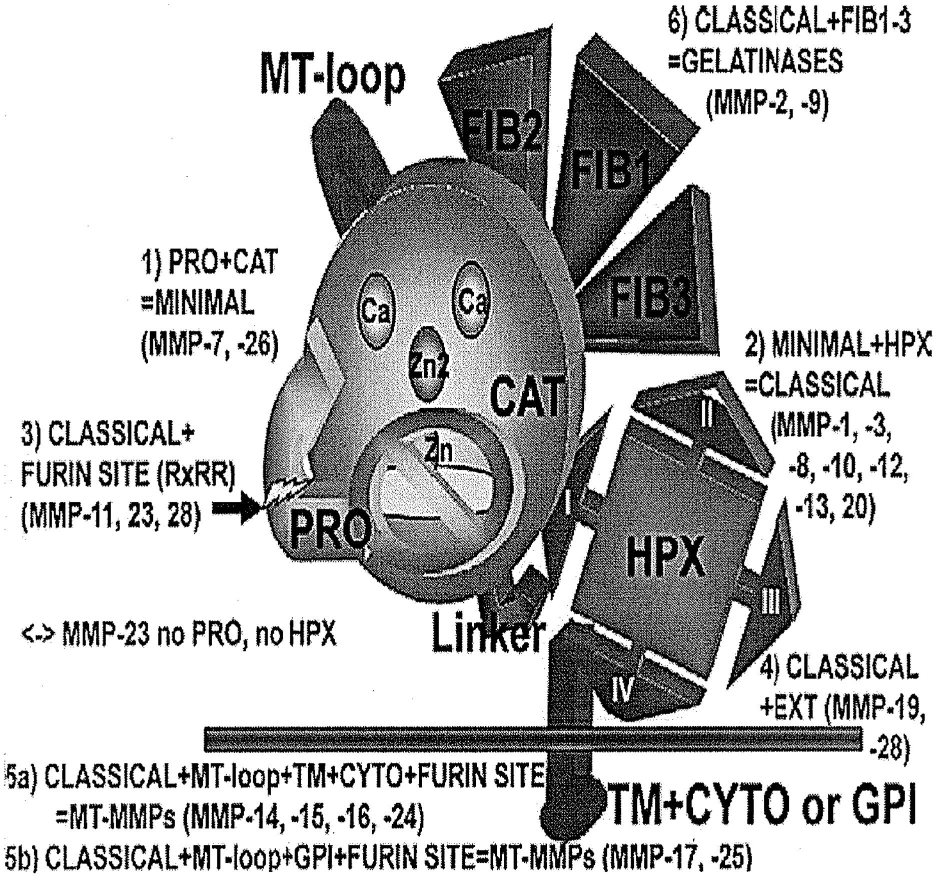


Figure 1: Schematic representation of the domain structure of human MMP family members. CAT: the catalytic domain with the two zincs (pink) and two calcium ions (yellow). PRO: the prodomain in dark yellow color which blocks the active site. LINKER: A linker peptide which connects the catalytic and the hemopexin-like (HPX) domain. Some classical MMPs show an extension (EXT) at the C-terminus which is not a membrane anchor. The MT-MMPs (MT-loop) show a membrane-anchor that is either be a transmembrane (TM) helix with small cytoplasmic part (CYTO) or a GPI-anchor (GPI), (*Klaus, 2005*).

2.8 Transcription

Matrix metalloproteinase gene expression and regulation is a subtle, complex, not fully delineated mechanism, which occurs under tightly regulated conditions that lead to cell - and tissue-specific expression of the individual genes *(Benbow et al., 1997).* It is the key regulating step for most of MMPs, because of their inducible gene expression *(Ye, 2000).* All MMPs, except MMP-2, have a TATA box located at -37 to -29 upstream from the transcriptional start-site. The promoter cis-acting elements of MMPs exhibit a high degree of similarity. Stimulants including 12-O-tetradecanoylphorbol-13-acetate, cytokines and growth factors, induce MMP gene expression, due to the interaction between the two most critical cis-elements, the tumor promoter-responsive element (TRE) and the polyoma enhancer A binding protein-3 element (PEA3). The TRE has a TGAGTA consensus sequence and is located approximately -80 to -74 bp upstream of the transcriptional start site. It binds the activator protein-1(AP-1) transcription factor, which is composed of dimers of the products encoded by the families of the oncogenes *Jun* and *Fos*. The PEA3 element is found either adjacent to the TRE site, or further distally and it interacts with the Ets family of transcription factors *(Mauviel A, 1993; Benbow et al., 1997).* Down-regulators, like transforming growth factor (TGF-3), retinoids and glucocorticoids, inhibit matrixin gene transcription, primarily but not exclusively, via the same elements *(Benbow et al., 1997).* The gene regulatory effects of distant located cis-elements, such as the stromelysin-1 platelet derived growth factor responsive element (SPRE) found in MMP-3 gene, silencer sequence (Sil) and AP-2 elements, may play a role in MMP gene expression as well *(Kirstein et al. 1996; Benbow et al., 1999).*

2.9 Secretion

After gene transcription, the highly stable (12-150 hour long half life) mRNAs translation, proMMPs are secreted through:

2.9.1 *The constitutive secretory pathway, default pathway (Woessner and Nagase, 2000).*

Most MMPs are transported directly to the cell surface once translated *(Sternlicht et al., 2001).* It takes about 35 minutes from the initiation of zymogen synthesis to its secretion *(Woessner and Nagase, 2000).*

2.9.2 *The regulated secretory pathway*

Secretion through this pathway occurs in response to signals, such as inflammatory ones. In the case of gelatinases, which are packaged into tertiary-vesicles in the *trans*-Golgi network of neutrophils, clathrin-coated secretory vesicles will bud, fuse with the cell-membrane and exocytose their gelatinase contents to the extracellular space *(Borregaard et al., 1997).*

The result of both pathways is membrane- bound or extracellular secreted MMPs.

2.10 Zymogen activation

It was in 1971, almost a decade after the Gross and Lapiere discovery of the tadpole's collagenolytic enzyme, that Harper and colleagues demonstrated that collagenases occur as zymogens and that they could be activated by trypsin *(Harper et al., 1971*).

MMP activation takes place intracellularly, as well as extracellularly through the "*Velcro mechanism"* (*Woessner and Nagase, 2000*). This mechanism is based on the disruption of the latent enzyme, propeptide Cys73 sulfhydryl coordination to the catalytic domain active-site zinc atom, which blocks the active pocket. This dissociation, by proteolytic and nonproteolytic agents, exposes the active site and results in Zn-water association, which is necessary for peptide hydrolysis. Therefore, the propeptide Cys73 containing sequence is called the "cysteine-switch", which is a highly conserved sequence among MMPs and is crucial to MMP activation *(Springman et al., 1990, Van Wart and Birkedal –Hansent, 1990).*

MMPs are mainly activated by *proteolytic* and non *proteolytic* agents

2.10.1 *Proteolytic activation pathway:*

Proteolytic enzymes, such as plasmin activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13. Activated MMPs can participate in processing other MMPs (*Sternlicht et al., 2001*).

Proteinases attack the bait region (exposed loop-region between the first and second helices of the propeptide) resulting in propeptide instability, including its cysteine switch, with concomitant zinc-water interaction and partial MMP activation. Full activation is obtained after complete removal of the propeptide domain, by partially- activated MMP intermediates or fully activated MMPs, through a poorly understood intermolecular maturation *(Visse et al., 2003).*

2.10.2 *Non-proteolytic pathway:*

Chemical activators modify the cysteine switch sulfhydryl-Zn association and remove a portion of the propeptide domain, resulting in a partially activated intermediate. Complete elimination of this domain by intermolecular processing by an active or intermediate MMP leads to full activation. Nonproteolytic agents include sodium dodecyl sulfate, thiol-modifying agents (4-aminophenylmercuric acetate, HgCl2, and N-ethylmaleimide), heavy metals as Au compounds, oxidants and disulfide compounds. In addition to chaotropic agents, reactive oxygen, low pH and heat treatment which can also lead to MMP activation (*Visse et al., 2003)*.

2.10.3 Non-Specific MMPs activations

Nagase et al., (1990) demonstrated that ProMMP-3 initial cleavage by proteinases such as neutrophil elastase, plasma kallikrein, plasmin, or chymotrypsin, occurs within the propeptide and results in three intermediates. Two intermediate-intermolecular interactions result in full activation of the enzyme. On the other hand, the nonproteolytic agent, APMA (4-aminophenyl mercuric acetate) generates only a short-lived intermediate of Mr = 46,000, which is then converted to a stable MMP-3 of Mr = 45,000 by autocatalytic activation. The fully activated MMP-3 does not to activate proMMP-3 *(Nagase et al., 1990)*.

Similar to other MMPs, MT1-MMP, a 61 kDa protein, is synthesized as an inactive precursor, which is activated intracellulary or extracellularly. Intracellular activation by furin, a proprotein convertase present in the Golgi apparatus, leads to cleavage of the enzyme into 39-kDa and 22-kDa fragments. The catalytic domain is in the 22-kDa fragment, which will be inserted into the membrane concomitant to activation (*Sato et al., 1996)*. Its extracellular matrix activation is demonstrated by *Will and colleagues (1996)*. Only plasmin, of a number of extracellular trypsin-like proteases, was capable of activating the enzyme, by cleaving off the prodomain at two processing sites. The TIMP-

2 N-terminal domain then binds the membrane-bound, activated TM1-MMP and maintains latency *(Will et al., 1996).*

The rest of the MT-MMPs, proMMP-11 (stromelysin-3), MMP-23 and epilysin (MMP-28), have a similar furin-recognition motif in their propeptide domains and therefore are activated in a similar pattern *(Sternlicht et al., 2001).*

Complete activation of MMP-2 is a two-receptor-mediated event, where integrin (αvβ3) and MT1-MMP/TIMP-2 complex are involved. TIMP-2 plays a dual role in MMP-2 regulation, being an inhibitor as well as an essential component of the MMP-2 activation complex. Two MMP-2 molecules are involved. The first ProMMP-2 binds the C-terminal domain of the bound TIMP-2 of the membrane-associated complex MT1-MMP/TIMP-2, which acts as a receptor for the hemopexin(PEX) domain of ProMMP-2 *(Will et al., 1996).* The second associates via the same domain with integrin (αvβ3), which is the most efficient among the integrins in the MMP-2 activation. Integrins mediate the clustering of MMP-2 intermediates in close proximity to the MT1-MMP/TIMP-2.proMMP-2 multimolecular activation complex, thus supplying it with additional MMP-2 intermediates. They also facilitate the autocatalytic 64 to 62 kDa maturation of MMP-2 in *trans*. Furthermore, they release mature MMP-2, so the enzymes accumulate extracellularly, diffuse in the matrix and function at distant sites. Mature MMP-2 can be released from the cell surface, or bind to it by another cell surface MMP-2-docking protein. It can also be inhibited by another TIMP molecules or left in an uninhibited active state depending on local MMP:TIMP molar ratios *(Deryugina et al., 2001).*

2.11 Clearance and metabolism

Clearance of matrixins is not a fully established mechanism, yet Yang and colleagues demonstrated the possibility of MMP-2/thrombospondin endocytosis by the low lipoprotein receptor-related protein LRP *(Yang et al., 2001).* Little is known about active MMPs autoproteolysis, but as demonstrated above, cleavage at certain sites inactivates MMPs, or decreases their substrate binding and degrading capacity. This is especially true of those that remove the hemopexin domain *(Woessner and Nagas,e 2000).* Such factors can alter MMP concentrations and availabilities to perform their function.

3. TOBACCO SMOKE

3.1 History of tobacco use

Tobacco is the product of the *Nicotiana tabacum* plant, which is one of the sixty-four species of *Nicotiana* genus. It is a tall, annual, broad-leafed plant, native to the Americas, where it was first encountered 18,000 years ago. How human developed interest in tobacco is not clearly known, however archaeologists believe that it was tobacco's biphasic effect, being a relaxant analgesic and an irritant at the same time, what attracted and trapped native people. Tobacco was mainly sniffed, then other practices evolved including chewing, eating, drinking, incorporating into eye-drops and enemas. Smoking, which was considered as a form of profound meditation, was certainly the most important practice that soon prevailed and replaced all other practices. Ironically during that time, tobacco was mostly used in medicine. Owing to its mild analgesic properties, tobacco was applied on aching teeth, wounds and snake bites. It was also used for dulling the feeling of hunger and in curing asthma, fever and serious ailments such as cancers. Tobacco was also a crucial player in Shamans practices *(Boyle et al., 2004)* due to its meditating effects and its "incense evil-emancipating abilities". During that time, Europeans knew nothing about tobacco, whose first entry to Europe was toward the end of the sixteenth century. Shortly after, the "*holy herb*" swept the whole continent, settled in its *materia medica* and was highly extolled for its medicinal properties and remedial abilities. Europeans used tobacco for the treatment of many conditions, including uterine, spleen, liver and colic pain, they applied it on wounds and burns and exchanged this precious and divine gift on special occasions. In England tobacco was praised for its appetite-dulling ability, while Italians considered tobacco a "*herba panacea*".

Nevertheless, it was not long before tobacco lost most of its medicinal values, as Europeans overindulged themselves in its consumption. Tobacco became a symbol of aristocratism and fashion and a source of pleasure and recreation during the end of the seventeenth century. In 1881, the situation was greatly aggravated, as the hand-rolled human-made cigarettes were replaced by John A Bosack's first machine-made cigarettes. Cigarettes flooded the markets resulting in a further surge in their consumption all over the world and cigarette smoking replaced all other forms of tobacco practices and usages *(Lock et al., 1998; Routh et al., 1998; Gately, 2001; Boyle et al., 2004).*

3.2 Tobacco Abuse

It was not until the seventeenth century, following tobacco over consumption and abuse, that its deleterious effects started to attract attention. Anti-smoking activists including King James I of England (1604) *(Cain, 1964)* and religious leaders, such as the Catholic clergies considered tobacco a devil and its usage an immoral and an unhealthy act. Several countries including Turkey, Russia and Persia imposed death penalties on tobacco users *(Castiglioni, 1942; Routh et al., 1998; Boyle et al., 2004)*. Tobacco real health hazards were first noticed 200 years ago by *Sommering, (1795)* who attributed lip cancer to smoking. *Hammer, (1878)* described a smoke-related coronary thrombosis, a condition first diagnosed by *Herrick, (1912)* in Chicago and which was considered by *Hoffman, (1920)* a consequence of increased cigarette consumption. In the same year *Adler, (1912)* was the first to suggest that lung cancer may be attributed to cigarette smoke. Nonetheless the lack of supporting scientific evidence, as pathologists failed to reproduce cancer experimentally made these findings controversial and unbelievable. It was not until the mid-twentieth century that *Doll, (1954)* pooled the results of the study of 34000 American doctors and showed an increase in coronary artery thrombosis among smokers. This was supported by another large scale study that demonstrated an increase in mortality of smokers less than 65 years of age due to coronary thrombosis *(Hammond et al., 1954)*. Meanwhile laboratory-experimental results proved that tar is carcinogenic *(Cooper et al., 1955)* and can result in skin cancer when applied on the skin for several months *(Wynder et al., 1953; Doll, 1998),* thus supporting the early findings. Now it is fully accepted and documented that smoke is a major health risk which causes, or exacerbates a myriad of disorders.

3.3 Tobacco composition and the carcinogens mechanism of action

Tobacco smoke is a complex-mixture of more than 4500 compounds, 300 of which have been proven to be carcinogenic. Carcinogens such as the polyaromatic hydrocarbons (PAH) present in tar, hydrazine, arsenic and benzpyrene (20-40 ng/cigarette) have been identified in cigarettes. Furthermore, carcinogenic metals such as the hexavalent chromium, the most abundant and notorious N-nitrosamines (TSNA), including nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and many other compounds were also found in cigarettes *(Johnson, 2001)*. Following their metabolic activation, these carcinogens covalently bind DNA forming adducts. Healthy cells detoxify these compounds. However long term exposure to insults jeopardizes cellular repair-mechanisms resulting in DNA miscoding. If miscoding occurs in a critical DNA region, such as an oncogene or a tumor suppressor gene, it may alter cellular growth and consequently lead to the development of pathologies, such as cancers.

Brenna, (1995) has shown a P53 tumor-suppressor factor mutation in tobacco and alcohol exposed-patients. Furthermore, cigarette smoke releases free radicals that cause oxidative DNA-damage and hence derangement of cellular functions *(Pryor, 1997; Arora et al., 2001).*

3.4 Tobacco and diseases

Tobacco, the sixteenth century *panacea* to all ailments, is paradoxically this millennium's devil that claims the lives of millions around the world. It is considered a 20th century epidemic and a major cause of death, where 1 billion deaths are expected in 21th century compared to 0.1 billion in the 20th century and half of these will be between 35-69 years of age *(Boyle et al., 2004).* Smoking pathogenicity, carcinogenicity and health adverse effects have gained worldwide attention, as no system is immune to smoke insults and its detrimental effects.

3.5 Tobacco and the respiratory system

Early lung exposure to smoke results in a typical picture of airway inflammation, with hyperplasia of the lining epithelium, increase in its permeability, muscular hypertrophy and fibrosis *(Buist et al., 1976-1979; Cosio, 1978; Cosio et al., 1980).* It also stimulates the mucous-secreting cells resulting in reflex-chronic coughing with increased sputum production. The permeabilized epithelium-release of proinflammatory mediators leads to the infiltration of airways with inflammatory cells. Among these cells are neutrophils and macrophages which secrete proteases, such as MMPs, that digest lung elastin and collagen fibers, thus increasing chances of alveolar wall rupture and emphysematous dilation of the alveolar spaces. This may be aggravated by the down-regulated antiprotease secretion by cigarette smoke-oxidants *(Barnes, 2000).* These changes may be attributed to the noxious byproducts formed following the rapid chemical change that some of tobacco constituents undergo after inhalation. Furthermore, they may be due to the direct deposition of the 0.1-1.0 µm aerosol particles of smoke on the alveolar and airway surfaces. These particles are potent lung irritants that trigger the above mentioned inflammatory responses *(Stratton et al., 2001).* Other constituents may paralyze the cilia of the airway-lining epithelium, thus diminishing the lung clearance capacity and inducing more irritation. Continuous exposure to irritants, carcinogens and biologically active free-radicals, formed following cigarette burning, interact with lung cells for long periods, increase their susceptibility to DNA-damage and consequently deranging their functions. *Lannan et al., (1994)* and *Leanderson et al., (1992)* have shown a single DNA-strand breaks, altered proliferation, attachment and repair of type II alveolar pneumocyte. *Wirtz et al., (1996)* and *Subramaniam et al., (1999)* demonstrated a decrease in surfactant associated-lipid formation and consequently diminished surfactant secretion. Collectively, these changes contribute to lung injury, decrease in function and development of various diseases such as asthma, COPD and cancers, which are one of the leading causes of cigarette smoke associated-death around the globe.

3.6 Tobacco and embryonic development

Although smoking is still perceived as a male habit, the WHO (1999) estimates that there are 200 million smoking-women around the world and tobacco claims the lives of more than half a million women each year. As a matter of fact, smoking may have a higher impact on women's health as in addition to the health-risks they share with men, female smokers' are susceptible to gender specific diseases such as cervical cancers and cardiovascular problems such as coronary thrombosis. Compared to nonsmokers, they may also experience irregular menstrual cycles, early menopause, and infertility *(Estner, 2001; Jacobs, 2001)*. Moreover, they have increased risks of abortion, ectopic pregnancy and preterm delivery Owing to smokers' increased cortisol levels, babies born to smoking mothers are more likely to suffer from altered pulmonary development and lung growth, precocious lung maturity. Furthermore, these babies are more likely to suffer from low-birth weight, sudden infant death syndrome and stillbirth, *(Wen et al., 1990; Hanrahan et al., 1992; Cliver et al., 1995; Cunningham et al., 1994; Mathew, 1998; Higgins, 1992. Gilliland et al., 2003; Hofhuis et al., 2003; Joad, 2004; Janoff et al., 1987).*

3.7 Tobacco and Oral diseases

In addition to the obvious pulmonary sites of cigarette smoke induced disease, the tissues of the oral cavity are similarly pries sites of smoke induced disease. Smoke inflicts changes ranging from merely cosmetic such as halitosis, bad oral hygiene, staining of the dental tissues and prostheses *(Asmussen et al., 1986; Murray et al., 1986)*, which is believed to be worse than that caused by tea and coffee consumption *(Ness et al., 1977)*, to pathological disorders such as inflammation and tumors. Smokers' inferior oral hygiene contributes to excessive plaque and calculus accumulation increasing their susceptibility to gingivitis, periodontitis and caries *(Johnson et al., 2000)*. *Bergström et al., (1994)* and *Salvi et al., (1997)* suggest that smoking is a significant risk for periodontal disease, with increased prevalence and severity of periodontitis among smokers. Smokers display deeper periodontal pockets, severe attachment and marginal bone loss and furcation involvement. They are also less responsive to non-invasive treatment as well as surgical approaches *(Preber et al., 1990; Kaldahl et al., 1996)*. This may be in part attributed to the nicotine-induced peripheral vasoconstriction, thus accounting for the increased cases of refractory (non-responsive to treatment) periodontitis among smokers *(MacFarlane, 1992)*. Furthermore, smokers display disturbed immunoglobulin and cytokine levels, lymphocyte counts, neutrophil impaired-functions (*Kenney et al., 1977; Johnson et al., 1990; Boström et al., 1998*). Injury to fibroblasts may jeopardize their host-defense mechanisms and increase their susceptibility to sever forms of infections *(Tipton et al., 1995; Salvi et al., 1997)*, such as *acute necrotizing ulcerative gingivitis (ANUG)*. ANUG is a severe form of gingival inflammation and ulceration accompanied by fever and lympadenopathy *(Kardachi et al., 1974)*. Moreover, epidemiological studies have shown that tobacco smokers have a higher risk of developing oral cancers, which account for 11% of cancers in women and 46% in men (*Pakin et al., 2000)*. Together with pharyngeal cancers, oral cancers are considered to be the sixth most common cancer in both sexes *(Boyle et al., 1990a-1995; La Vecchia et al., 1997; Franceschi et al., 2000)*.

