THE RESPONSES OF HUMAN NEUTROPHILS TO TOBACCO SMOKE COMPONENTS

Nouf Khider Al-Shibani

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the School of Dentistry,
Indiana University

December 2012

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

L. Jack Windsor, Ph.D., Chair

_____

Fengyu Song, D.D.S., M.S., Ph.D.

Doctoral Committee

_____

Michael Kowolik, B.D.S., Ph.D.

_____

John Goodpaster, Ph.D.

_____

September 7, 2012

Danise Rogers Subramaniam, Ph.D.

ACKNOWLEDGMENTS

My most sincere thanks go to my advisor and mentor, Dr. L. Jack Windsor for his guidance, encouragement and support during the development of this work. He has taught me all about self-discipline when doing laboratory work and about writing a scientific communication.

I am indebted to my committee members (Dr. Michael Kowolik, Dr. Fengyu Song Dr. John Goodpaster, and Dr. Danise Rogers Subramaniam) for their kind insights and suggestions.

I would like to thank my husband (Nawaf Labban) for his support and everything he has done for me. I also want to thank my colleagues (Weiping Zhang, Jun Sun, and Eman Allam) for their help in the lab and their support.

Last but not least, I would like to express my eternal gratitude to my parents and my children (Tala, Talal and Sultan) for their everlasting love and support.

ABSTRACT

Nouf Khider Al-Shibani

THE RESPONSES OF HUMAN NEUTROPHILS TO TOBACCO SMOKE COMPONENTS

Tobacco smoking is considered a major modifiable risk factor for periodontal disease. Tobacco contains about 6700 compounds and almost 4000 compounds of these have been identified in tobacco smoke. Nicotine is the addictive ingredient in tobacco and has been shown to affect multiple cellular processes. Cigarette smoke condensate (CSC) is the particulate matter of smoke. It is believed to be a powerful inducer of inflammatory responses.

Neutrophils are the first line of host defense and are critical cells in the maintenance of periodontal health through their role in the control of bacteria, but they can also contribute to the progression of periodontal disease by the production and release of reactive oxygen species (ROS). Virulence factors from periodontal pathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*), stimulate the respiratory burst of neutrophils. In this dissertation, three studies aimed at understanding the oxidative activity of neutrophils when stimulated with either nicotine, cigarette smoke condensate (CSC) or four other components of tobacco smoke (2-naphthylamine, hydroquinone, acrolein, and acetaldehyde) with or without *P. gingivalis* supernatant. The release of matrix metalloproteinase-9 (MMP-9) was also examined.

ROS production increased significantly when the neutrophils were stimulated with nicotine. *P. gingivalis* induced the maximum ROS production when compared to all the other components examined. The combination of nicotine and *P. gingivalis* did not have an additive effect on ROS production. Nicotine significantly increased the MMP-9 release from the neutrophils. On the contrary, CSC inhibited ROS production at all the concentrations examined. The combination of CSC and *P. gingivalis* resulted in the inhibition of ROS production. MMP-9 release was also increased from the CSC-treated neutrophils. The four other tobacco smoke components examined affected ROS production and MMP-9 release differently.

These projects demonstrated that CSC inhibited the ROS production from neutrophils, which can be attributed to several components in tobacco smoke that may include acrolein and hydroquinone. More research is needed to determine the mechanisms of inhibition and if other tobacco components are involved in ROS inhibition.

L. Jack Windsor, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Introduction

Periodontal disease is an inflammatory process of bacterial origin that affects the periodontal tissues and can result in the destruction of the supporting tissues of the teeth (Alpiste Illueca, Buitrago Vera et al. 2006). This destruction is the consequence of interactions between the oral microflora and host defense mechanisms (Alpiste Illueca, Buitrago Vera et al. 2006). These oral microflora or microorganisms are reported to cause tissue destruction in one of two ways: (i) directly, through the production of harmful substances that can cause tissue necrosis and cell death or (ii) indirectly, where these microorganisms will activate inflammatory cells that produce mediators that will act on the host cells and cause tissue destruction (e.g., cytokines) (Bascones-Martinez, Munoz-Corcuera et al. 2009). It has also been reported that some bacteria can interfere with the host defense mechanisms in two other ways: (i) by inhibiting the action of phagocytic cells or (ii) by deactivating specific antibodies (Williams 1990).