4. RATIONALE

Cigarette smoke contains a plethora of molecules which collectively may inflict changes or induce or exacerbate numerous pathological conditions in various systems. Of these systems, the oral cavity is the first route of exposure to tobacco smoke, while the respiratory system large surface area presents a region of maximum exposure are mostly. The disturbance of basic cellular functions including viability, proliferation, migration and secretion of important factors or enzymes such as matrix metalloproteinases (MMPs) following exposure to tobacco smoke or various components has gained tremendous attention, as no system seems to be immune to its detrimental effects. Moreover, it is becoming increasingly clear that cigarette smoke is considered a major agent in augmenting connective tissue destruction in numerous pathologies. For example chronic obstructive pulmonary diseases (COPD), which are characterized by widening of the airway spaces and periodontitis whose hallmark is loss of the supporting periodontal ligament fibers binding the tooth to the alveolar bone are associated with smoking. It is accepted fully that degradation of numerous structural and extracellular matrix proteins is carried out by proteases of which a group of Zn-dependent proteinases the MMPs are major players. MMPs are involved in various physiological as well as pathological conditions. Cigarette smoke modulated MMP-activities and secretions and altered proteases/anti-proteases balance has attracted considerable attention. In this regard the release and activation of two MMPs, MMP-2 and MMP-9 also known as gelatinases are of particular interest, as they are expressed in most mesenchymal cells during tissue development as well as repair and regeneration. Furthermore they may play important roles in pathological conditions such as inflammation. Mesenchymal cells mainly fibroblasts predominate the extracellular matrices (ECM) in the tooth-supporting apparatus (periodontium) and lung parenchyma. In both systems their secreted collagen fibers are the major constituent of the ECM. Furthermore, the periodontium has a rapid remodelling and high turnover rate. Similarly, approximately 5% of an adult lung collagen is turned over on daily basis. These high turnover rates reflect the dynamic nature of these tissues and the ECM in particular. Therefore highly regulated and efficacious ECM-turnover mechanisms maintain the integrity and homeostasis and to ensuring proper remodelling and repair of both systems. Furthermore, the lung grows by what is known as branching morphogenesis. This dictates precise dichotomous branching of the duct system and necessitates accurate degradation of the surrounding and investing connective tissues. Collectively, the dynamic nature of these processes in both systems underscores MMPs role in tissue remodeling in both the peridontium and the respiratory system.

Taking all the aforementioned data, we hypothesized that cigarette smoke extract may alter basic functions of the mesenchymally derived fibroblasts of the periodontium and the fetal rat pulmonary cells (fibroblasts and type II alveolar pneumocytes). This in turn may have an impact on MMP-2 and MMP-9 gelatinases secretion and thereby may be related to resulting pathologies in these systems.

Approaches

To investigate the effects of cigarette smoke extract on the viability of rat periodontal ligament fibroblasts and fetal rat pulmonary cells we will:

- Prepare cigarette smoked extract medium (CSE) following a modification of the method of Janoff and Carp (1977)
- Treat the cells of interest with increasing concentration of CSE for 24 hours
- Examine cells using a phase contrast microscope
- Investigate cellular viability by:
    - Trypan-blue cellular exclusion assay as a measure of cellular viability
    - Formazan assay
    - Crystal violet assay
- Examine cellular gelatinases (MMP-2 and MMP-9) secretion by
    - Zymography
    - Enzyme Linked Immuno-Sorbent Assay (ELISA)
    - Immunohistochemistry

Collectively the results of these studies will provide information on any changes in cellular functions including viability, proliferation and MMP-secretion after exposure to CSE.

5. MATERIALS AND METHODS

5.1 Materials

Timed pregnant Sprague-Dawley rats were obtained from the Central Animal Care facility, University of Manitoba. Animals were treated following protocols approved by the Canadian Council on Animal care and their local representative agencies. Cell culture reagents, including media, antibiotics, fungizone, new born calf serum (NCS) were bought from Life Technologies-Gibco/BRL (Burlington, ON). Plastic culture flasks were from Fisher Scientific (Edmonton, AB). Chemicals used in zymograms were obtained from Sigma Chemicals (St Louis, MO). Electopheresis equipment was from BioRad Laboratories. Primary Antibodies were produced by Calbiochem (California, USA), while the goat anti mouse HRP conjugated secondary antibodies were from Santa Cruz (California, USA). Universal Block was purchased from KPL (Gaithersburg, Maryland,USA) and 3' diaminobenzidine (DAB) immunohistochenmistry staining kit from vector laboratories (Burlingame, CA, USA). ELISA kits were purchased from R&D Systems (Minneapolis MN. USA). Research grade cigarettes, 2R1, were obtained from Kentucky Tobacco Research and Development Center at the University of Kentucky, Lexington, KY, USA. Formazan assay (CellTiter 96® AQ$_{\text{ueous}}$ Non-Radioactive Cell Proliferation Reagent) was purchased from Promega (ON. Canada)

5.2 Methods

5.2.1 *Lung fibroblast and type II cell isolations*

Twenty-one gestational day timed pregnant, 360-420g, Sprague-Dawley rats (time of mating designated as day 0, term = 23.5 days) were euthanized with 2ml of Euthanyl

(240 mg/ml sodium pentobarbital) injected intraperitoneally. Fetuses were removed by hysterotomy. Under a laminar flow hood and using sterile techniques fetal heads were removed and the bodies were placed in ice-cold, sterile Hanks Balanced Salt Solution (HBSS) (pH 7.1). The thorax and abdomen of each fetus was cut opened with a scalpel, lungs were removed, cleaned and placed in Petri dishes in HBSS. The lung tissues were minced using a Sorval Tissue Chopper (Sorval Instruments, Newton, CT). Tissue slurry was trypsinized in a continuously stirred trypsinization flask containing 100ml of digestion buffer (ice cold 90% HBSS and 10% trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%/0.02%)) at 37^0C for 40 minutes. The enzymatic reaction was stopped by adding minimal essential medium (MEM) containing 10% of newborn calf serum (NCS), 5% antibiotics and fungizone, to the tissue suspension in 3:1 ratio. The solution was filtered through three layers of 150μm Nitex gauze. The filtrate was centrifuged at 250 x g for 10 minutes at 20^0C. The supernatant was discarded and the pellet was resuspended in 10ml of MEM with 10% NCS, 5% antibiotics and fungizone. The cell suspension was plated into two $75cm^2$ tissue culture flasks for approximately one-hour at 37^0C in a humidified incubator (95% air/ 5% CO_2) for the fibroblasts to adhere to the plastic flasks. Media containing the unattached cells were collected. Cells were counted using a cell counter and plated into $25cm^2$ flasks at a density of $8.0x10^5$ cells/flask and 96 well plates at $5.0x10^5$ cells/well with MEM containing 10% newborn calf serum stripped of its growth factors (sNCS), 5% antibiotics and fungizone. Five mls MEM/sNCS per $25cm^2$ flask were used to culture the fetal rat lung type II alveolar cells. The media for the fibroblasts as well as type II cells were changed after 24 hours and every 48 hours afterwards. Fibroblasts and type II alveolar cells reached confluence within 3-5 days after

isolation. At confluence fibroblasts, but not type II cells, were passaged at a ratio 1:4 flasks. To detach the cells, one ml trypsin/EDTA per 75cm^2 flask was added to cover the cells and incubated at 37^{0}C for five minutes. Detachment of cells was determined using phase contrast microscopy and the enzymatic reaction was stopped by adding five mls of MEM/NCS. The cell suspension was centrifuged at 250x g for 10 minutes at 20^{0}C. To collect the cells, the pellet was re-suspended in the same MEM/ NCS medium after discarding the supernatant.

5.2.2 *Rat periodontal ligament fibroblasts isolation*

Rat periodontal ligament fibroblasts were isolated following the established method outlined by *Lekic et al., (2005)*. In brief, after sacrificing the pregnant rat a block of the mandible containing the first molar root, the supporting periodontal ligament and a small stretch of the supporting alveolar bone was obtained. The segment was minced into small pieces following 4x washing with MEM containing 5% antibiotic and 0.5 µg/ml of fungizone. The small bony fragments were then placed into 25 cm^2 flask containing 10 % NCS, 5% antibiotics and fungizone in MEM. After approximately one week, cells from the bone fragment migrate to the base of the culture vessel. The periodontal ligament progenitor cells obtained by this technique included precursor cells from the root and bone related compartment of the PDL, as well as precursor in the endosteal spaces of the surrounding alveolar bone (*Lekic et al., 2005)*. Cells were passaged following the same procedures used for the lung fibroblasts.

5.2.3 *Aqueous cigarette smoke extract (CSE) preparation*

CSE preparation was performed following a modification from Janoff and Carp method (*Janoff and Carp, 1977*). Briefly, the smoke of one 100mm non filtered cigarette was bubbled through 50 ml of minimum essential medium (MEM) using a syringe driven apparatus, see figure: 1. A two-second application of suction via a 40 ml syringe plunger was followed by 20 seconds of rest after evacuation of the air. This cycle was repeated until combustion of the cigarette was complete. The obtained CSE, which was defined as 100% was filtered using 0.22 um pore filters (Millipore) to ensure removal of bacteria and contaminants before use. Desired treatment concentrations were obtained by diluting the 100% CSE stock.

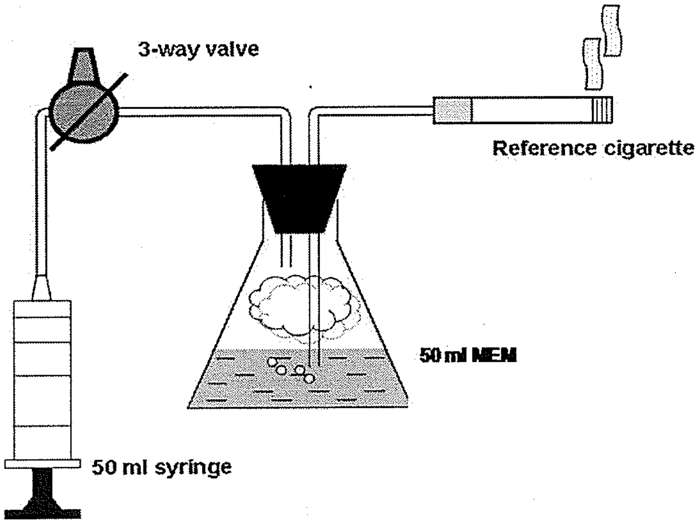


Figure 2: Cigarette smoked media preparation apparatus (Scott DA, 2006)

5.2.4 *Treatments*

Lung fibroblasts, type II alveolar cells and PDL fibroblasts were exposed to increasing concentrations of CSE (1 %, 5%, 10% or 30%) with or without the gram-ve bacterial endotoxin lipopolysaccharide (LPS 1 μg/ ml) or (1 μg/ ml) LPS-alone. All samples were solubilized in serum-free MEM with 5% antibiotics and fungizone and in all cases cells were exposed for 24 hours. Conditioned media were collected, filtered through 0.22 um Millipore filters to remove bacteria and other debris and stored at – 80 ^{0}C until needed.

5.2.5 *Phase-contrast microscopy cellular examination*

Following the 24 hour CSE with or without LPS treatments of the cells, phase contrast microscopy examination was carried out to assess visually any signs of cellular toxicity displayed as morphological changes or detachment from plates.

5.2.6 *Cells proliferation & viability*

Cellular proliferation and viability were determined employing three different assays, trypan-blue viability assay, formazan and crystal violet assays

5.2.6.1 Trypan-blue Viability Assay

This assay is based on staining of dead cells with disrupted plasma membranes by a negatively charged vital chromophore; viable cells exclude the stain (*Freshney, 1987)* Cells viability was expressed as the ratio of cells that excluded the trypan-blue to the total number of cells in every field of a grid haemocytometer. Dead cell numbers were expressed as a ratio of the number of blue stained cells to the total number of cell.

5.2.6.2 Formazan Assay

In vitro cellular vitality, after exposure to LPS and CSE, was determined using the CellTiter 96® AQ$_{ueous}$ Non-Radioactive Cell Proliferation Reagent, from Promega (ON. Canada) and following manufacturer's instructions. Briefly, lung fibroblasts, Type II alveolar pneumocytes and PDL-fibroblasts were grown on 96-well culture plates. Cells were treated with 1%, 5%, 10% or 30% CSE with or without LPS or LPS-alone solubilized in serum free-MEM in a total volume of 250µl/well for 24 hours. 150µl of the medium was removed to standardize the supernatant to 100µl/well. 20 µls of MTS-formazan reagent were added to each well. Plates were incubated for one to four hours at 37^0C, 5%CO_2 and 100% humidity. Absorbance at 490nm was measured every hour for four hours using a Biorad 550 Microplate Reader with Biorad Microplate Manager 5.1 plate reader software.

5.2.6.3 Crystal Violet Assay

This assay enables the quantitative analysis of the relative density of cells adhering to multi-well dishes, by measuring the dark blue-crystal violet stain bound to cellular-protein, which reflects cells number and viability (*Vendetti et al.*, 2002; *Alfa* et al., 1998). In brief, lung and PDL fibroblasts together and type II alveolar cells were grown in 96 wells and treated following the same procedures and protocols as described in section 5.2.6.2 Formazan assay. After 24 hours of treatment culture media were removed from wells. Plates were washed gently with HBSS (Hank's Balanced Salt Solution, pH 7.5). 100 µls of crystal violet solution [0.25 %(w/v) crystal violet, 20 mM Tris-HC1, pH 7.5, 0.9% (w/v) NaCl in 20% (v/v) methanol] were added to each well after removal of the HBSS and incubated for 10 minutes. Plates were placed vertically and washed gently under slow running tap water and drained upside down on a paper towel. This was repeated three times. Cells were fixed with 250 µl of 100%methanol/well for 20 minutes. Absorbance was read at 590 nm using a BioRad 550 micro-plate reader *(Alfa et al., 1998).*

5.2.7 *Protein assay*

Bradford protein assay, which depends on the binding of Comassie Blue G-250 stain to proteins, was used to determine the protein concentrations in media collected from cells exposed to LPS and CSE with or without LPS *(Bradford, 1976)*. Bovine serum albumin (BSA) was used as a protein standard for all assays and BIO-RAD DC Protein Assay Kit was utilized. The optical density of protein samples at 595 nm was measured on a Beckman DU series 640 spectrophotometer within 15 minutes of preparation.

5.2.8 *Zymography*

Zymography is established as a simple, sensitive, quantifiable electrophoretic method used to detect the presence of matrix metalloproteinases (MMPs) by the degradation of their preferential substrate. This technique permits the differentiation between active and latent forms of the enzyme. Furthermore, this method eliminates of the tightly bound endogenous tissue inhibitors of metalloproteinases (TIMPs), thus producing a measure of the total MMPs existing in the samples. The procedure is based on electrophoretic separation of proteins on a sodium dodecyl sulfate (SDS) gel impregnated with a specific protein substrate. This substrate is degraded by the proteases after they are resolved on the gels during the incubation process. Coomassie blue staining and destaining of the gels reveals sites of proteolysis as white bands on a dark blue background *(Leber et al., 1997)*.

5.2.8.1 *Zymography Steps*

An aliquot of medium from cells exposed to LPS and CSE with or without LPS was mixed 4:1 with non-reducing 4x Laemmli sample buffer, [62.5 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 4% (w/v) SDS and 0.01% (w/v) bromophenol blue], sat at room temperature for 30 minutes, loaded onto gels without boiling. A BIO-RAD mini-gel vertical electrophoresis apparatus was used for all applications. Non-reducing 10% gels containing an acrylamide-copolymerized substrate of choice (ex: 1 mg/ml gelatin I), [10% (w/v) acrylamide, 375 mM Tris-HCl, pH 8.8 and 0.1% (w/v) sodium dodecyl sulfate (SDS)] with 4% stacking gels [4% (w/v) acrylamide, 125 mM Tris-HCl, pH 6.8, and 0.1% (w/v) SDS] was employed. Electrophoretic separation of the proteins was carried out for an hour and twenty minutes at 125 V, in the cold room at 4^0C. Denaturation of the proteins results in unfolding and partial activation of the enzymes, through the cysteine switch: Cys- zinc dissociation *(Beurden et al., 2005, Hawkes et al., 2001)*. Gels were washed (2 x 30 minute) washings of the zymograms with 2.5 % (v/v) Triton X-100, a nonionic weak detergent to renature the proteins. Gels were incubated in a developing buffer (50 mM Tris-HCl, pH7.4, 0.2 M NaCl & 5 mM CaCl2) containing 1mM PMSF (a general protease inhibitor) or 20mM EDTA (a specific MMP inhibitor) overnight at 37 °C and stained for 60 minutes with 0.25% Coomassie blue solution [30% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie brilliant blue R 250]. Gels were destained for 30 minutes in [10% (v/v) glacial acetic acid, 30% (v/v) methanol]. MMPs activity was detected as clear bands against a blue background of undegraded substrate (*Beurden et al., 2005, Hawkes et al., 2001*). Gels were scanned using the Fluorchem HD2 (Alpha Innotech, CA, USA) and proteolytic activity was quantified by densitometry using Alpha Ease FC analysis software.

5.2.9 *Enzyme Linked Immuno-Sorbent Assay (ELISA)*

MMP-2 and MMP-9 released by the various cell types under investigation into the medium was quantified using 96-well sandwich ELISA kits (R&D Systems Minneapolis. MN. USA). Undiluted medium collected from cells treated with LPS and CSE with or without LPS were used for the ELISA assay. Optical densities were determined using BioRad model 550 microplate reader with the wavelength set to 450 nm and 570nm wavelength to correct for plate imperfections. Readings were taken within 30 minutes of the addition of the stop solution according to the manufacturer's instructions. MMP concentrations were extrapolated from a standard curve generated for each assay.

5.2.10 *Immunohistochemistry (IH)*

This technique was employed to localize MMP-9 expression in paraffin embedded, 21 gestational days fetal rat embryonic lung-sections to identify pulmonary cells contributing to this enzyme secretion. This technique permits the localization of fixed tissue-immobilized antigens of interest, by the use of labelled antibodies. The antibodies/antigenic interactions will be visualized by markers such as horse raddish peroxidase (HRP), which was used in our current study. Two IH techniques may be followed either direct IH, in which the primary antibody is tagged with a marker or an indirect technique when a secondary antibody is conjugated with a marker. In the present study, 21gestational day embryonic lung tissues were removed after animal euthanization and tissue were fixed with 10% formalin at 4°C for 24-48 hours. Fixed tissues were placed in Shandon Citadel 1000 Tissue Processor (Thermo Fisher Scientific, MA. USA) overnight and afterward embedded in paraffin for 24 hours at 4°C to harden and permit sectioning. Lung sections were deparaffinised, alcohol-dehydrated, blocked with Universal Block (KPL, Maryland, USA) for 10 minutes and incubated with the primary mouse monoclonal MMP-9 antibody (2μg/ml) for two hours, washed, incubated with an HRP conjugated, goat anti-mouse monoclonal secondary antibody for 10 minutes and stained with 3' diaminobenzidine-kit (DAB: Vector Laboratories, Burlingame, CA, USA) for 2-10 minutes in a dark chamber. Copious 1xPBS washings were performed between steps. Slides were dehydrated and sealed with cover-slips. Negative-controls were processed similarly, but no primary antibody was used.

5.3 Statistical Analysis

Data was analyzed statistically with a one-way analysis of variance test followed by Duncan's new multiple range post hoc test (Ott,1977), assuming a significant difference at $p < 0.05$.

RESULTS

Phase contrast microscopy was used initially to examine the effects of the gram –ve bacterial endotoxin (LPS) and the cigarette smoke extract (CSE) with or without LPS on isolated fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament (rPDL) fibroblasts. Fetal rat lung fibroblasts and type II alveolar pneumocytes showed signs of detachment from culture dishes and a marked change in morphology upon exposure to doses of CSE (20% CSE and above with or without LPS) [Figures 3 and 4]. In contrast, 1%, 5% or 10% CSE with or without LPS did not appear to alter cellular morphology or cause changes in attachment. On the other hand, rPDL fibroblasts exhibited signs of morphological changes upon exposure to concentrations of CSE of 10% or higher with or without LPS [Figure 5]

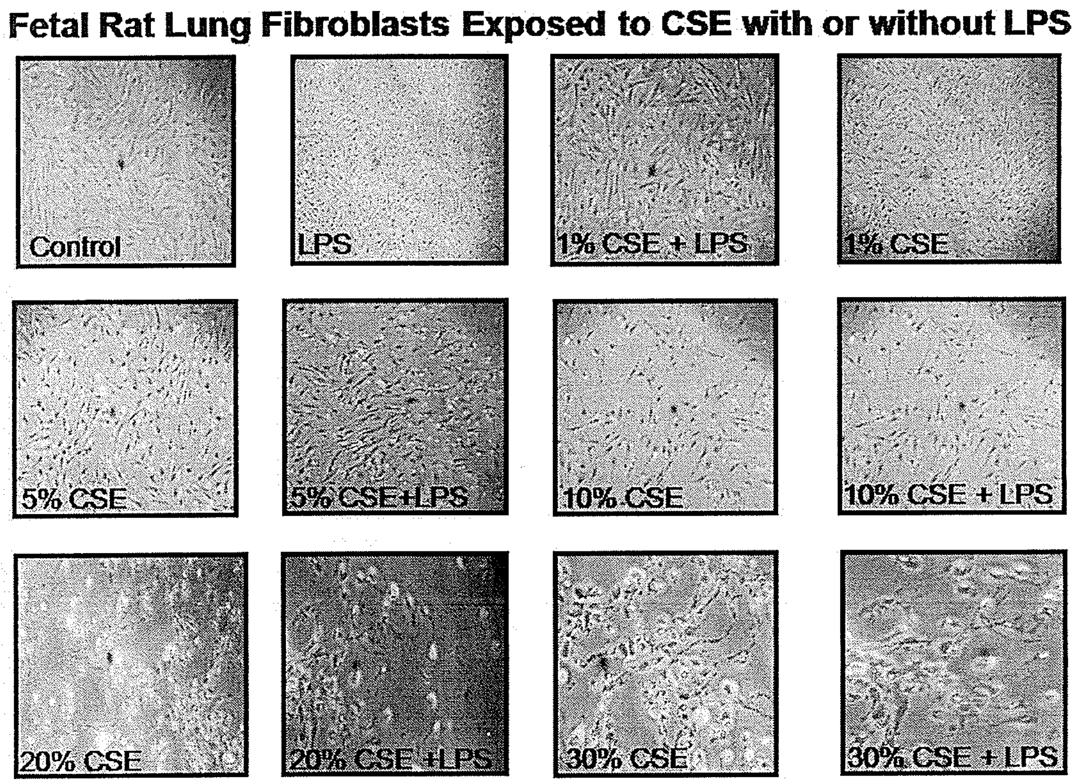


Figure 3: Phase contrast microscopic images of fetal rat lung fibroblasts after a 24 hour treatment period with CSE with or without LPS, (Scale 4500 μm, magnification 4x).