Numerous bacteria can degrade tissue directly and indirectly but Birkedal-Hansen (Birkedal-Hansen 1993) reported interestingly that the host connective tissue (collagen) is mainly degraded by host mechanisms in periodontal disease. This degradation is a result of the host attempting to protect itself from the microorganisms. The host responses involve the activation of both the innate and adaptive immunities (Kornman, Page et al. 1997).

In 1976, Page and Schroeder (Page and Schroeder 1976) classified the development of periodontal disease in animals into a series of stages. **Stage 1:** *The initial lesion* occurs within the first four days following the beginning of plaque accumulation. It is characterized by the formation of edema, increased gingival fluid, and the accumulation of neutrophils. It is also characterized by the loss of connective tissue. With the increased gingival fluid, activation of the complement system through the alternative pathway can occur. Neutrophils migrate to the gingival tissues and into the gingival sulcus to play protective roles. They form a barrier between the subgingival plaque and the underlying epithelium (Dixon, Bainbridge et al. 2004). The lesion occurs in no more than 5-10 percent of the connective tissue and is still not clinically evident. **Stage 2:** *The early lesion* arises between 4-7 days of plaque accumulation. It is characterized by an increased number of lymphocytes and macrophages. There is a subsequent increase in the flow of gingival crevicular fluid. Neutrophil numbers increase four-fold within the junctional epithelium. In this stage, 60-70 percent of the collagen within the infiltrated zone is degraded. **Stage 3:** *The established lesion* occurs 14-21 days after plaque accumulation. The established lesion can be readily identified by the presence of plasma cells and this lesion may remain stable for extended periods of time, thus this lesion might or might not develop into periodontal disease. **Stage 4:** *The advanced lesion* is characterized by bone loss and is called a periodontal lesion or disease.

The bone loss seen in periodontal disease results from the secretion of enzymes and the release of oxygen radicals (Palmer 2010). Furthermore, the imbalance between the osteoclasts and osteoblasts can favor osteoclastic bone resorption (Assuma, Oates et al. 1998). This happens from the increased osteoclastogenesis which is stimulated by the receptor activator of NFkB ligand (RANKL) that are expressed on the surfaces of the osteoblasts. Another factor that can increase osteoclastogenesis is the increased expression of cytokines such as interleukin-1 (Assuma, Oates et al. 1998). With the increased resorption found in periodontal disease, the tooth becomes mobile in its socket and is eventually lost.

Periodontal disease is a multi-factorial disease with many host risk factors. These include systemic diseases, age, sex, smoking, nutrition, stress, and environmental exposure. All of these have been reported to exacerbate the severity of the disease (Kinane 2001). Epidemiological studies have also linked periodontal disease to many chronic diseases, thus suggesting that oral diseases impact the individual's general health (Williams, Barnett et al. 2008). Periodontal disease has been reported to be a risk factor for diabetes mellitus (Saremi, Nelson et al. 2005), rheumatoid arthritis (Mercado, Marshall et al. 2003), kidney disease (Shultis, Weil et al. 2007), cardiovascular diseases (Bahekar, Singh et al. 2007) and respiratory infections (Garcia, Nunn et al. 2001).

Porphyromonas gingivalis

The role of specific gram-negative bacteria in the etiology and pathogenesis of periodontal disease has been well established (Holt and Bramanti 1991; Tokuda, Duncan et al. 1996). *Porphyromonas gingivalis* (*P. gingivalis*)*, Prevotella intermedia, and Aggregobacter actinomycemcomitans* have been confirmed as being more virulent than other related pathogens (Haffajee and Socransky 1994). *P. gingivalis* is an anaerobic, non-motile, nonsporulating coccobacillus (Haffajee and Socransky 1994). There is increasing evidence that *P. gingivalis* is the major etiological agent in the severe forms of periodontal disease (Lamont and Jenkinson 1998; Slots and Ting 1999). *P. gingivalis* contain many virulence factors that facilitate the colonization of the gingival sulcus and the initiation of periodontal disease. These virulence factors include lipopolysaccharides (LPS) (Pussinen, Paju et al. 2007), gingipains (Travis, Pike et al. 1997), polysaccharide capsule, fimbriae, immunoblogulin A and G proteases, and outer membrane proteins.