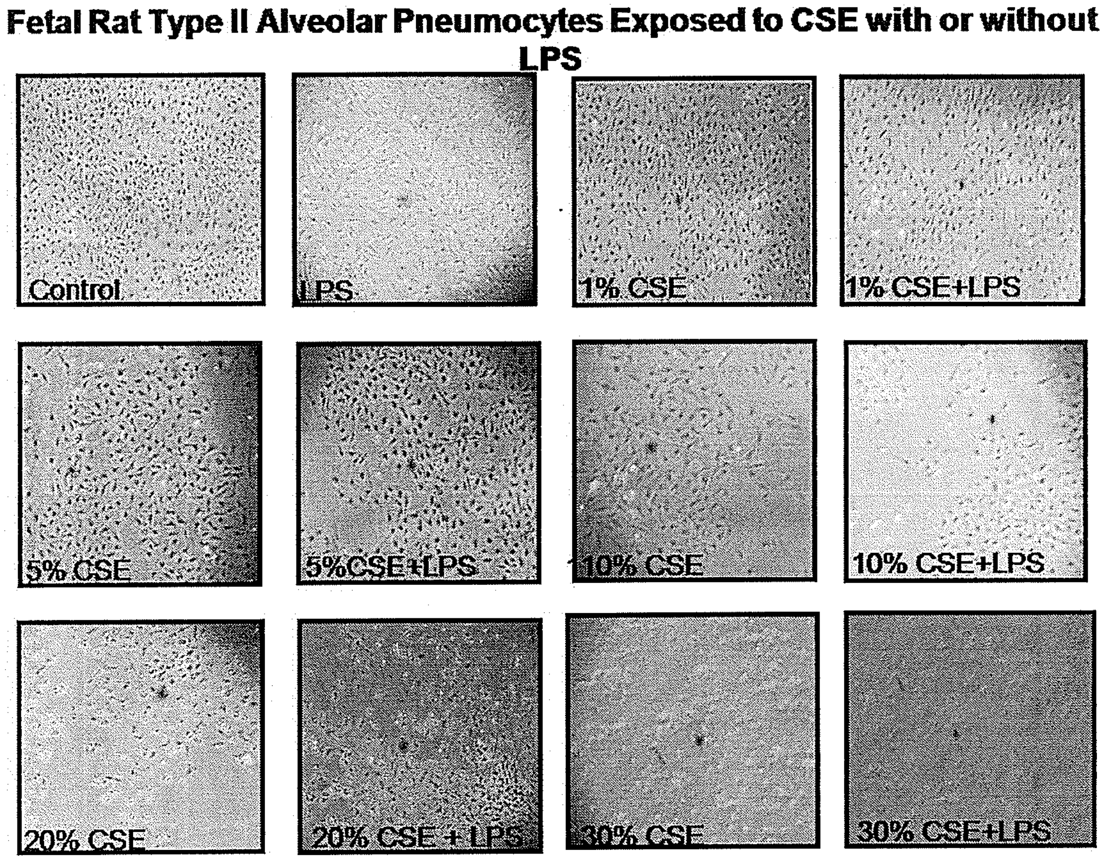


Figure 4: Phase contrast microscopic images of fetal rat lung type II alveolar pneumocytes after a 24 hour treatment with CSE with or without LPS (Scale 4500 μm, magnification 4x).

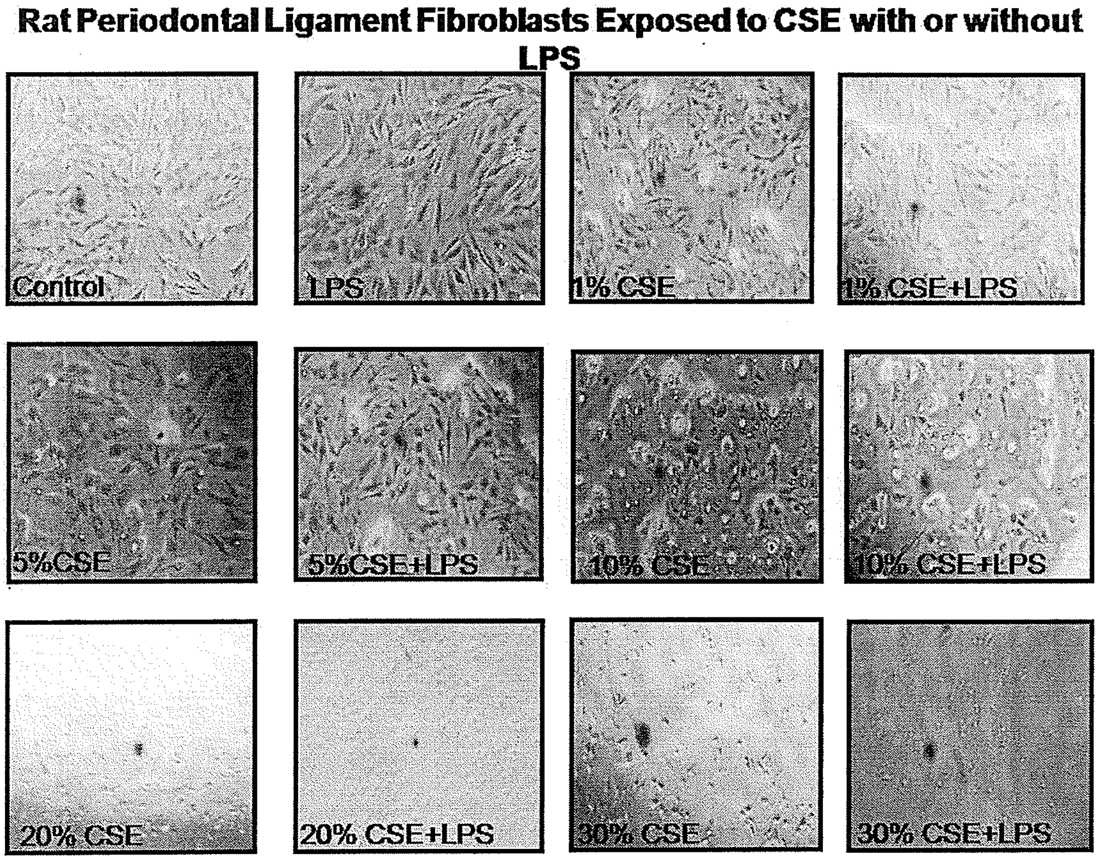

Rat Periodontal Ligament Fibroblasts Exposed to CSE with or without LPS

Figure 5: Phase contrast microscopic images of rat periodontal ligament fibroblasts after a 24 hour treatment with CSE with or without LPS (Scale 4500 μm, magnification 4x, 10x).

Trypan blue viability assay

Trypan blue was used to assess cellular viability as defined by stain exclusion in cell cultures exposed to CSE with or without LPS *(Freshney, 1987)*. Data was analyzed statistically as described in the Materials and Methods. The results are presented in Figures 6, 7 and 8.

Fetal rat lung fibroblasts cultured under conditions with 10% serum displayed close to 98% dye exclusion, while their serum-starved counterparts exhibited approximately 90% dye exclusion [Figure 6]. Exposure to LPS did not alter significantly the level of dye exclusion compared to the serum starved control cultures. Fibroblasts exposed to 10% or 30% CSE with or without LPS excluded trypan blue at a significantly lower level ($p<0.01$) compared to the serum starved control samples or LPS treated cells. Conversely, exposure to several low concentrations of CSE did not alter significantly cellular dye exclusion compared to the serum starved control cultures. Concurrent exposure to CSE and LPS did not alter significantly cellular dye exclusion compared to cells treated with CSE alone

Fetal rat lung type II alveolar pneumocytes exhibited a significantly reduced ($p<0.01$) dye exclusion upon exposure to 10% or 30% CSE with or without LPS compared to the serum starved control samples or LPS-alone treated cells. On the contrary exposure to 1% or 5% CSE with or without LPS did not alter significantly cellular dye excluded compared to their serum starved control counterparts or LPS-treated cells. Simultaneous cellular exposure to 30% CSE and LPS reduced significantly ($p<0.05$) the trypan blue exclusion compared to cells treated with 30% CSE alone [Figure 7].

Rat periodontal ligament fibroblasts excluded trypan blue at a significantly lower level ($p<0.01$) after exposure to 10% or 30% CSE with or without LPS compared to the serum starved controls or the LPS-treated counterparts. Those samples treated with concentrations of 1% or 5% CSE with or without LPS exhibited no significant changes in dye exclusion. Similarly LPS-treated fibroblasts did not show any significant changes in dye exclusion compared to the serum starved control samples. Combined LPS and CSE treatments did not reduce significantly dye exclusion rPDL fibroblasts compared to cells treated with LPS alone [Figure 8].

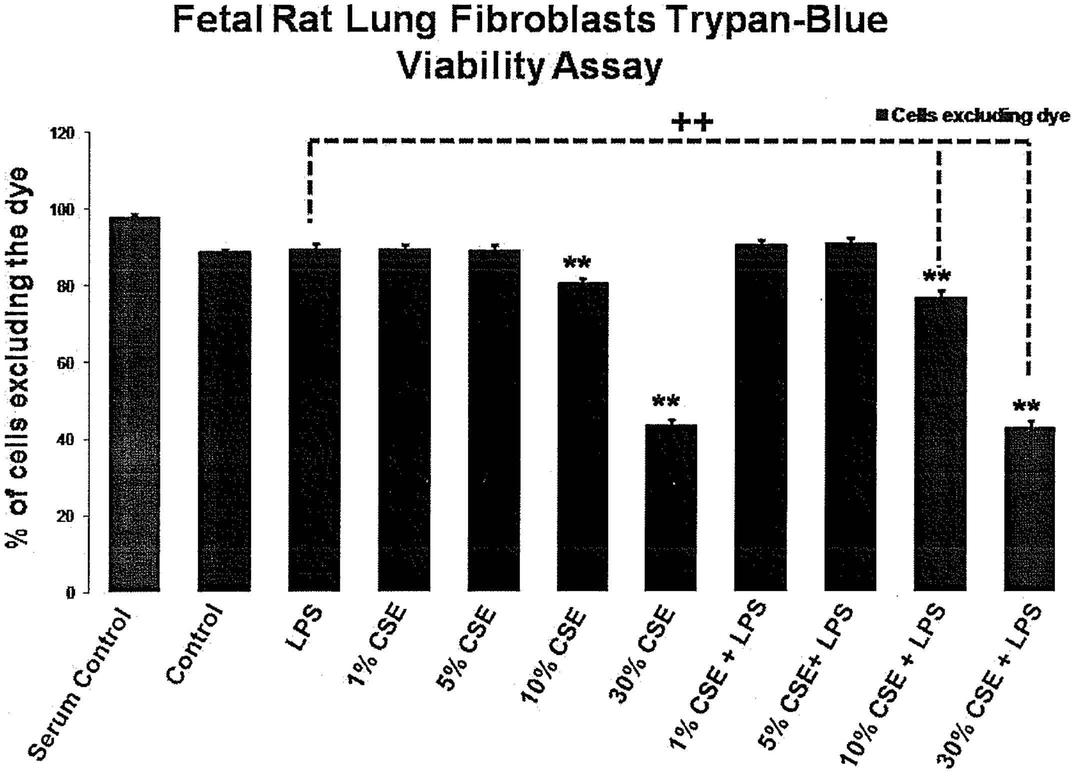


Figure 6: Effects of CSE with or without LPS on trypan blue exclusion in isolated fetal rat lung fibroblasts. Results are expressed as the mean +/- SD of eleven assays for each condition. **: ($p<0.01$) significantly different from values obtained for the serum starved control group. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

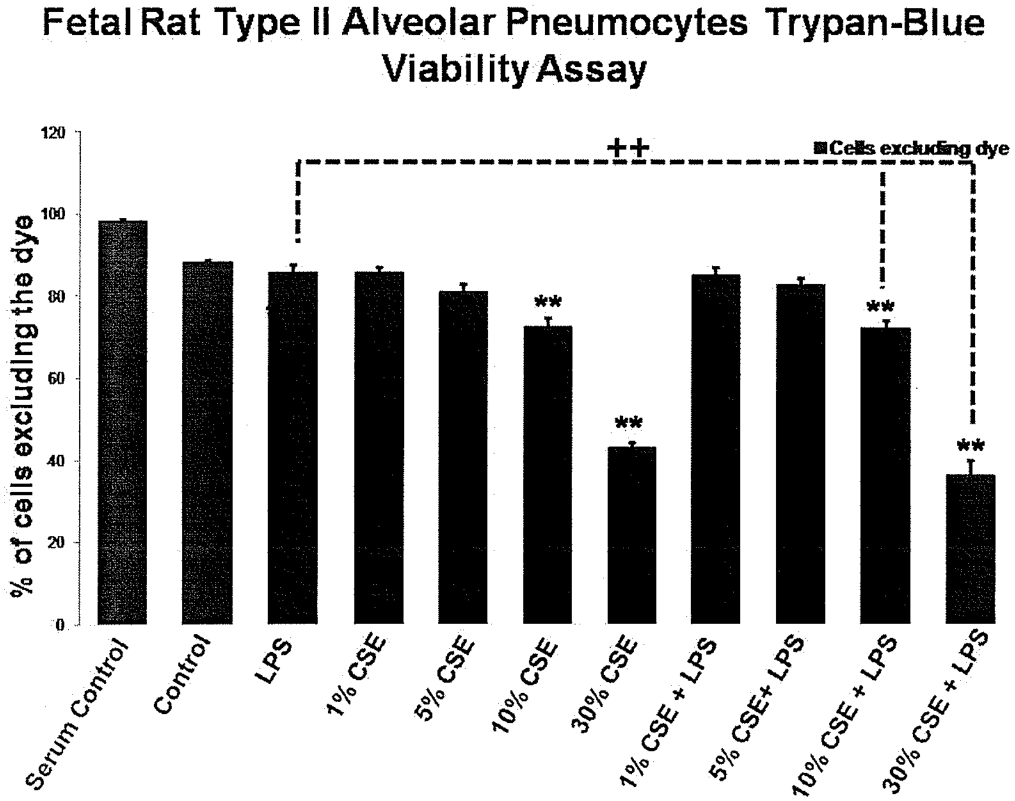


Figure 7: Effects of CSE with or without LPS on trypan blue exclusion in isolated fetal rat lung type II alveolar pneumocytes. Results are expressed as the mean +/- SD of eleven assays for each condition. **: ($p<0.01$) significantly different from values obtained for the serum-starved control group. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

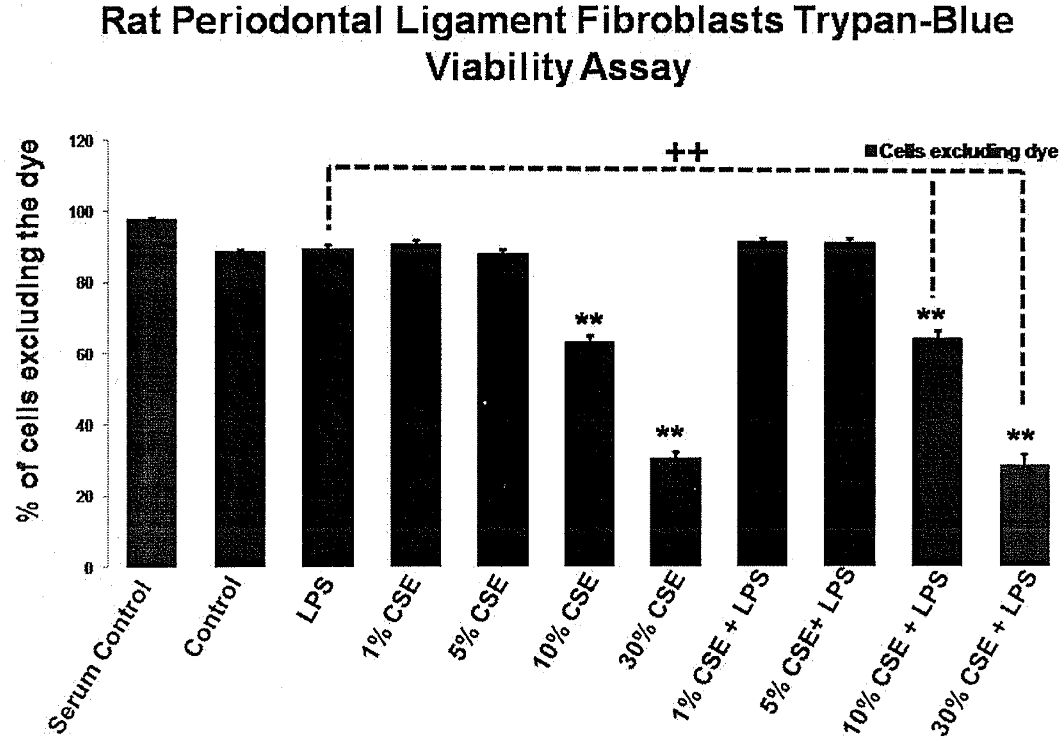


Figure 8: Effects of CSE with or without LPS on trypan blue exclusion in isolated rat periodontal ligament fibroblasts. Results are expressed as the mean +/- SD of eleven assays for each condition. **: ($p<0.01$) significantly different from values obtained for the serum-starved control group. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

Formazan Assay

Formazan assay was performed to determine the *in vitro* viability and proliferation of cells after exposure to various concentrations of CSE with or without LPS. The formazan assay was first described by Mosmann (1983). It is based on the capacity of cellular mitochondrial dehydrogenase to cleave the tetrazolium rings of the pale yellow MTT compound [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] thereby producing dark blue formazan crystals, which accumulate within cells. Release of these crystals upon solubilization reflects the number of metabolically active cells and can be quantified by measuring absorbance at 490 nm *(Mosmann, 1983).* **MTS,** a novel tetrazolium compound [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS] from Promega (ON. Canada) was used to perform this assay. Like the MTT compound, the MTS is also bio-reduced by cellular mitochondrial dehydrogenase into an aqueous formazan product which is released into the tissue culture medium, resulting in a color change. Absorbance is measured at a wavelength of 490nm and is directly proportional to the number of living cells in culture.

Rat PDL fibroblasts, fetal rat lung fibroblasts and type II alveolar pneumocytes were exposed to increasing concentration of CSE with or without LPS for 24 hours. At the end of this period the formazan reagent was added and its conversion was measured at one, two, three and four hours. Results are shown in Figures 9, 10 and 11.

Fetal rat lung fibroblast mitochondrial dehydrogenase activity as determined by formazan assay was reduced significantly ($p<0.01$) over a four hour incubation period upon exposure to 10% or 30% CSE with or without LPS compared to corresponding control cells. A similar pattern was observed in cells treated with 5% CSE with or without LPS. An opposite trend was observed in fibroblasts treated with 1% CSE with or without LPS, where cellular mitochondrial dehydrogenase activity was significantly increased ($p<0.05$ and $p<0.01$respectively) at most of the time points observed. Similarly formazan conversion by LPS-treated cells was increased significantly ($p<0.05$) during the second hour of incubation compared to the serum starved control samples. In contrast to the LPS treated fetal rat lung fibroblasts, formazan conversion was significantly less ($p<0.01$) in cells exposed to 5%, 10% or 30% CSE with LPS as measured at one, two, three and four hours [Figure 9: a and b].

Formazan conversion by fetal rat lung type II alveolar pneumocytes was significantly ($p<0.01$) reduced after 24 hours of exposure to 5%, 10% or 30% CSE compared to the serum starved control samples. A similar reduction was observed in cells treated with 1% CSE during the first hour of incubation with formazan ($p<0.01$). On the contrary, LPS-exposed cells did not display any statistically significant changes in mitochondrial dehydrogenase activity compared to the serum starved control samples. Exposure to 5%, 10% or 30% CSE with LPS reduced significantly ($p<0.01$) cellular formazan conversion measured at one, two, three and four hours as compared to their LPS-alone treated counterparts [Figure 10: a and b].

Mitochondrial dehydrogenase activity in rPDL fibroblasts as measured over a four hour incubation period with formazan reagent was reduced significantly ($p<0.01$) upon exposure to 10% or 30% CSE. Similarly cells treated with 10% or 30% CSE with LPS also displayed significant reductions ($p<0.01$) in mitochondrial dehydrogenase activity compared to the serum starved controls as well as those cells exposed to LPS alone. On the contrary exposure of cells to lower concentrations of CSE with or without LPS in several cases induced significant increases ($p<0.01$, $p<0.05$) in mitochondrial dehydrogenase activity compared to the serum starved control samples or the LPS-alone counterparts. Concurrently CSE and LPS treatments did not induce any significant changes in rPDL fibroblast formazan conversion compared to cells treated with CSE alone over a four hour incubation period [Figure 11: a and b].

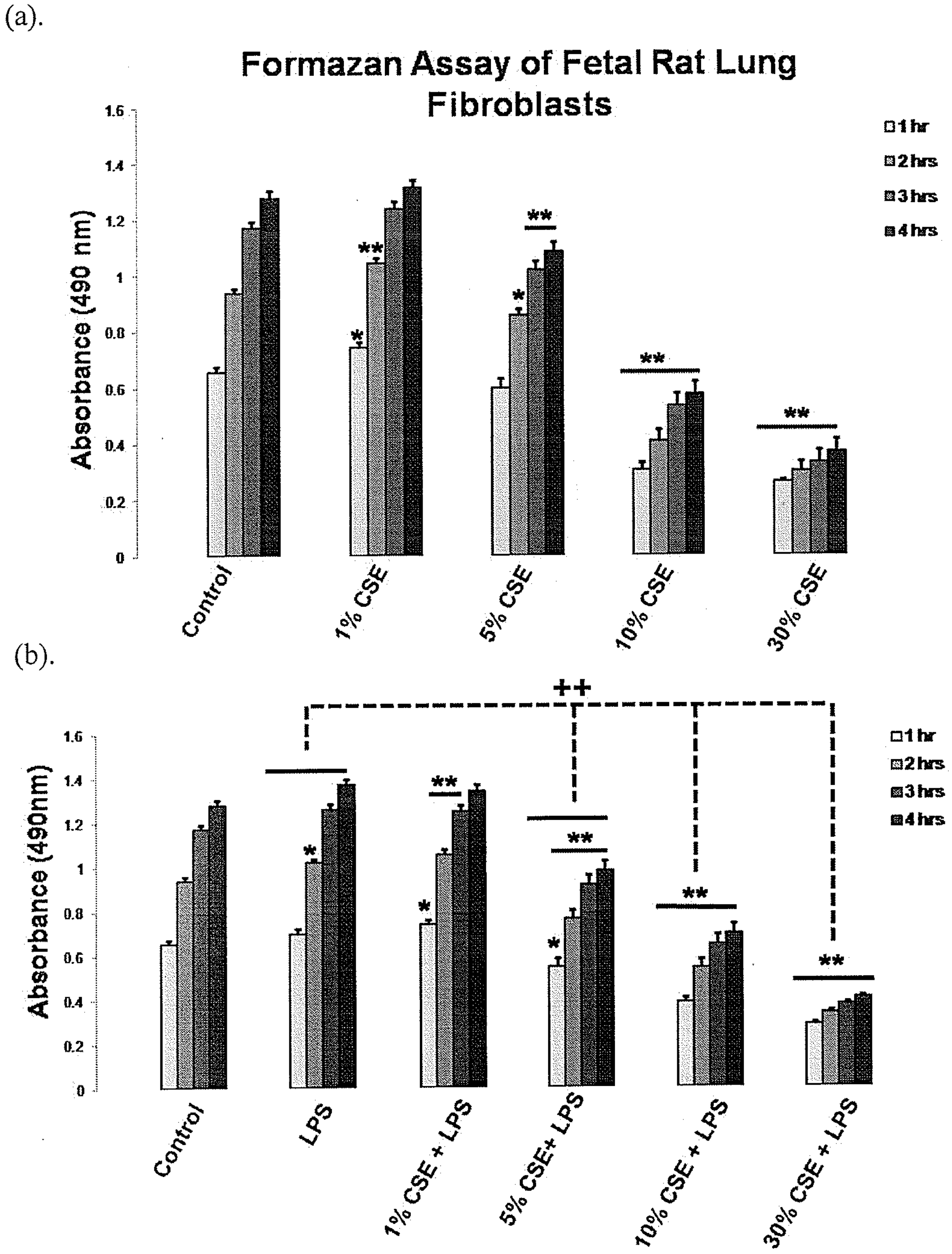


Figure 9: Effects of CSE without (a) or with (b) LPS on mitochondrial dehydrogenase activity in isolated fetal rat lung as determined by formazan assay. Absorbance was measured at 490nm using Biorad 550 Microplate Reader. Results are expressed using the mean absorbance values +/-SD of eighteen assays for each condition. **: ($p<0.01$), *: ($p<0.05$): significantly different from values obtained for the serum starved controls. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

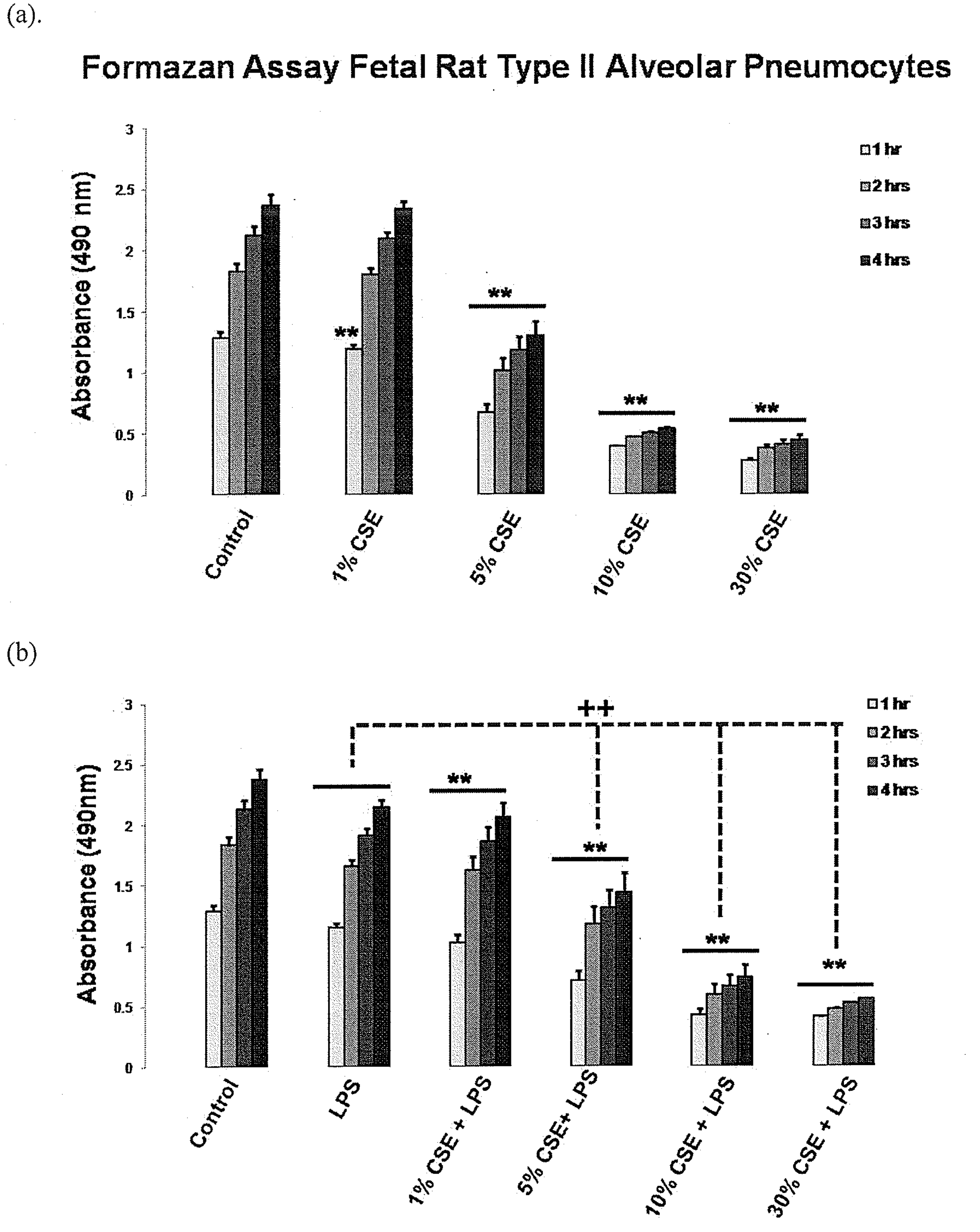


Figure 10: Effects of CSE without (a) or (b) with LPS on mitochondrial dehydrogenase activity in isolated fetal rat lung type II alveolar pneumocytes as determined by formazan assay. Absorbance was measured at 490nm using Biorad 550 Microplate Reader. Results are expressed using the mean absorbance values + SD of sixteen assays for each condition. **: ($p<0.01$), *: ($p<0.05$) significantly different from values obtained for the controls. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

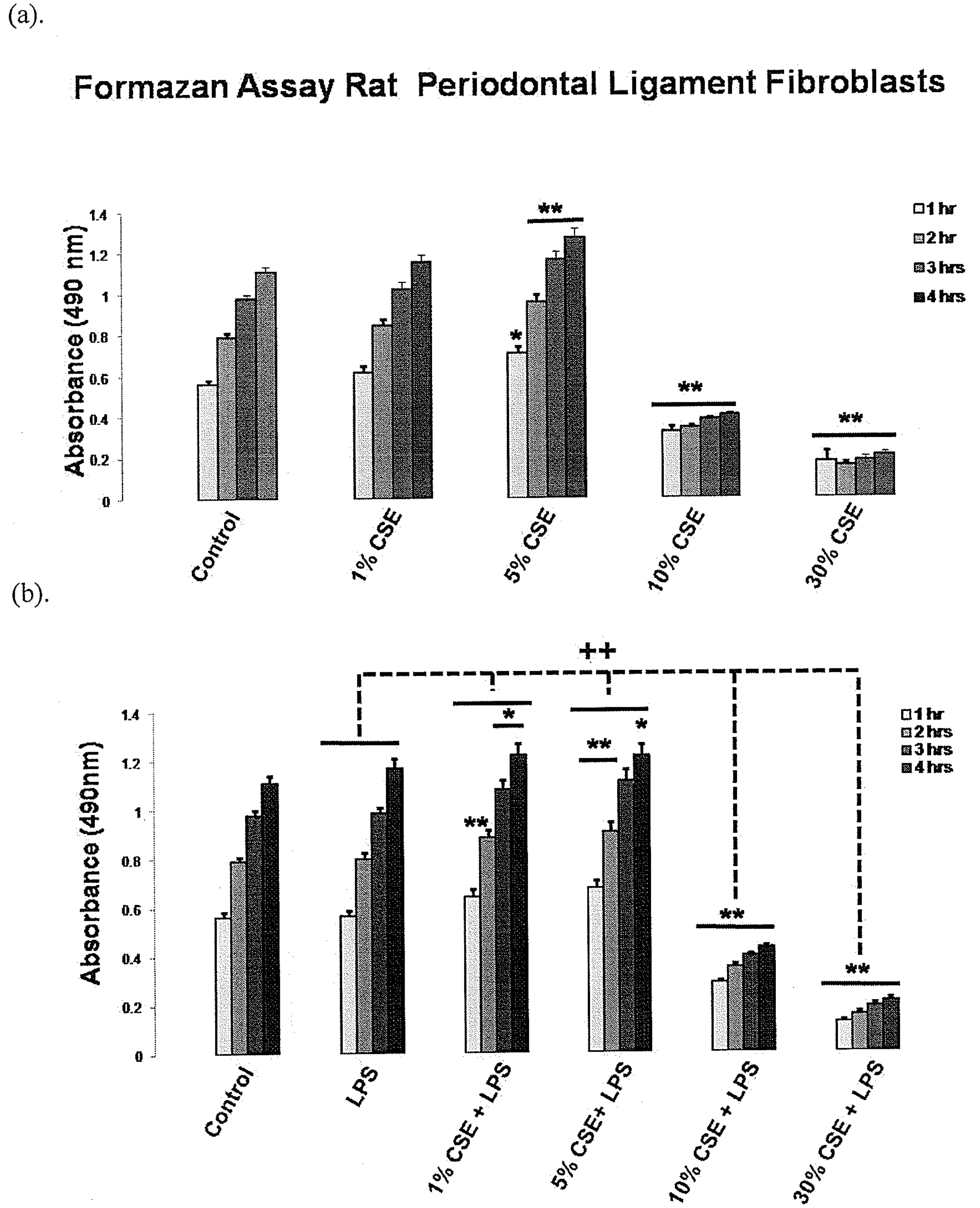


Figure 11: Effects of CSE without (a) or with LPS (b) on isolated rat periodontal ligament fibroblasts-mitochondrial dehydrogenase activity as determined by formazan assay. Absorbance was measured at 490nm using Biorad 550 Microplate Reader. Results are expressed using the mean absorbance values + SD of fifteen assays for each condition. **: ($p<0.01$), *: ($p<0.05$) significantly different from values obtained for the serum starved controls. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

Crystal Violet Assay

Crystal violet assay was carried out to investigate further the effects of CSE or LPS on cellular viability. This assay uses an analysis of binding of crystal violet dye to cellular proteins as a measure of cellular viability (*Alfa, 1998*) [Figures 12, 13 and 14].

Compared to cells cultured under serum starved control conditions, the absorbance values obtained from fetal rat lung fibroblast cultures exposed to LPS or 1% CSE increased significantly ($p<0.01$ and $p<0.05$ respectively) [Figure 12]. Cells exposed to 5%, 10% or 30% CSE with or without LPS displayed significantly reduced ($p<0.05$, $p<0.01$) absorbance at 590nm compared to cells cultured under serum starved control conditions or those treated with LPS-alone ($p<0.01$). Similarly exposure to 1% CSE with LPS was associated with diminished absorbance values ($p<0.01$) compared to the LPS-treated counterparts. Addition of LPS to the CSE treatments did not alter significantly the absorbance values obtained from cells exposed to CSE alone except in the case of those treated with 1% CSE.

In fetal rat lung type II alveolar pneumocytes absorbance values obtained by the crystal violet were reduced significantly ($p<0.05$, $p<0.01$) after exposure to 5%, 10% or 30% CSE with or without LPS compared to the serum starved control samples[Figure 13]. Similarly a significant reduction ($p<0.05$ and $p<0.01$ respectively) in absorbance was observed in cells treated with 10% CSE or 30% CSE with LPS compared to LPS alone treated cells. On the other hand, exposure to LPS or 1% CSE with or without LPS did not alter significantly the absorbance values compared to the serum starved control group (p>0.05). Addition of LPS to the 30% CSE treatment was associated with significantly greater ($p<0.05$) cellular absorbance values compared to cells exposed to 30% CSE alone.

In rPDL fibroblasts the absorbance values for cells exposed to 10% or 30 % CSE with or without LPS were decreased significantly ($p<0.01$) compared to those obtained from cells cultured under serum starved control conditions and the LPS-alone treated fibroblasts. Cells exposed to 1% CSE with or without LPS or 5% CSE with LPS displayed significantly augmented ($p<0.05$) absorbance values compared to the corresponding serum starved control group or the LPS-treated cells. LPS-exposed cells displayed a similar significant increase ($p<0.01$) in absorbance values compared to the serum starved controls [Figure 14].

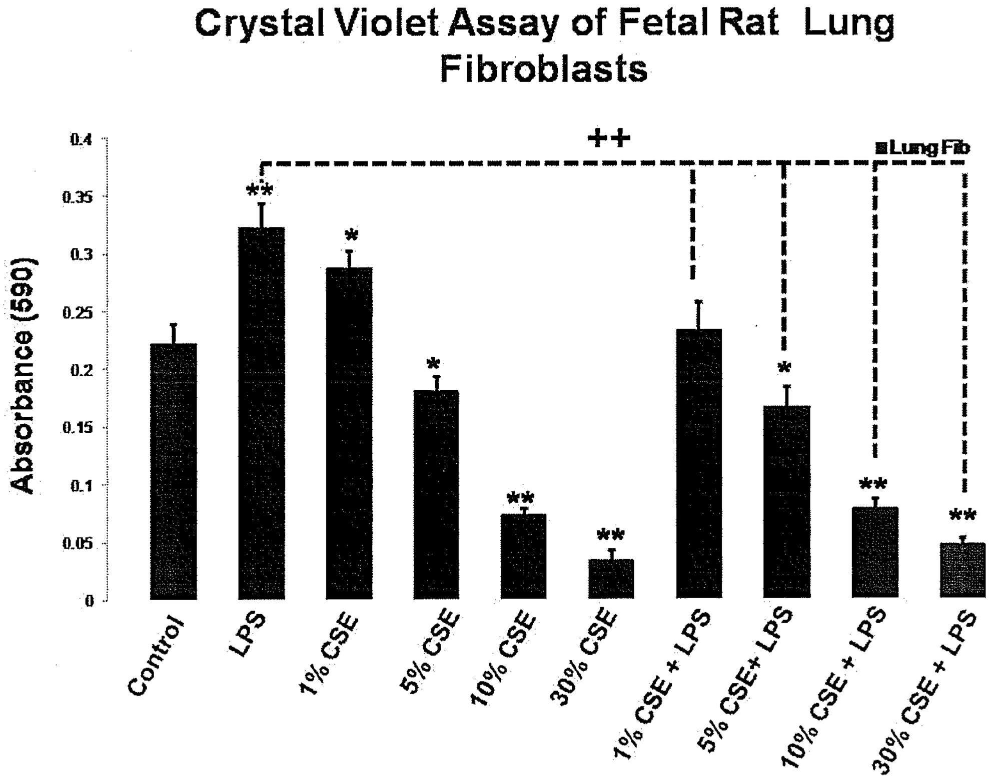


Figure 12: Viability of fetal rat lung fibroblasts as determined by crystal violet assay after a 24 hour exposure to CSE with or without LPS. Absorbance at 590 nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD of twelve assays for each condition. **: ($p<0.01$), *: ($p<0.05$) significantly different from the values obtained for the serum starved control group. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

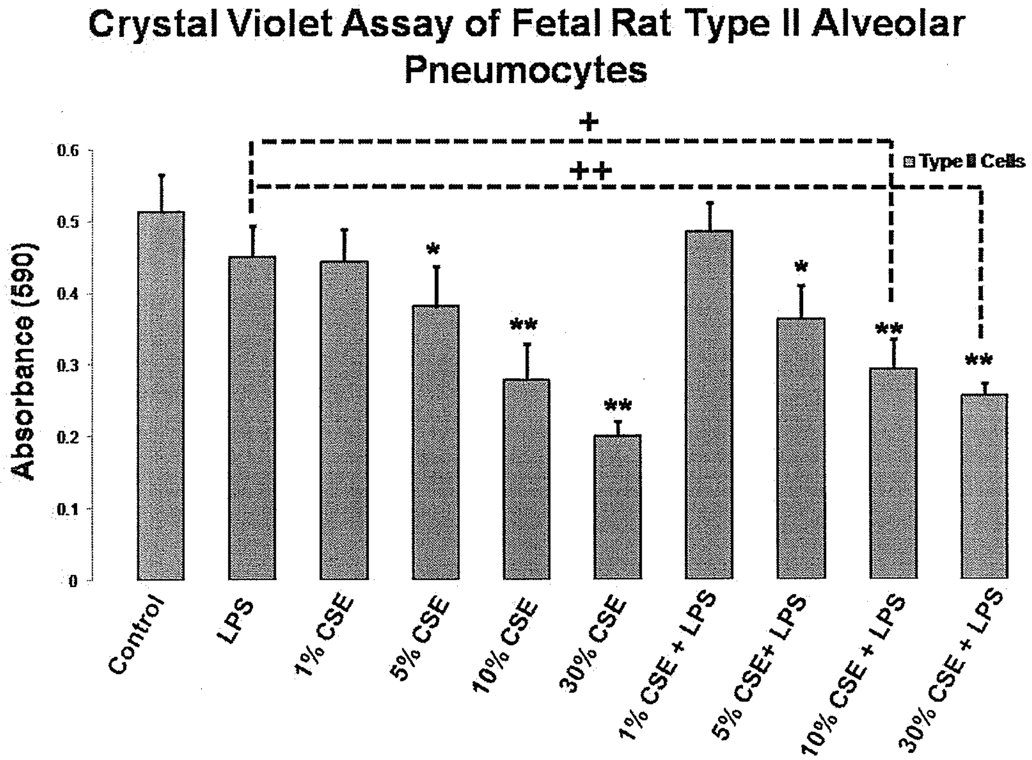


Figure13: Viability of fetal rat lung type II alveolar pneumocytes as determined by crystal violet assay after a 24 hour exposure to CSE with or without LPS. Absorbance at 590 nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD of seventeen assays for each condition. **: ($p<0.01$) *: ($p<0.05$) significantly different from the values obtained for the serum starved control group. ++: ($p<0.01$), +: ($p<0.05$) significantly different from values obtained for the LPS treated cells.

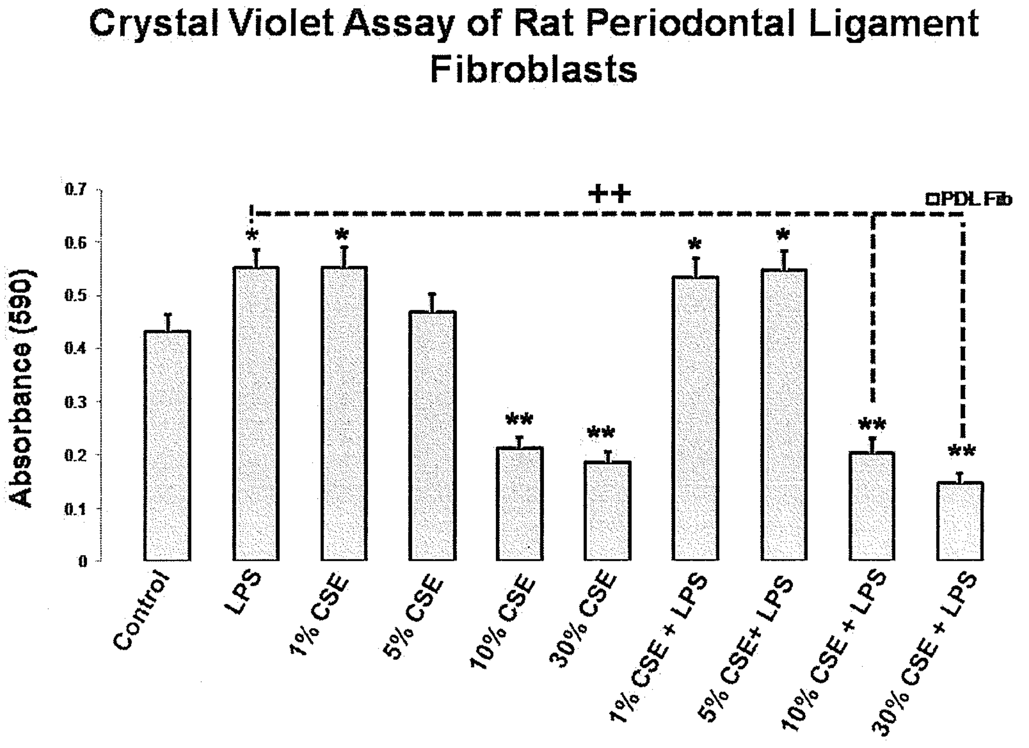


Figure14: Viability of rat PDL fibroblasts as determined by crystal violet assay after a 24 hour exposure to CSE with or without LPS. Absorbance at 590 nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD of twenty assays for each condition. **: ($p< 0.01$) *: ($p<0.05$) significantly different from the values obtained for the serum starved control group. ++: ($p<0.01$) significantly different from values obtained for the LPS treated cells.