LPS is composed of three components: lipid A, antigen O, and an oligosaccharide that binds them together (Bascones-Martinez, Munoz-Corcuera et al. 2009). It is known that lipid A can trigger significant inflammatory responses. LPS can activate the innate system by stimulating and interacting with the Toll-Like Receptor-2 (TLR-2) and -4 (Darveau, Pham et al. 2004), and this is due to the multiple forms of lipid A found in the *P. gingivalis* LPS (Darveau, Pham et al. 2004). TLRs are cell surface proteins that recognize bacterial

products and play important roles in the induction of antimicrobial responses in different host cell types.

Gingipains play important roles in the development of periodontal disease (Imamura 2003). The gingipains are trypsin-like cysteine proteinases that have been reported to contribute to 85% of the overall proteolytic activity and 99% of the trypsin-like activity associated with *P. gingivalis* (Potempa, Sroka et al. 2003). They are cysteine proteinases that cleave the C-terminal peptide bonds of either arginine (Arg-X) or lysine (Lys-X) residues (Potempa, Sroka et al. 2003). Arg-gingipains (RgpA and RgpB) are encoded by two genes *rgpA* and *rgpB* respectively (Curtis, Thickett et al. 1999). Lys-gingipain (Kgp) is encoded the gene *kgp* (Curtis, Kuramitsu et al. 1999). Reports describing *P. gingivalis* mutants that were deficient in *rgpA* and/or *rgpB* and/or *kgp* genes demonstrated that RgpA, RgpB, and Kgp are major virulence factors of *P. gingivalis* (Nakayama, Kadowaki et al. 1995; Tokuda, Duncan et al. 1996). Gingipains have been shown to degrade a range of tissue components, as well as host matrix and plasma proteins (Cutler, Kalmar et al. 1995). They have also been shown to be involved in producing fimbriae (Tokuda, Duncan et al. 1996) and activating pro-thrombin to thrombin (Imamura, Tanase et al. 2001). Arg-gingipains have been also shown to activate latent matrix metalloproteinase-2 (MMP-2) in vivo, which may contribute to periodontal tissue destruction (Grayson, Douglas et al. 2003). In addition, Al-Shibani and Windsor demonstrated that Arg-gingipain could enhance the collagen degrading ability of human gingival fibroblasts through the activation of multiple MMPs (Al-Shibani and Windsor 2008).

P. gingivalis, as well as other periodontopathogenic bacteria, have proteolytic activity that can degrade some of the components of the complement system (e.g., C3 and C5). With this degradation, the bacteria prevent the opsonization process (Bascones-Martinez, Munoz-Corcuera et al. 2009).

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of proteinases whose primary purpose is believed to be the degradation of the extracellular matrix (Johnson, Dyer et al. 1998). The MMPs contribute to both normal and pathological tissue remodeling. The physiological roles for MMPs include cell migration and tissue remodeling during growth, wound healing, and angiogenesis. They play major roles in pathological conditions such as arthritis, periodontal disease and cancer.

Most of the MMPs are secreted by multiple cell types as inactive precursors (pro-MMPs) (Ryan and Golub 2000). They are divided into several subfamilies, which include the collagenases (MMP-1,-8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3,-10, and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) and others (MMP-12, -20, and -23) (Table 1.1). The collagenases have the ability to cleave Types I, II, and III fibrilar collagens into characteristic ¾ and ¼ fragments, but they can also digest other extracellular matrix molecules and soluble proteins (Visse and Nagase 2003). The gelatinases can degrade denatured collagens (gelatin). Gelatinase A (MMP-2) can also cleave Type I collagen, but at a slower

rate than the collagenases. The gelatinases digest a number of extracellular molecules including Types IV, V, and XI collagens. The stromelysins digest a number of extracellular molecules and participate in the activation of some of the pro-MMPs. Membrane-type MMPs are also able to degrade Types I, II, and III collagens, but at a slower rate than the collagenases and they play important roles in angiogenesis.