Release of MMP-2 and MMP-9 by fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament fibroblasts upon exposure to CSE or LPS: Zymography results and analysis

To examine the effects of CSE on the release of gelatinases A (MMP-2) or B (MMP-9), zymographic gelatin A-impregnated gels were employed. Fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament fibroblasts were grown to subconfluence and treated with increasing concentrations of CSE with or without LPS for 24 hours. Conditioned media were collected, filtered through 0.22 μm Millipore filters, aliquoted and stored in -80 ^{0}C until needed. 20 μls of conditioned media/sample buffer (4:1) were applied without boiling to the substrate containing gels. Gels were run at 125V for an hour and twenty minutes and incubated overnight with developing buffer containing either 1mM PMSF or 20mM EDTA. Subsequently, gels were stained for 60 minutes in 0.25% Coomassie blue solution [30% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie brilliant blue R 250], destained for 30 minutes in 10% (v/v) glacial acetic acid, 30% (v/v) methanol and scanned as described in the Material and Methods.

Fetal rat lung fibroblasts secreted a 72 kDa gelatinase A and a 92 kDa gelatinase B, as indicated by the presence of clear bands due to digestion of gelatin [Figure 15: a and b], in contrast to gels incubated in EDTA where no bands were detected.

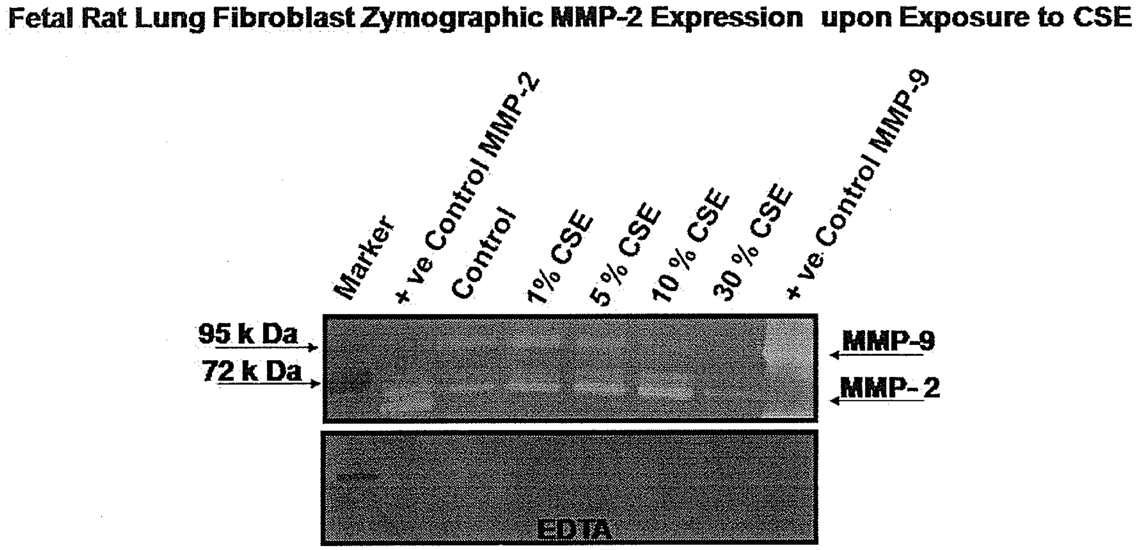
Quantitation of bands seen in figure 15: (a and b) is shown in figure 15: (c and d). Exposure of cells to 1%, 5% or 10% CSE resulted in a significant increase ($p<0.01$) in MMP-2 band size as quantified by Alpha Ease FC analysis software compared to the bands obtained from the serum starved control samples [Figure 15 c]. Similarly exposure of the cells to 1% CSE plus LPS resulted in a significant increase ($p<0.01$) in band size compared to either the serum starved controls or LPS-treated cells. Fibroblasts treated with 1% or 5% CSE with LPS showed significantly greater ($p<0.05$) band sizes compared to the LPS-treated cells. On the other hand, cells exposed to 30% CSE with or without LPS displayed significantly reduced ($p<0.01$) band size compared to the corresponding control samples. Band size of samples cultured with 10% CSE with LPS also showed significantly reduced ($p<0.01$) band size compared to both controls.

Fetal rat lung fibroblasts MMP-9 exposed to 10% or 30% CSE with or without LPS displayed significantly reduced ($p<0.01$) MMP-9 bands compared to those obtained from the serum starved control samples or LPS control samples. Similarly treatment with 5% CSE with LPS resulted in a significantly reduced band size ($p<0.01$) compared to the LPS treated cells. On the contrary, exposure to LPS or 1% CSE with or without LPS resulted in significantly greater ($p<0.05$, $p<0.01$) MMP-9 band sizes. Addition of LPS to the CSE treatment did not reveal a statistically significant effect on MMP-9 band sizes compared to those obtained from cells treated with CSE alone ($p>0.05$) [Figure 15 d]

In fetal rat lung type II alveolar pneumocytes, gelatinases A and B activities were also investigated using gelatin zymography which revealed indistinct MMP-9 bands thus hindering their densitometric quantification [Figure 15 (a and b)]. On the contrary, MMP-2 bands were well-identified and they were significantly greater ($p<0.01$) in cells exposed to LPS compared to those obtained from the serum starved control group [Figure 16 (a, b and c)]. On the other hand, cells exposed to 1% or 5% CSE with LPS displayed significantly reduced ($p<0.01$, $p<0.05$) band sizes compared to those of the serum starved control cultures or LPS-alone treated cells. A similar pattern was observed in cell cultures treated with 10% or 30% CSE with or without LPS compared to the LPS-alone treated counterparts or the serum starved control cultures ($p<0.01$) [Figure 16 c].

rPDL fibroblasts MMP-9 zymographic bands, although existing, were hardly detectable and unquantifiable [Figure 17 (a and b)]. On the other hand, MMP-2 bands were well identified and their sizes were significantly reduced ($p<0.01$) in cells exposed to 1% or 5% CSE compared to the corresponding serum starved control samples [Figure 16 c]. A similar significant reduction ($p<0.01$) in band sizes were observed in cells exposed to 1%, 5% or 10% CSE with LPS compared to those of the LPS-alone treated cells or serum starved control cultures.

(a).


Fetal Rat Lung Fibroblast Zymographic MMP-2 Expression upon Exposure to CSE

(b).

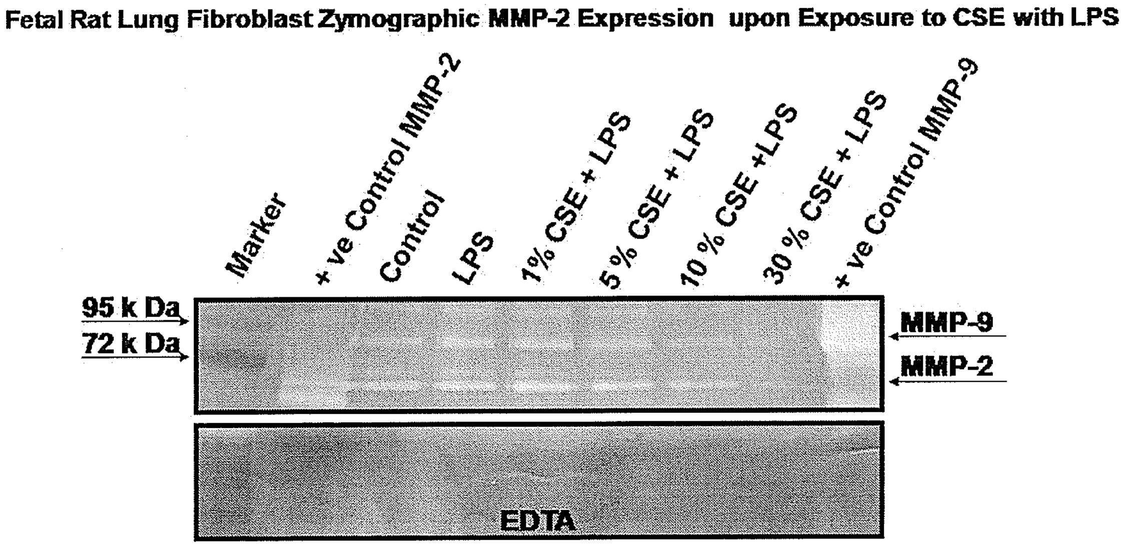

Fetal Rat Lung Fibroblast Zymographic MMP-2 Expression upon Exposure to CSE with LPS

Figure 15: **MMP-2 and MMP-9 gelatin zymography of fetal rat lung fibroblast cultured media.** (a,b). Representative gelatin zymographic gel images indicating the modulation of the conditioned media gelatinase-A, MMP-2 (72 kDa), secretion by fetal rat lung fibroblasts following the 24 hour treatment with increasing concentrations of CSE without LPS [(a). lanes 4, 5, 6 and 7)], with LPS [(b) lanes 5, 6, 7 and 8] and LPS-alone [(b) lane 4].

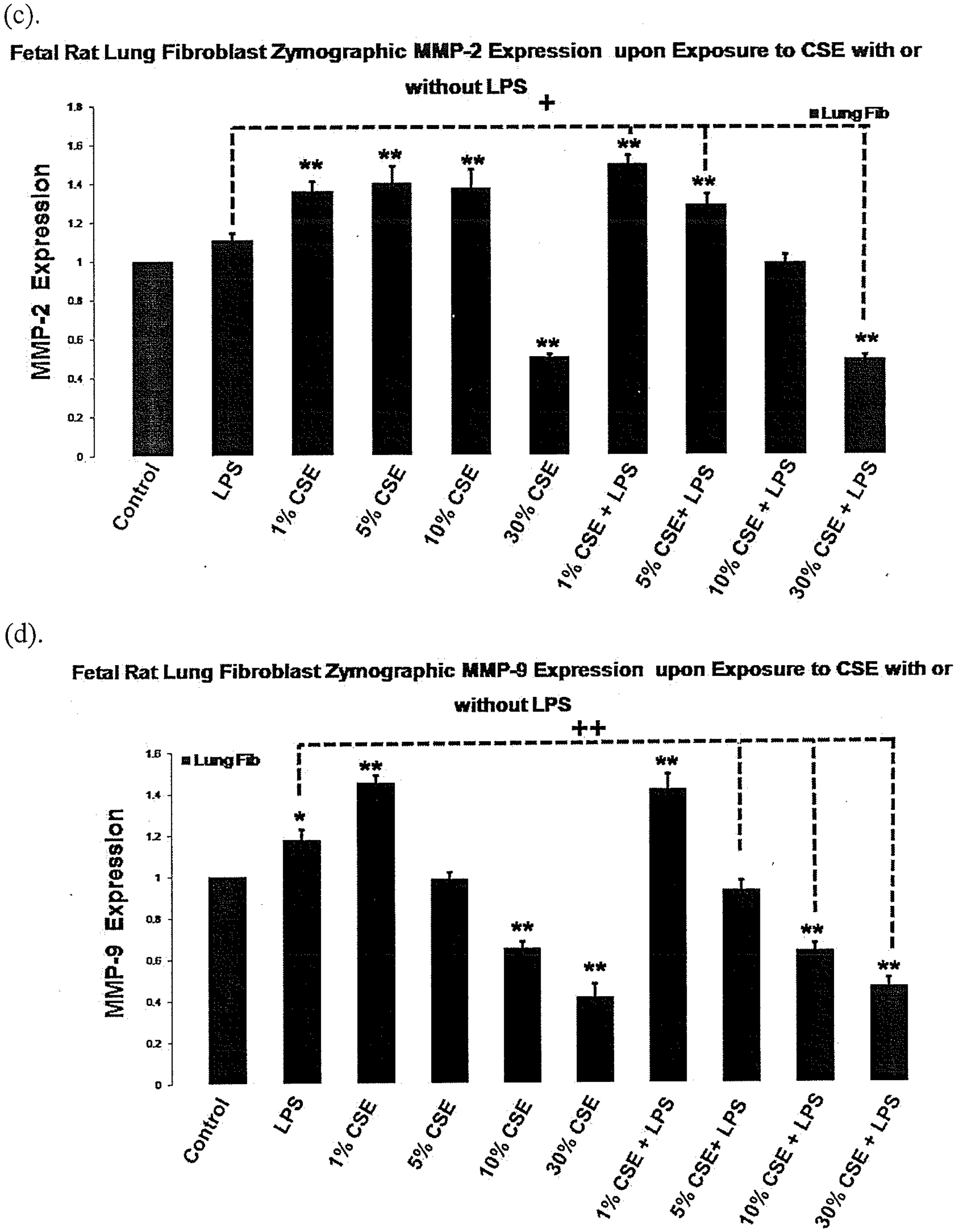
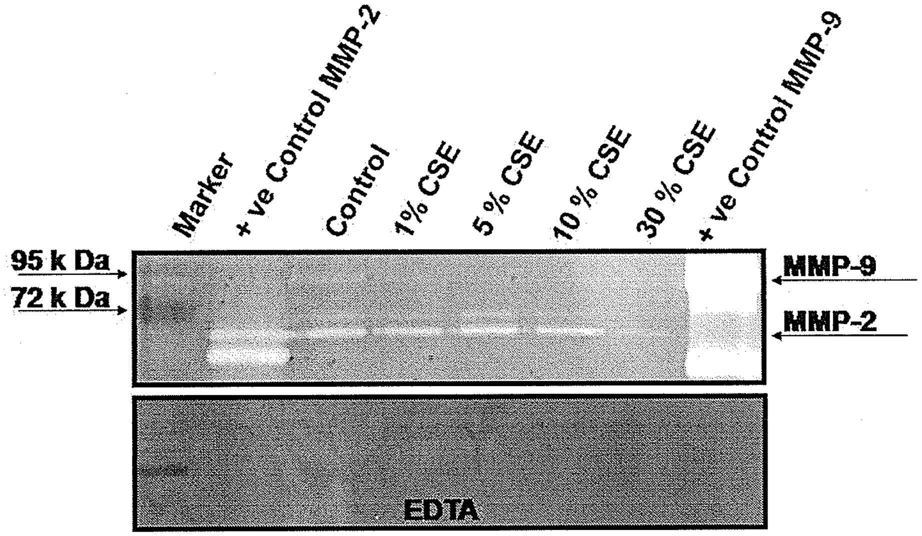


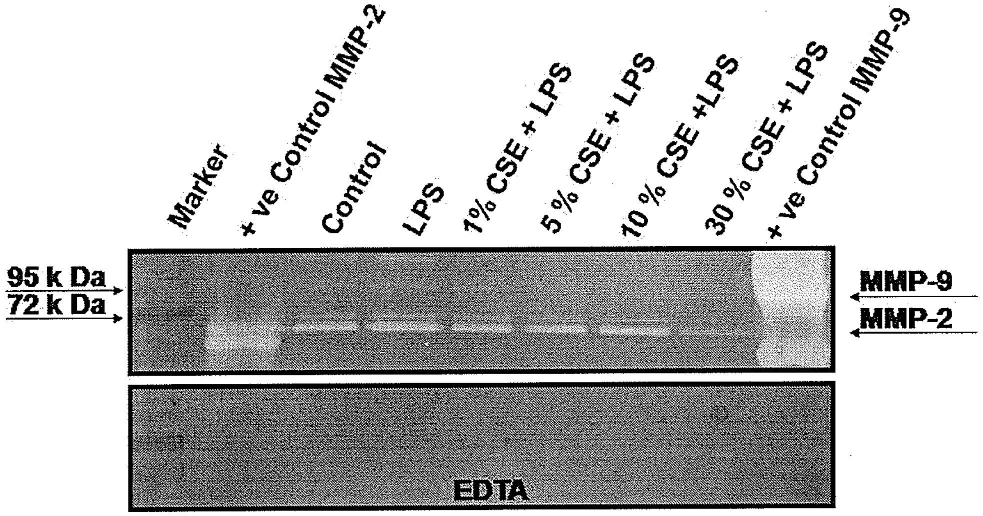
Figure 15 (c, d): Quantification of MMP-2 and MMP-9 for cultured media of fetal rat lung fibroblasts. Histograms plotted following densitometric quantification of MMP-2 and MMP-9 proteolytic activity detected as clear bands on gelatin-zymograms of twelve assays for each condition. (c) MMP-2 expression and release from cells exposed to CSE or CSE with LPS. (d). MMP-9 expression and release from cells exposed to CSE or CSE with LPS. **: ($p < 0.01$) *: ($p<0.05$) significantly different from the values obtained for the control group. ++: ($p<0.01$), +: ($p<0.05$) significantly different from values obtained for the LPS treated cells.

(a).



Fetal Rat Lung Type II Alveolar Pneumocyte Zymographic MMP-2 Expression upon Exposure to CSE

(b).



Fetal Rat Lung Type II Alveolar Pneumocyte Zymographic MMP-2 Expression upon Exposure to CSE with LPS

Figure 16: MMP-2 and MMP-9 gelatin zymography of fetal rat lung type II alveolar pneumocytes cultured media. (a,b). Representative gelatin zymographic gel images indicating the modulation of the conditioned media gelatinase-A, MMP-2 (72 kDa), secretion by fetal rat lung type II lung alveolar cells, following the 24 hour treatment with increasing concentrations of CSE without LPS [(a). lanes 4, 5, 6 and 7] and with LPS [(b). lanes 5, 6, 7 and 8] and LPS-alone [(b). lane 4].

(c).

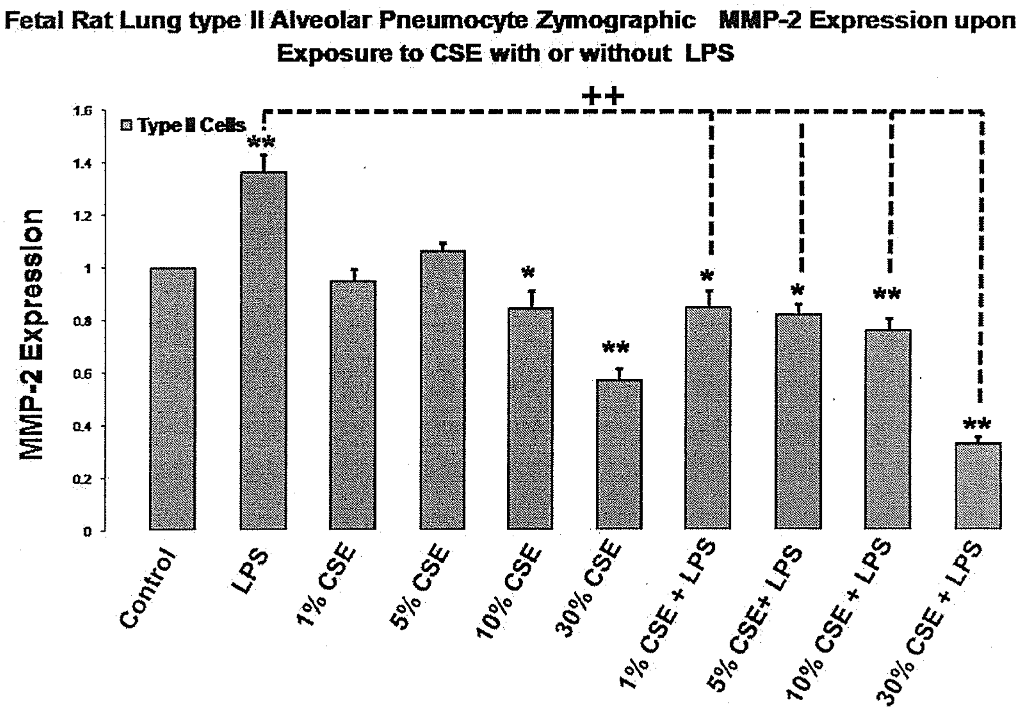
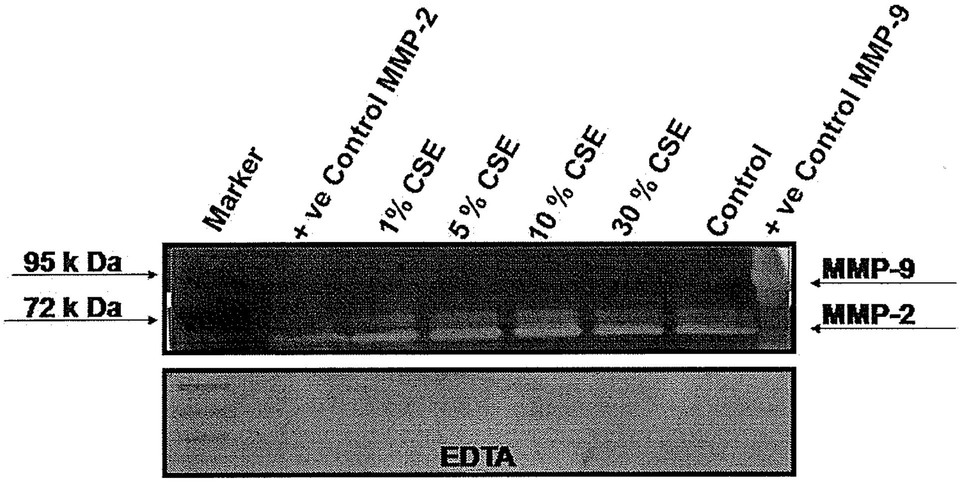


Figure 16: Quantification of MMP-2 for cultured media of fetal rat lung type II alveolar pneumocytes (c). Plotted statistical analysis results following densitometric quantification of MMP-2 proteolytic activity of twelve assays for each condition. **: ($p< 0.01$) *: ($p<0.05$) significantly different from the values obtained for the control group. ++: ($p<0.01$), +: ($p<0.05$) significantly different from values obtained for the LPS treated cells.

(a).

Rat Periodontal Ligament Fibroblast Zymogrphaic MMP-2 Expression upon Exposure to CSE



(b).

Rat Periodontal Ligament Fibroblast Zymographic MMP-2 Expression upon Exposure to CSE with LPS

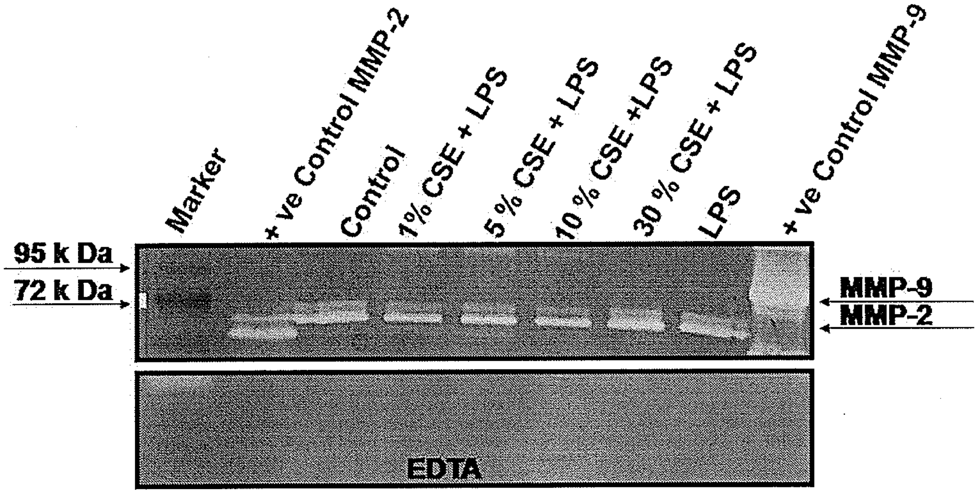


Figure 17: **MMP-2 and MMP-9 gelatin zymography of rat periodontal ligament fibroblast cultured media** (a,b). Representative gelatin zymographic gel-images indicating the modulation of the conditioned media gelatinase A, MMP-2 (72 kDa) secretion by rPDL fibroblasts, following the 24-hour treatment with increasing concentrations of CSE without LPS [(a) lanes 3, 4, 5 and 6) and with LPS [(b) lanes 4, 5, 6 and 7] and LPS-alone [(b) lane 8].

(c).

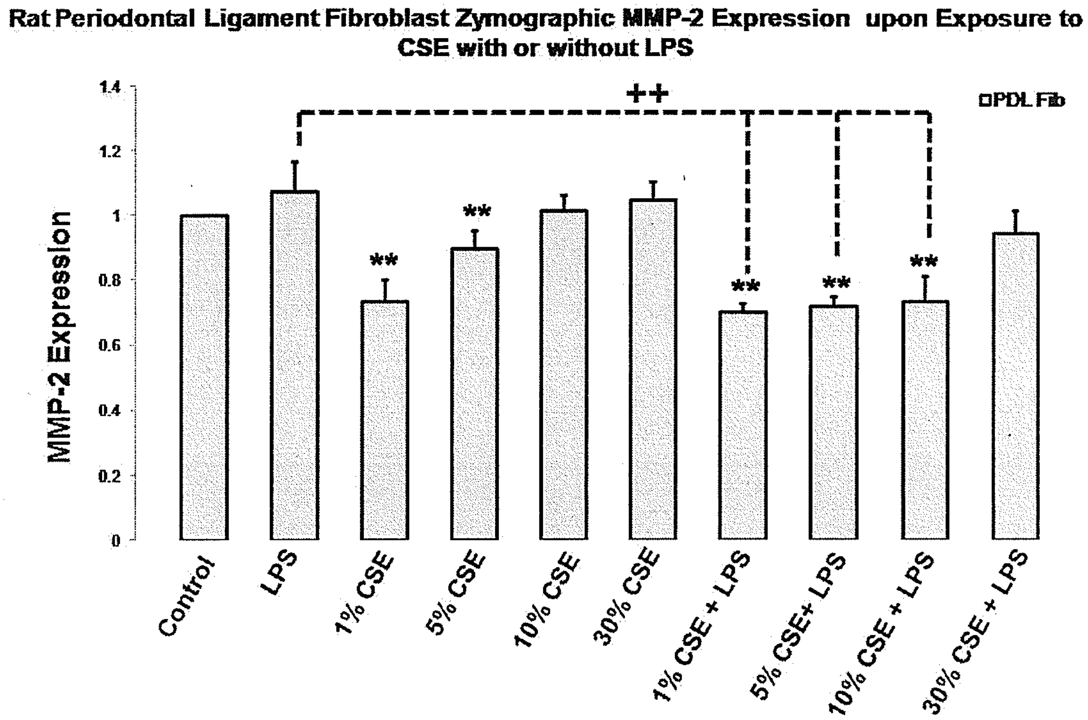


Figure 17: **Quantification of MMP-2 for cultured media of rat periodontal ligament fibroblasts (c)**. Plotted statistical analysis following densitometric quantification of MMP-2 proteolytic activity of twelve assays for each condition **: ($p < 0.01$) *: ($p<0.05$) significantly different from the values obtained for the control group. ++: ($p<0.01$), +: ($p<0.05$) significantly different from values obtained for the LPS treated cells.

Enzyme Linked Immuno-Sorbent Assay (ELISA)

ELISA is a biochemical technique that was performed to investigate the release of the gelatinases (MMP-2 and MMP-9) by fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament fibroblasts. ELISA against rat MMP-2, mouse MMP-9 and human MMP-8 antibody coated 96-well plates were purchased from R&D Systems (Minneapolis, MN. USA). Conditioned media collected after treating the three cell types under investigation with CSE with or without LPS, were applied following the manufacturer's instructions and incubated for two hours. Secondary antibodies were applied for two hours as well. Incubation with the tertiary enzyme-linked antibodies revealed detectable signals after the addition of a specific chromogen. MMP concentrations were extrapolated from a standard curve generated for each assay after measuring the absorbance at 450nm with a 570nm correction wavelength as recommended by the manufacturer using BioRad model 550 microplate reader.

In fetal rat lung fibroblasts the MMP-2 absorbance values obtained were increased significantly ($p<0.01$) after exposure of the cells to LPS or 1% CSE compared to those of the serum starved control samples [Figure 18]. On the contrary, cells exposed to 30% CSE with or without LPS displayed a significant reduction ($p<0.01$) is absorbance values compared to the serum starved controls or the LPS-alone treated cells. Similarly cells treated with 5% or 10% CSE with LPS displayed significantly reduced ($p<0.05$, $p<0.01$) absorbance values compared to the serum starved controls or the LPS- alone treated counterparts. Fetal rat lung fibroblasts treated with 1% CSE with LPS revealed significantly reduced ($p<0.05$) absorbance values compared the LPS-alone treated cells.

Fetal rat lung type II alveolar pneumocytes displayed a significant reduction ($p<0.05$, $p<0.01$) in absorbance values after exposure to 5%, 10% or 30% CSE with or without LPS compared to the serum starved controls [Figure 19]

Absorbance values of rPDL fibroblasts were reduced significantly ($p<0.05$) after exposure to 1% CSE compared to the serum starved control samples. [Figure 20]. No other significant changes were observed.

The same experiment was performed to examine MMP-9 or MMP-8 secretion but using anti-mouse MMP-9 and anti-human MMP-8 ELISA kits, as mouse and human have more than 80% amino acid homology for rat MMP-9 and MMP-8 respectively. In fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rPDL fibroblasts results were uninterpretable and statistically insignificant [Figures 21, 22, 23, 24, 25 and 26].

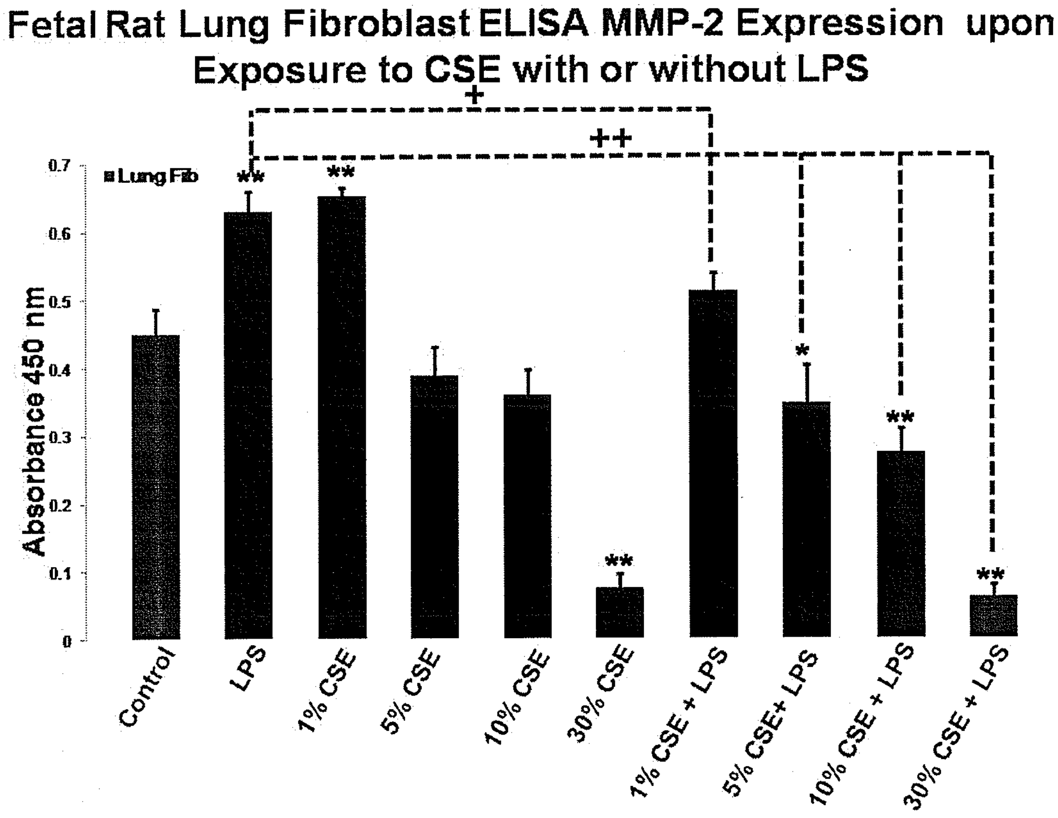


Figure 18: Fetal rat lung fibroblasts MMP-2 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD. **: ($p < 0.01$) *: ($p < 0.05$) significantly different from the values obtained for the serum starved control group. ++: ($p < 0.01$), +: ($p < 0.05$) significantly different from values obtained for the LPS treated cells.

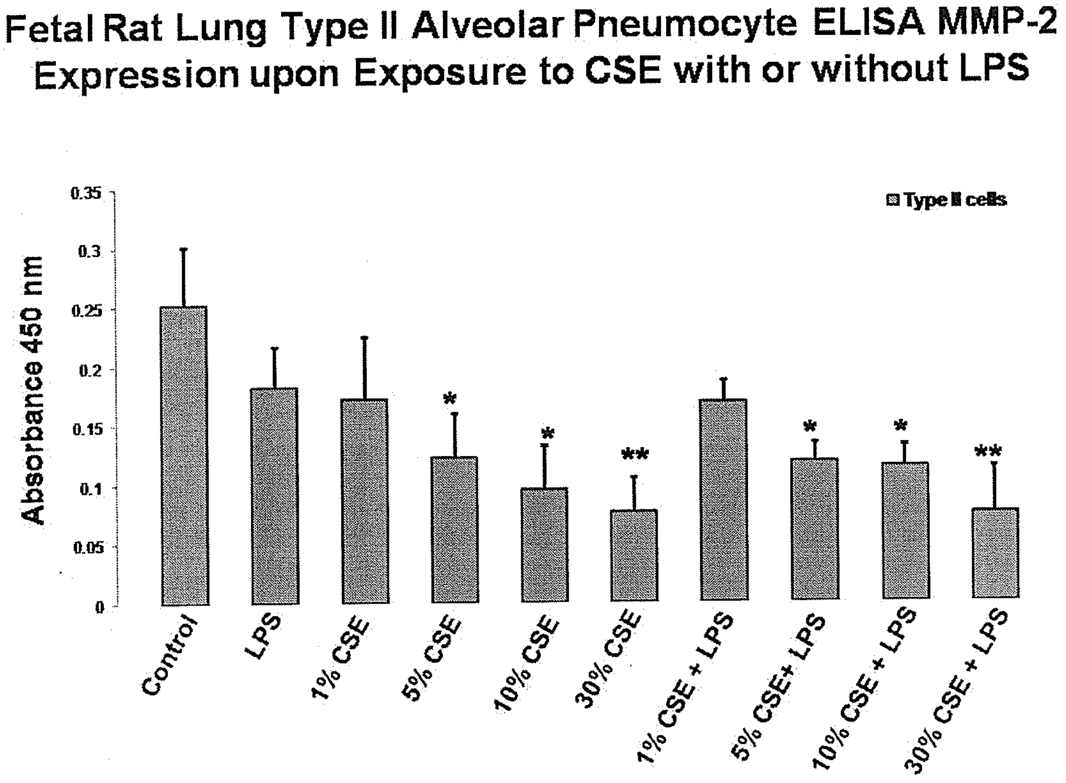


Figure 19: Fetal rat lung type II alveolar pneumocytes MMP-2 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD. **: ($p < 0.01$) *: ($p < 0.05$) significantly different from the values obtained for the serum starved control group.

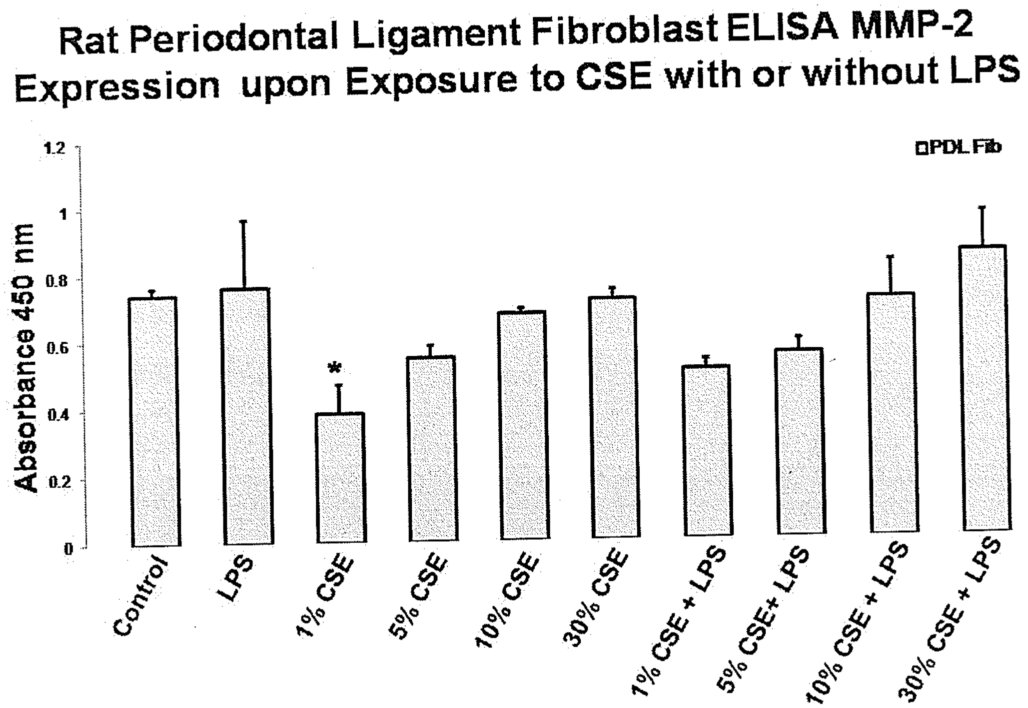


Figure 20: Rat periodontal ligament fibroblasts MMP-2 enzyme linked immunosorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader. Results are plotted using the mean absorbance values +SD. **: ($p < 0.01$) *: ($p < 0.05$) significantly different from the values obtained for the serum starved control group.

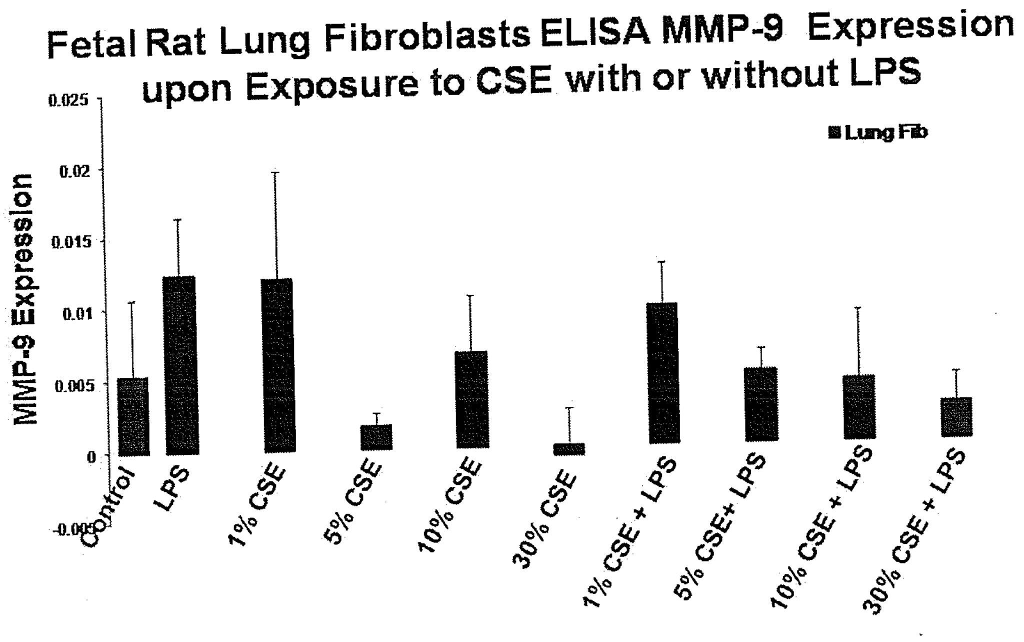
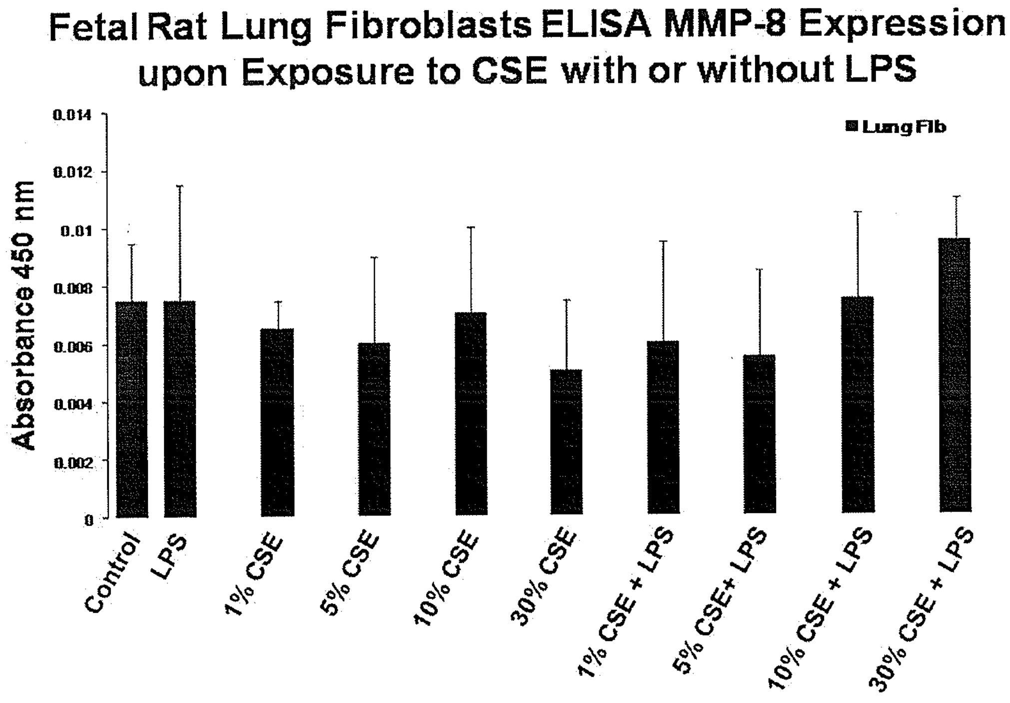


Figure 21: Fetal rat lung fibroblasts MMP-9 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader.



Fetal Rat Lung Fibroblasts ELISA MMP-8 Expression upon Exposure to CSE with or without LPS

Figure 22: Fetal rat lung fibroblasts MMP-8 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader.