The MMPs have been highly correlated with periodontal disease and are believed to be the major players in the collagen breakdown that occurs during periodontal tissue destruction. The MMPs are expressed by inflammatory host cells (neutrophils, monocytes, macrophages, and lymphocytes) and by resident host cells (fibroblasts, epithelial cells, osteoblasts, and endothelial cells) (Birkedal-Hansen 1993). The MMPs are regulated at the level of expression and activation, as well as by the tissue inhibitors of metalloproteinases (TIMPs). The TIMPs bind to the active sites of the MMPs and inhibit them at a 1:1 ratio. The imbalance between the MMPs and TIMPs can lead to tissue destruction.

MMP-8 and -9 play central roles in the turnover and degradation of periodontal tissue. MMP-8 is mainly expressed by neutrophils (neutrophil collagenase), but it is also expressed by other cells (Bentwood and Henson 1980). MMP-8 is stored in the secondary granules of neutrophils and is released upon recruitment to inflamed lesions and activated. MMP-9 was first discovered in neutrophils but is also produced by other cells including macrophages and monocytes (Lepidi, Kenagy et al. 2001). It is stored in the tertiary granules of the neutrophils and is released upon neutrophil activation.

Evidence has documented that the MMPs are the most important enzymes involved in periodontal tissue destruction (Birkedal-Hansen 1993). It has been reported that the collagenases and gelatinases are not only found in crevicular gingival fluid (Teng, Sodek et al. 1992) and saliva (Makela, Salo et al. 1994), but also in biopsy specimens of inflamed periodontal tissues in higher amounts than in non-inflamed control tissues (Ejeil, Igondjo-Tchen et al. 2003). A study by Marcaccini et al. (Marcaccini, Meschiari et al. 2010) demonstrated that MMP-8 and -9 levels decreased significantly after non-surgical periodontal therapy, while they were reported to be at higher levels in chronic periodontal patients (Sorsa, Uitto et al. 1988; Sorsa, Ding et al. 1995; Kinane, Darby et al. 2003).

MMP-9 forms dimers consisting of covalently bonded monomers with a disulfide bond that allows these dimers to be found in tissues. This is a unique feature of MMP-9 (Olson, Bernardo et al. 2000). Both monomeric and dimeric forms of MMP-9 can cleave gelatin, as well as can be activated by MMP-3. The only difference between the monomeric and dimeric forms of pro-MMP-9 is that the dimeric catalytic efficiency of MMP-9 is 10-fold lower than that of the monomeric MMP-9. Also, it has been reported that the dimeric form is more stable than the monomer form (Olson, Bernardo et al. 2000) thus leading to the belief that they might behave differently in vivo such as the monomeric form being activated much faster than the dimeric form.

The role of MMP-8 in periodontal destruction has been well documented in the literature (Sorsa, Tjaderhane et al. 2004; Giannobile 2008). MMP-8 levels decreased significantly after scaling and root planing in periodontal patients, thus suggesting that this enzyme may be useful as a marker of current periodontal disease status and a predictor of future disease (Kinane, Darby et al. 2003).

P. gingivalis has been reported to affect the expression and activation of several MMPs expressed by periodontal fibroblasts (DeCarlo, Windsor et al. 1997). When HGFs were treated with *P. gingivalis*, MMP-15 mRNA increased 1.41 fold and MMP-2 mRNA increased slightly (1.25 fold), while MMP-14 mRNA decreased 0.67 fold. The mRNA expression of TIMP-1 and TIMP-2 increased 1.58 and 1.68, respectively (Zhou and Windsor 2006). Also, MMP-9 production was disrupted in gingival epithelial cells following contact with *P. gingivalis,* which may interfere with extracellular repair and organization (Fravalo, Menard et al. 1996; Grayson, Douglas et al. 2003). Purified gingipains from *P. gingivalis* upregulated MMP-8 and MMP-3 expression in rat epithelial cells (DeCarlo, Grenett et al. 1998) and also activated the latent forms of MMP-1, -3, and -9 (DeCarlo, Windsor et al. 1997).