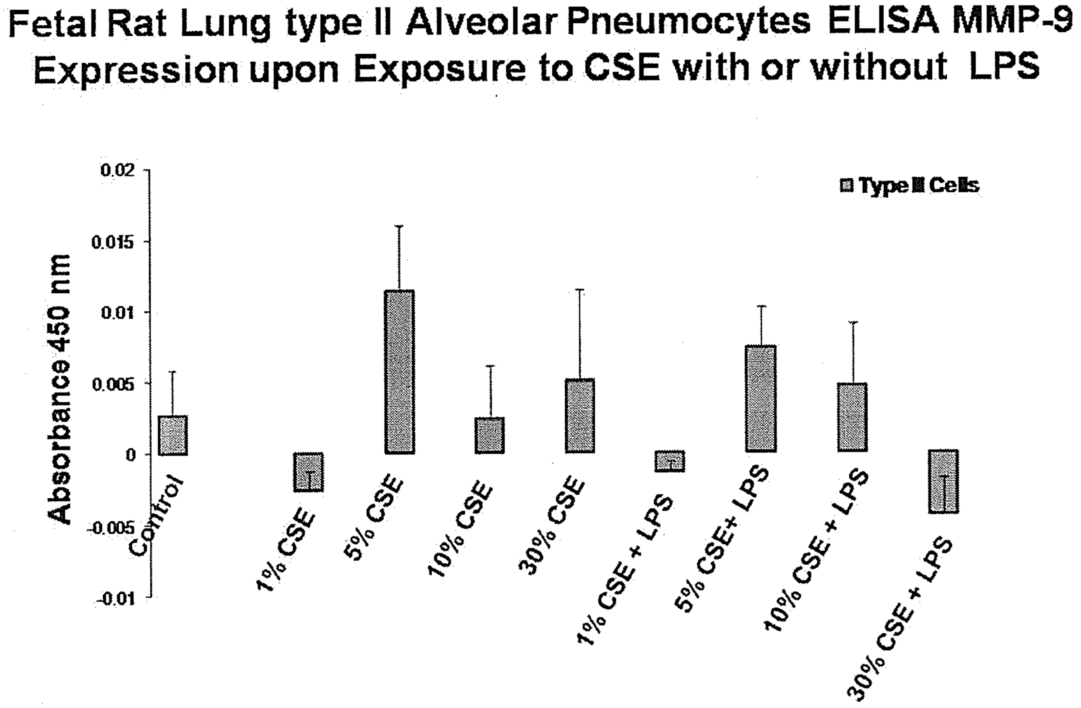


Figure 23: Fetal rat lung type II alveolar pneumocytes MMP-9 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader.

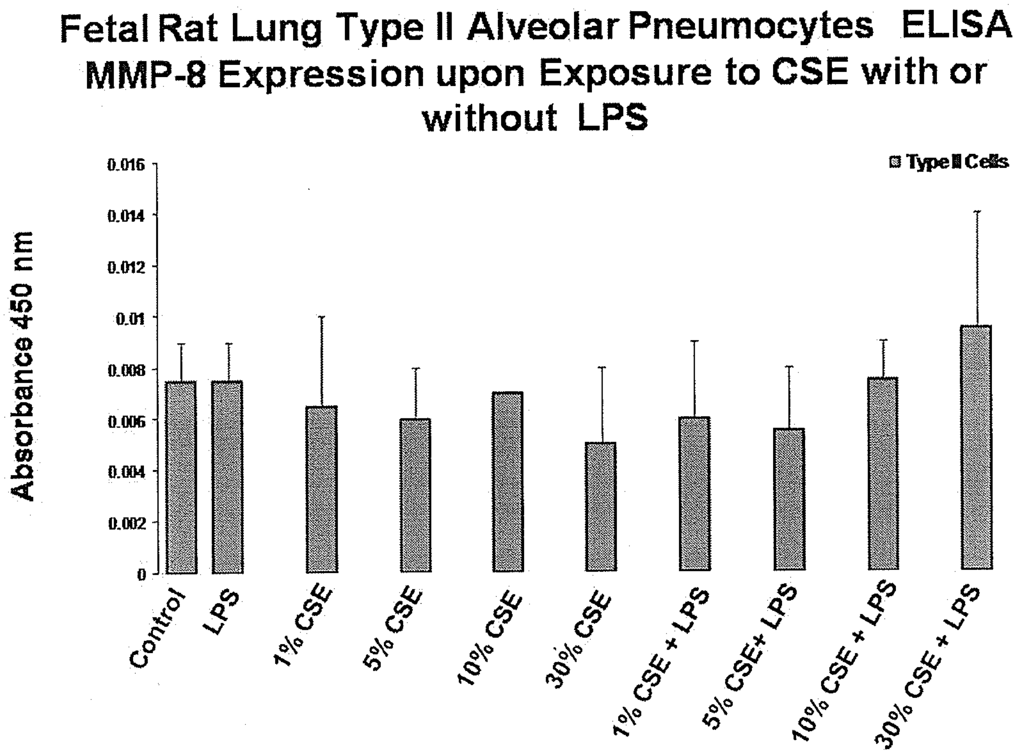


Figure 24: Fetal rat lung type II alveolar pneumocytes MMP-8 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader.

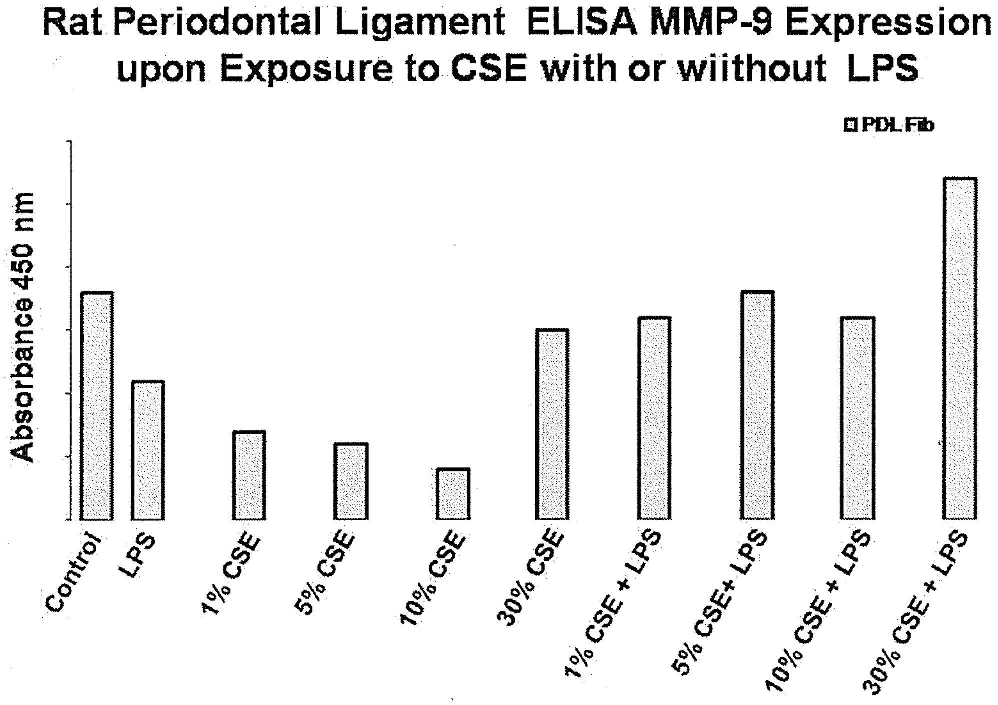


Figure 25: Rat periodontal ligament fibroblasts MMP-9 enzyme linked immunosorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader.

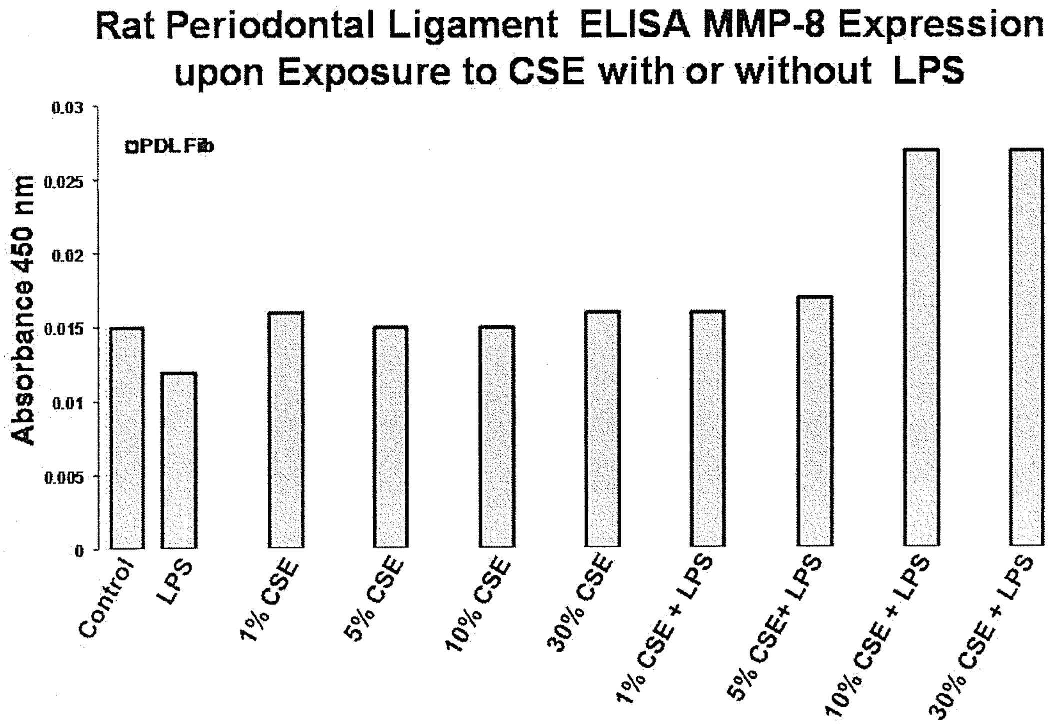
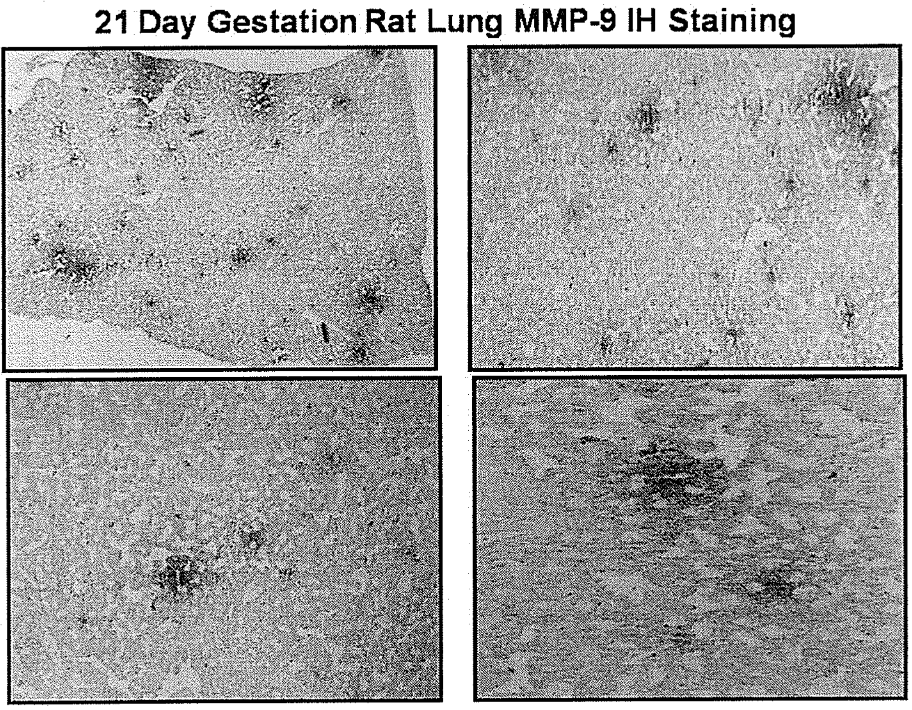


Figure 26: Rat periodontal ligament fibroblasts MMP-8 enzyme linked immuno-sorbent assay (ELISA) expression following a 24 hour treatment with increasing concentrations of CSE with or without LPS. Absorbance at 490nm was measured using a BioRad 550 microplate reader

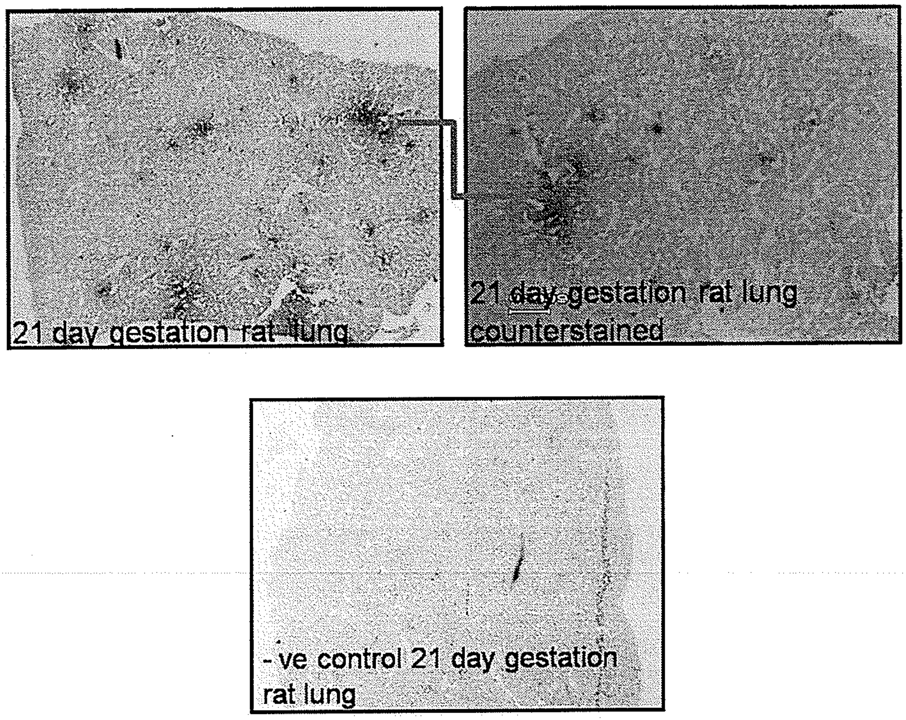
Immunohistochemistry (IH)

Immunohistochemistry was performed to localize MMP-9 antigens in fetal rat lung paraffin embedded tissue sections to identify pulmonary cells containing this enzyme. Expression of MMP-9 was observed in fetal lung tissues on the 21st gestational day [Figure 27 (A)]. Staining occurred in localized pockets distributed evenly throughout the lung. This staining appeared to be associated with canalicular regions but not the developing acini [Figure 27 (A)]. The control samples reacted without the primary antibody did not display any reaction product [Figure 27 (B, D)]. A similar localized pattern of staining in the developing mandibular and maxillary areas were also detected [Figure 27 (C)].

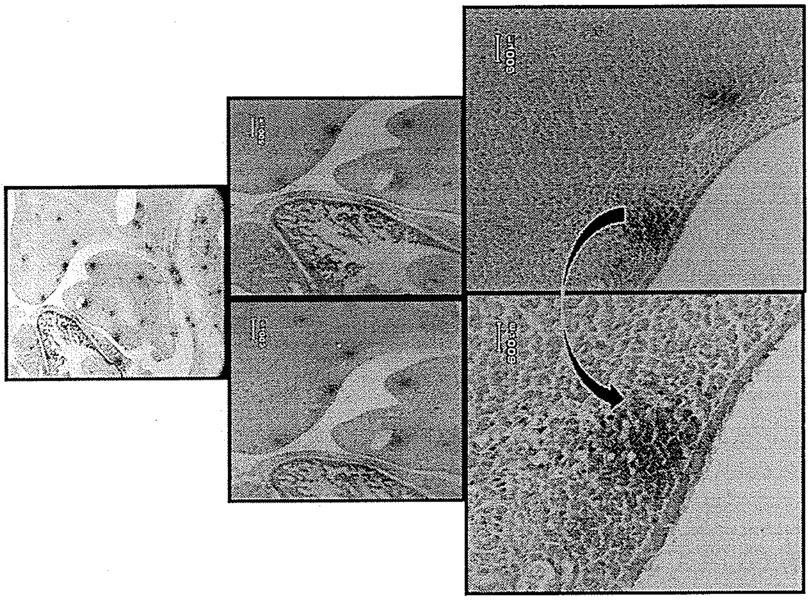
(A).


21 Day Gestation Rat Lung MMP-9 IH Staining

(B).


21 day gestation rat lung
21 day gestation rat lung counterstained
- ve control 21 day gestation rat lung

(C).



(D).

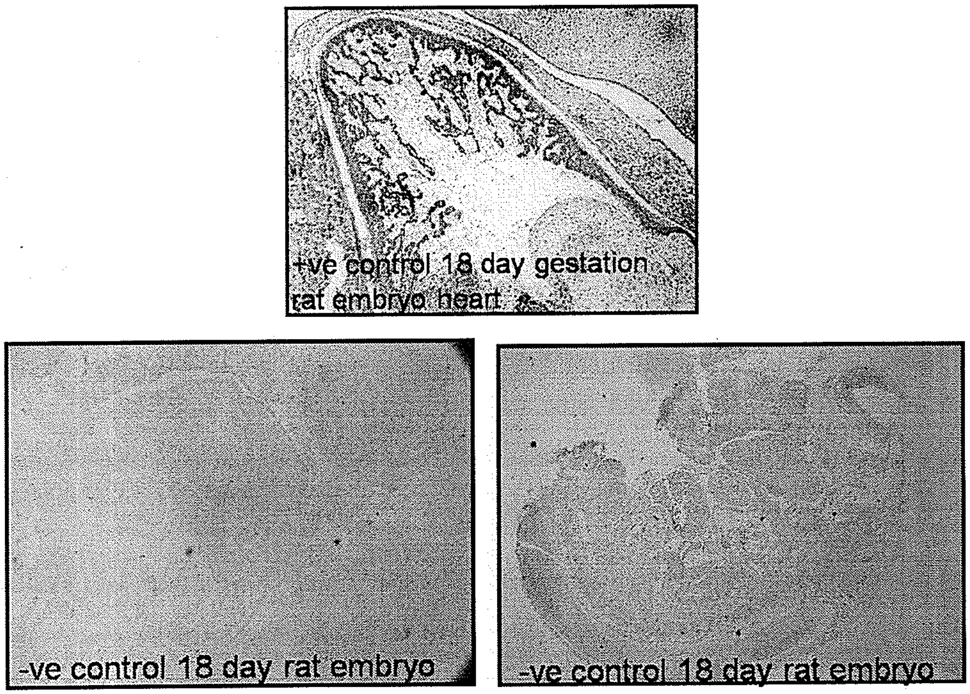


Figure 27: 21 day gestation rat lung and 18 day gestation full embryo sections of MMP-9 immunohistochemical staining. (A). 21 day gestation rat lung MMP-9 IH staining with (B). Counter-staining and the negative control (C). 18 day gestation rat full embryo section with enlarged head and jaw regions displaying MMP-9 IH staining and counter-staining (D). Shows the positive and negative controls. (10x and 40x magnification)

DISCUSSIONS, CONCLUSIONS and FUTURE DIRECTIONS

Tobacco is a complex mixture of over 4500 chemicals; including a plethora of toxic components such as free radicals, polycyclic aromatic hydrocarbons, nitrosamines, oxidants, carcinogens and other constituents like the highly addictive nicotine. Many of these compounds are metabolized further into more harmful chemicals. The World Health Organization estimates that there are 1.3 billion cigarette smokers worldwide, a number that will rise to 1.6 billion by 2020, bringing the tobacco death-toll to 10 million by 2030. Tobacco smoke exposure induces or exacerbates a myriad of pathological conditions such as cardiovascular pathologies, inflammatory conditions such as pneumonia, COPD, periodontitis, rheumatoid arthritis and peptic ulcers. Smokers also have a higher risk of developing cancers in various organs than non-smokers (*Stanescu, 2003; Thorley et al., 2007*). Children born to smoking mothers are more likely to suffer from respiratory disorders and may succumb to sudden infant-death syndrome at a higher rate *(DiFranza and Lew, 1995)*. Furthermore, fetal developmental defects, abnormal pulmonary alterations, abortion, preterm delivery, stillbirth and other disorders are plausible detrimental effects of maternal smoking during pregnancy. All these may be attributed to the dual exposure of the fetus to smoke components through the circulation and the amniotic fluid, where slow removal rates lead to their accumulation. This is particularly true of the lungs, whose normal development and distention depend in part on the amniotic fluid brought into the lung by fetal breathing movements. Consequent prolonged fetal exposure to tobacco smoke constituents increases and aggravates the effects of tobacco on the fetus *(Janoff et al., 1987; Wen et al., 1990, Hannrahan et al.,* *1992; Higgins, 1992; Cunningham et al., 1994; Cliver et al., 1995; Mathew, 1998; Gilliland et al., 2003; Hofhuis et al., 2003; Joad, 2004).*

Cigarette smoke evokes inflammatory response, which triggers the accumulation of inflammatory cells and the release of mediators such as chemokines like CXCL8, inflammatory cytokines including IL-1β and TNF *(Walters et al., 2005)* and proteases. Proteolytic enzymes are not only secreted by the inflammatory cells, but also by non inflammatory epithelial and stromal cells such as fibroblasts.

The respiratory system is one of the most complex organs in the body. It has a dual developmental origin, being partially of epithelial or mesenchymal origin and its organogenesis commences prenatally and continues postnatally. The major organ of the respiratory system is the lung, which is composed of more than 40 specialized cell types. It has the largest body surface area exposed to the external environment *(Harding et al., 2004)*, which increases the lung exposure to infections from various pathogens and external insults, thus triggering its repair mechanisms. Interestingly, about 5% of lung collagen can turnover in an adult animal on a daily basis, thus demanding an intricate balance between synthesis and degradation of the ECM components *(Laurent, 1982).*

The main function of the lung is to deliver oxygen and remove carbon dioxide from the blood stream. This takes place within specialized air filled sacs called the alveoli, whose walls are lined by two distinct cell types, type I and type II alveolar pneumocytes. Type II alveolar pneumocytes are the most prevalent cells in the alveoli. They are cuboidal in shape and are believed to be the precursors of type I cells. Moreover they are the producers of surfactant, which lower the surface tension on the lung preventing its collapse in neonates. Type I alveolar pneumocytes are considered to be terminally differentiated cells, which line the alveoli providing an extremely attenuated gas exchange surface and a mechanical barrier that prevents the passage of toxins and particulate matters. Furthermore, the lung is enveloped with a connective tissue continuum, which supports it and provides a mechanical scaffold on which the alveoli and the capillaries are mounted. Within this parenchyma, like most organs, connective tissue components are embedded, such as collagen and elastin fibers, proteoglycans, fibronectins and a mélange of connective tissue cells of which pulmonary fibroblasts are predominant. Lung fibroblasts are the major cellular population of the lung parenchyma *(Carnevali et al., 2006).* They play crucial roles in its remodeling, repair and development where epithelial/mesenchymal interactions are paramount *(Wong et al., 2004).* Besides the scaffold they provide for the epithelial alveolar cells to grow on, their secretions including growth factors and proteases are crucial to this E/M interaction during lung morphogenesis. External stimuli and insults, although first targeting the mechanical barrier of the lung epithelium, may percolate through the basement membrane into the underlying connective tissue. This is particularly true of the aqueously-soluble compounds, such as these components found in cigarette smoke Indeed, cigarette smoke may interact with the fibroblasts (*Ishii et al., 2001; Kim et al., 2004; Chen et al., 2005)* triggering DNA damage, inhibiting migration and proliferation *(Nakamura, 1995)* and inducing apoptosis *(Carnevali et al., 2003),* thus resulting in an overall change in the lung interstitium and the epithelial compartment leading to defective pulmonary development and functions.

The periodontium

The periodontium comprises the tooth-supporting and surrounding tissues including the alveolar bone proper, the periodontal ligament and cementum. The periodontal ligament (PDL) is a unique, multifunctional, soft connective tissue that connects the tooth-cementum to the investing and surrounding alveolar bone. It comprises different cell-populations embedded in a mostly collagenous matrix. Periodontal ligament fibroblasts predominate in the periodontium and are considered to be its basic building block. They maintain this unique connective tissue integrity, secure its continual turnover, remodeling and regeneration and protect it against myriad oral cavity insults *(Park et al., 2001)*. PDL fibroblasts synthesize most of the extracellular matrix components including collagen fibers, which are the major PDL fibers. They also engage in breaking down the extracellular matrix through their direct degradative capacity or by secreting proteases such as MMPs *(Birkedal-Hansen, 1993),* as demonstrated in orthodontic tooth movement *(Takahashi, 2006).*

Many studies have provided convincing evidence that tobacco users have a much higher risk of developing oral pathologies ranging from minor tissue discoloration, connective tissue destruction, particularly associated with the tooth-attachment apparatus to carcinomas *(Bergstrom et al., 2000; Calsina G et al., 2002; Mullally, 2004; Baljoon, 2005).* Degradation of the tooth supporting and surrounding peridontium results in increased tooth mobility and a plausible subsequent tooth loss *(Krall et al., 1999).* Consequently, deranging the masticatory apparatus and decreasing response to periodontal treatment, resulting in what is clinically known as refractory periodontitis *(Ah et al., 1994; Haffajee et al., 1997)*. Moreover, the prevalence of periodontitis among smokers *(Calsina et al., 2002)* is a potential cause of bacteremia and spread of infection to other organs, thus increasing the potential of developing systemic diseases *(Vellappally et al., 2007),* such as cardiovascular ailments, respiratory disorders like pneumonia or rejection of transplanted organs *(Ioannidou et al., 2006).*

Lipopolysaachride is a gram-ve bacterial endotoxin that resides in the outer membrane of bacteria. Exposure to LPS triggers the host inflammatory response by activating various intracellular signalling pathways, such as the inhibitor of kappa B kinase (IKK) and mitogen activated kinase pathways (MAKP). Following exposure, LPS binds to the LPS-serum binding protein (LBP) which delivers it to the glycosyl-phosphatidylinositol-linked membrane protein CD14 and enhances LPS binding affinity to its receptor (*Meng et al., 1997*). Subsequently, the LPS-CD14 complex will interact with toll-like receptor 4 (TLR4) and its accessory protein MD2. The intracellular adaptor proteins MyD88 and Mal will be recruited to the Toll/IL-1 receptor (TIR) cytoplasmic domain of TLR4, thus transducing the signal intracellularly and activating the above mentioned signalling pathways (*Mackman, 2003*). Consequently, pro-inflammatory cytokine secretion by various cells is increased *(Moore et al., 1994; Rossa et al., 2007),* and hence that of proteases like MMPs resulting in connective tissue destruction *(Butler et al., 1989)*

Taking all this information into account, we examined the *in vitro* effects of whole cigarette smoke extract with or without the gram –ve bacterial endotoxin LPS on the adult oral mesenchymally-derived rat periodontal ligament fibroblasts and the pulmonary developing fetal lung cells (fetal rat lung fibroblasts and fetal rat lung type II alveolar pneumocytes). Subsequently, we investigated cellular proteases (MMP-2 and MMP-9) release following exposure to CSE and LPS.

Our phase contrast microscopic images of the fetal rat lung fibroblasts and fetal rat lung type II alveolar pneumocytes exposed to (1%, 5% or 10%) of CSE with or without LPS or LPS-alone showed no signs of cellular detachment or change in morphology, thus accord with *Lannan et al., (1994)* and *La Rocca et al., (2007)*. On the contrary, cellular exposure to doses of CSE (20% and above) was associated with alteration in the morphology and attenuation of attachment to culture dishes. These observations are in keeping with several published reports *Ishii et al., (2001)*.

To the best of our knowledge we were the first to investigate the *in vitro* effects of whole cigarette smoke extract on rPDL fibroblasts. Our phase contrast microscopic images showed signs of cellular detachment in cells exposed to concentrations of CSE (10% and above) with or without LPS, whereas 1% or 5% CSE or LPS-alone did not alter cellular morphology or cause detachment from culture dishes.

The observed cellular detachment of all cell types upon exposure to high CSE doses suggests that CSE induces irrevocable cellular damage. This damage might result in cellular apoptosis and/or necrosis, that *in vitro* might be reflected as a form of cellular detachment. These intriguing observations triggered further scrutiny to investigate further the effects of CSE on cellular viability and proliferation. To do so, three different assays were performed trypan-blue, crystal violet and formazan.

Trypan blue viability assay, which is based on dye exclusion by living cells *(Freshney, 1987)* revealed a significant reduction in trypan blue exclusion in fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament fibroblasts exposed to 10% or 30% CSE with or without LPS. Conversely, low doses of CSE (1% or 5%) with or without LPS or LPS-alone did not alter significantly dye exclusion in the three types of cells under investigation.

Crystal violet dye binding to cellular protein was evaluated to examine also cellular viability. Fetal rat lung fibroblasts and fetal rat lung type II alveolar pneumocytes displayed significant reduction in absorbance after exposure to 5%, 10% or 30% CSE with or without LPS. In rPDL fibroblast crystal violet absorbance values were reduced significantly after exposure to 10% or 30% CSE with or without LPS. This provides strong evidence that higher concentrations of CSE reduce cellular viability. On the contrary, exposure to 1% CSE with or without LPS or 5% CSE with LPS significantly increased the absorbance values suggest little or no effect on cellular viability. Similarly our rPDL fibroblast crystal violet assay absorbance values suggest that under certain circumstances rPDL fibroblast and fetal rat lung fibroblasts may be stimulated to proliferate after LPS exposure thus accord with *Kim et al., (2007)* who showed enhanced gingival fibroblast proliferation after LPS treatment.

The formazan assay cellular mitochondrial dehydrogenase activity is often interpreted as a measure of cellular viability and proliferation *(Mosmann, 1983)*. Cells were examined following exposure to increasing concentrations of CSE with or without LPS. Our results were consistent with those of the crystal violet assay. Mitochondrial dehydrogenase activity in fetal rat lung fibroblasts and fetal rat lung type II alveolar pneumocytes was reduced significantly after exposure to 5%, 10% or 30% CSE with or without LPS. These findings accord with those of *Carnevali et al., (2003)* who demonstrated an increase in oxidative stress and apoptosis of human lung fibroblasts in a CSE dose dependent-manner, as well as those of *Hoshino et al., (2001)* who showed a reduction in epithelial cell viability in a dose response relationship to CSE-concentrations. On the other hand, fetal rat lung fibroblasts exposed to 1% CSE or the LPS-alone displayed significantly increased formazan conversion, possibly relating to the different tissue of origin of the cell types.

In rPDL fibroblasts a significant reduction in formazan conversion was observed only in cells exposed to higher CSE levels (10% or 30%) with or without LPS, whilst cells treated with 1% CSE with LPS or 5% CSE with or without LPS displayed high absorbance values. These outcomes follow the trend of the crystal violet assay and suggest that lower CSE levels did not alter cellular metabolism.

Collectively, the outcomes of the above mentioned viability assays suggest that cigarette smoke might modulate the cellular proliferation rate and viability. Although there is evidence that cigarette smoke induces cellular damage, yet the mechanisms and the nature of the damage are not clear.

In vivo fetal rat lung fibroblasts are closely associated with the interstitial connective tissue and may be shielded from insults by the mechanical barrier and basement membrane of type I alveolar pneumocytes. Nevertheless, they may be readily exposed to some insults including the aqueous smoke components, which pass the barrier and leach through the basement membrane into the underlying connective tissues. *Ishii et al., (2001)* suggested that lung fibroblast exposure to concentrations of CSE (5%-25%) for 20 to 24 hours induced their apoptosis and reduced their viability, while higher concentrations (50% and above) resulted in cellular necrosis, whereas according to *Kim et al., (2004)* low concentration of CSE induces severe, reversible DNA damage but not apoptosis. *Carnevali et al., (2003)* have demonstrated that CSE exposure increased human lung fibroblast oxidative stress and DNA damage resulting in cellular apoptosis. Such outcomes might affect these cells basic mesenchymal functions such as proliferation, migration and extracellular protein synthesis and degradation, which are paramount in the proper development and repair of tissues, hence increasing the chances of anomalous development. The previous observations support the trend that we have observed in our fetal lung fibroblast cell cultures in that the CSE-induced detachment and modulation in absorbance values of the viability assays, similar to the observations of *Ishii and colleagues, (2001)*. These results might also imply that CSE alters the cellular viability and proliferation potentials. Further investigation to elucidate the obscure mechanisms through which these changes occur is required.

Leanderson, (1992), Lannan, (1994), Hoshino, (2001) and their colleagues have demonstrated the detrimental effect of cigarette smoke on alveolar epithelial cells. These effects took the form of suppression of proliferation, attenuation of attachment, DNA single-strand breaks, diminishment of surfactant secretion and collagen production. Moreover, *Ramage et al., (2006)* have presented evidence that tobacco smoke exposure initiates apoptosis in lung epithelial cells resulting from mitochondrial damage, leading to cellular detachment and apoptosis. *VanDer Toorn et al., (2007)* believed that the CSE-induced alterations in mitochondrial membrane potential, oxygen consumption and production of ATP switch the energy dependent programmed cell death (apoptosis) to necrosis. On the other hand, *Tsuji et al., (2004)* suggested that cigarette smoke generated oxidative stress and DNA-damage result in alveolar epithelial cell stress-induced senescence. Therefore these changes may jeopardize cellular functions, such as their ability to proliferate, divide and migrate. They might also reduce physical defense capabilities by increasing alveolar space permeability and reducing surfactant production. These may in part explain the myriad findings of smokers' aberrant lung development, impaired repair capacity, re-epithelialization and increased susceptibility to infections.

Inhibition of PDL fibroblast attachment and growth by tobacco metabolites like nicotine and cotinine were extensively reported *(Tipton et al., 1995; Giannopoulou et al., 1999; James et al., 1999; Chang et al., 2002),* yet to the best of our knowledge and excluding our current report, whole cigarette smoke extract effects on PDL fibroblasts have not been investigated. Our viability and proliferation assays smoke outcomes, and in keeping with the above mentioned findings, suggest that tobacco smoke and its metabolites might endanger the integrity of these tissues, reduce their response to treatments, repair and regeneration capacity. The findings may also explain the decreased treatment-response and increased prevalence of periodontal disorders and connective tissue destruction among smokers *(Haber et al., 1993).*

Matrix metalloproteinases (MMPs) belong to a family of 26 proenzymes, which play significant roles in the extracellular matrix remodeling and turnover. Their expression in and secretion by cells, and their involvement in many developmental events and physiological mechanisms make their existence and proper functioning crucial. MMP-DNA transcription is an inducible, highly regulated mechanism, which occurs subsequent to stimulation by various molecules, including inflammatory cytokines, such as interleukins *(Benbow et al., 1997; Ye, 2000).* Upon translation the enzymes will be stored in vesicles in the Golgi apparatus. These vesicles are secreted into the extracellular matrix either automatically or through the regulated secretory pathway *(Borregaard et al., 1997; Frederick and Nagase 2000).* Zymogen activation is a highly regulated intricate mechanism. It occurs either intracellularly through furin convertases in the Golgi apparatus as in the case of MMP-11,-23,-28 and MT-MMPs, or in the extracellular environment. MMP-degrading arsenal, which resides in the catalytic domain is kept always under tight control either by masking it with the sulphahydryl bonding to Zn and the prodomain cysteine-switch on which activation is dependent or by MMP-inhibitors like the tissue inhibitors of metalloproteinases (TIMPs) (*Bauer et al., 1975*). TIMPs bind MMPs in a 1:1 ratio thus curtailing their accessibility to their substrates. MMP-degrading capacity is involved in many physiological events such as growth, development and wound healing. Yet their uncontrolled action is destructive due to tissue matrix lose potentially resulting in uncontrolled cellular growth and consequently tumor development. MMPs are divided into six groups on the basis of their anticipated function, substrate specificity, sequence similarity and domain organization.

MMP-2 and MMP-9 constitute a MMP-group known as gelatinases. These enzymes are expressed in most mesenchymal cells (fibroblasts) during development, tissue repair, regeneration and pathological conditions such as inflammation *(Morgunova et al., 1999).* They are also secreted by stromal cells surrounding various invading and metastasizing tumors *(Salo et al., 1983; Pykre et al., 1992)*, including head and neck cancers, lung, gastric, breast and ovarian malignancies where MMP-2 over expression is a prominent predictor of a poor prognosis *(Yoshizaki et al., 2002).* Moreover, *Shapiro, (1998)* has shown that MMP-2 is one of the major proteases involved in soft tissue degradation. Both gelatinases are engaged in breaking down collagen type IV, which is the major component of the basement membrane together with perlecan, laminins, integrins and other molecules. *Tsuruga et al., (2007)* have shown that MMP-2 degrade fibrillin-2, the major structural component of microfibrils, which together with tropoelastin form the elastic fibers. These fibers are an integral part of the ECM of the skin, blood vessels, periodontal ligament and lung *(Rosenbloom et al., 1993).* Therefore, in the respiratory system and particularly during lung development, gelatinases are crucial to ECM remodelling, adequate Epithelial/Mesenchymal (E/M) interaction and hence proper branching of the duct system (*Fukuda et al., 2000).* Furthermore, their involvement in various pathological conditions, such as asthma COPD and lung cancer, has been highlighted and investigated highly *(La Rocca, 2007)*. In the oral cavity, *Korstoff et al., (2000)* have shown that MMP-2 is increased in the gingival tissues of patients with periodontitis and according to *Neto et al., (2004)* its activity is augmented in smokers. Nevertheless, MMP-2 specific role in smoke induced-periodontitis is still obscure.

Gelatinases released by fetal rat lung fibroblasts, fetal rat lung type II alveolar pneumocytes and rat periodontal ligament fibroblasts upon exposure to CSE was investigated employing the sensitive substrate zymography technique. This technique is based on the ability of MMPs to degrade their preferential substrates, which are embedded in the SDS-based gels, resulting in clear gel bands. Zymographic bands were definitive of MMP-2 release by all cells under investigation. On the other hand, MMP-9 bands were well identified and quantifiable in fetal rat lung fibroblasts, whilst those of fetal rat lung type II epithelial cells were indistinct and barely detectable. This may be attributed to what *Fukuda et al.*, (*2000*) have demonstrated as little or no MMP-9 was found in epithelial cells during the late stages of lung development, whereas MMP-2 was detected in both epithelial cells and fibroblasts during each developmental stage. Interestingly, rPDL fibroblasts faint bands corresponding to MMP-9 were observed especially after LPS-stimulation. This contradicts with *Achong et al., (2003)* findings in gingival fibroblasts where MMP-2, but not MMP-9 was secreted.

The band sizes obtained from fetal rat lung fibroblast gelatinases were modulated by CSE. Our results revealed that MMP-2 band sizes were increased significantly in cells exposed to 1% CSE with or without LPS, 5% or 10% CSE. These outcomes are in keeping with those of *Ning et al., (2006)*. On the other hand, high CSE concentrations (30% and above) with or without LPS reduced MMP-2 band sizes. These findings agree with the recently published data of *La Rocca et al., (2007)* demonstrating a down regulation in MMP-2 activity in human lung fibroblasts exposed to CSE. MMP-9 expression was also investigated using gelatin zymography, which revealed reduced band sizes in cells exposed to 10% or 30% CSE with or without LPS. On the contrary, exposure to LPS or 1% CSE with or without LPS resulted in significantly increased MMP-9 band sizes.

Fetal rat lung type II alveolar pneumocyte MMP-2 bands were reduced in cells exposed to 1%, 5%, 10% or 30% CSE with LPS. An opposite trend was observed in cells exposed to LPS-alone. On the other hand, rPDL fibroblast MMP-2 band sizes were reduced significantly in cells exposed to 1% or 5% CSE with or without LPS, or 10% CSE with LPS compared to their controls. Nevertheless, these bands were increased approximately in a CSE dose dependent-manner, a trend that may be attributed to the CSE-induced cellular cytotoxicity, apoptosis and necrosis *(Carnevali et al., 2006)* and/or LPS-induced interleukin secretion where IL-1 α, IL-1β, IL-6 and IL-8, of which IL-6 and IL-8 are considered to be the major players in periodontal ligament inflammation *(Yamaji et al., 1995)*. Furthermore, based on what *Thirumangalakudi et al., (2007)* have demonstrated in neurons, the LPS-induced IL-8 transcription stimulates gelatinases secretion and augments the release of pro-apoptotic proteins *Bim* and cell-cycle protein cyclin D1, which in turn may increase cell death *(Thirumangalakudi et al., 2007).*

Taking collectively our zymography outcomes and referring to the published evidence, we conclude that CSE is definitely a modulator of gelatinases secretion. Yet, the disparity among the findings of other researchers hinders our capacity to fully explain the mechanisms that generated these observations. Nevertheless, to our understanding this discrepancy and variation may be attributed to the type of research cigarette used, the amount of solution or the solution itself through which the cigarette smoke was bubbled. Moreover, variations might also stem from the cells on which the experiments were conducted, whether they were from an immortal cell line or primary culture, as the latter, according *Ramage et al., (2006)* are likely more sensitive. Furthermore, the density of the plated cells and their culture environment may also be crucial modulators, as the density may affect the amount of antioxidants produced, which in turn may protect the cells against the harmful effects of CSE *(Kim et al., 2004).* On the other hand, the culturing as well as the extracellular matrix environment may alter the cellular response to treatment, *Kim et al., (2004)* have demonstrated that cells cultured in three dimensional collagen-gels react differently to the CSE treatment compared to those grown as monolayers in culture dishes.

In spite of these inconsistencies, there is generally a unanimous agreement that CSE is a modulator of MMP secretion. Such modulation may lead to alteration in proteolysis/anti-proteolysis balance, which in turn results in defective development or repair of the ECM.

Indeed, the importance of a physiologically balanced state of the ECM is underscored by the close association of cells and matrix and the presence of transmembrane signaling routes *(Ingber, 1991; Zimmerman, 1999)*. Consequently this effect of CSE on MMP secretion may alter basic cellular functions and contribute to the development of pathologies.

Gelatinases released by fetal rat lung fibroblasts, fetal rat lung type alveolar pneumocytes and rat periodontal ligament fibroblasts, were also examined by the highly sensitive antibody based ELISA assay. Our results showed that MMP-2 released by fetal rat lung fibroblasts was reduced after exposure to 5%, 10% CSE with LPS. A similar pattern was displayed by cells exposed to 30% CSE with or without LPS. On the other hand, 1% CSE and LPS-alone treatments augmented the release. In a similar fashion a higher levels of CSE (5%, 10% or 30%) with or without LPS were associated with reduced MMP-2 release. These results were consistent with those of demonstrated by zymography, thus suggesting that MMP-2 release as well as activity might be indeed affected by CSE.

In rPDL fibroblasts MMP-2 release was diminished significantly only in cells exposed to 1% CSE. Nevertheless, a similar trend was detected by zymography, where the MMP-2 release was augmented in a manner approximately dependent on the CSE concentrations.

The release of MMP-9 and MMP-8 was also investigated using ELISA with antibodies raised against human and rat antigens respectively from R&D Systems, due to the lack of specific antibodies raised against rat. Despite the fact that these enzymes have more than 80% interspecies amino acid homology and the experiments were technically successful, results were not clear. This might be due to the lack of antigen/antibody compatibility, which led to inconsistent readings.

Therefore, based on our cumulative results and taking all the aforementioned outcomes of our *in vitro* study, we conclude that cigarette smoke, with or without the gram-ve bacterial endotoxin LPS, may alter cellular viability and proliferation in a cell specific manner, as our cells of interest behaved and reacted differently to various concentrations of CSE with or without LPS. Furthermore, cellular exposure to CSE with or without LPS may alter cellular release of the gelatinases A and B, known as MMP-2 and MMP-9 respectively. Variations in zymogen secretion were observed in different cell types exposed to the same treatment and within the same cell-group exposed to different concentrations of CSE with or without LPS. Disturbing cellular functions such as viability, proliferation and secretion may result in derangement of the whole system in general and cellular secretions such as gelatinases in particular. Collectively, this may result in changes in the local conditions which in turn might adversely affect extracellular matrix formation, renewal and turnover rates, remodeling capacity and developmental interactions at the cellular level.

Although *in vitro* changes in cellular functions were obvious outcomes of our study, fully outlined scientific mechanisms and signaling pathways that explain these findings are still obscure. Therefore, investigating further the mechanisms through which tobacco smoke induces its effects would be intriguing future studies.

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