Human Neutrophils

Neutrophils are professional phagocytes and are the most abundant leukocytes in the circulation (Meng, Xu et al. 2007). They also help promote inflammatory resolution and tissue healing (Schenkein 2006). They respond to infections by the “3 R’s”: Recruitment, Response, and Resolution (Nussbaum

and Shapira 2011). Neutrophils are believed to be a key protective cell type in the periodontal tissues (Meng, Xu et al. 2007). Neutrophils are crucial for the maintenance of periodontal health as evident from the many severe periodontal diseases associated with the dysfunction of neutrophils including chronic/cyclic neutropenia and syndromes such as Papillon-Lefevere syndrome, Chediak-Higashi syndrome, and leucocyte adhesion deficiency syndrome (Cainciola, Genco et al. 1977; Carrassi, Abati et al. 1989; Delcourt-Debruyne, Boutigny et al. 2000; Inaloz, Harman et al. 2001).

After the neutrophils leave the bone marrow, they remain in the circulation for about 12 hours. When they are recruited, they adhere to the microvascular walls and then are attracted to the site of infection (Nussbaum and Shapira 2011). The neurophils will then penetrate the endothelial layer and migrate through the connective tissue to reach the site of infection. When the neutrophils enter the tissue, they undergo apoptosis (programmed cell death) after 1-2 days and then are cleared by the macrophages, which are induced to produce anti-inflammatory cytokines such as transforming growth factor β (TGF β) (Scott and Krauss 2012). The apoptosis process prevents tissue damage and is considered an anti-inflammatory process in contrast to necrosis where reactive oxygen species (ROS) and enzymes from the neutrophils are released in the periodontium and cause collateral damage to the surrounding connective tissue.

The neutrophil targets the bacteria through several cell-surface receptors including Fcγ receptors for the Fc regions of antibodies, pattern recognition receptors and also receptors for C3b, which is an opsonizing molecule of the

complement system (Garcia-Garcia 2005). After the binding of the neutrophils to one of these receptors, the neutrophils engulf the microorganisms and internalize them into a phagosome. The lysosome will then fuse with the phagosome to create a phagolysosome (Tapper 1996). In the phagolysosome, the neutrophil will begin the killing mechanisms to destroy the microorganisms by two general types of killing mechanisms: oxidative and non-oxidative. The oxidative mechanism involves the production of ROS, which are primarily released to kill the bacteria. However, the extracellular release of ROS also results in collateral damage of the surrounding tissues. ROS include oxygen derived free radicals such as the superoxide radical, hydroxyl radical, and nitric oxide radical species, as well as non-radical derivatives of oxygen such as hydrogen peroxide and hypochlorous acid (Waddington, Moseley et al. 2000). It has been reported that ROS, especially the active hydroxyl radical, can degrade a number of structurally and metabolically functional macromolecules in an attempt to balance its unpaired electronic state and thus causes tissue damage (Waddington, Moseley et al. 2000).

Following the activation of the neutrophils, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex generates superoxide radicals. The superoxide radical then dismutates to hydrogen peroxide, which forms a number of ROS species including hypochlorous acid through myeloperoxidase, hydroxyl radicals or peroxynitrite anions (Chapple and Matthews 2007).

In regard to the non-oxidative mechanisms, they include many components within the granules of the neutrophils which exert antimicrobial effects. These are the defensins, bacterial/permeability increasing protein, and the neutral serine protease family, which includes elastase, proteinases 3, azurocidin, and cathepsin G. Elastase, which is released extracellularly during neutrophil activation, can degrade multiple proteins in the extracellular matix. This complex interaction between the pathogenic bacteria and the host immune response give rise to tissue damage (Canakci, Cicek et al. 2005).

Specific Aims

The overall goal of this study was to examine the ROS production and MMP-9 release from neutrophils when stimulated with different cigarette tobacco smoke components. The study was accomplished by the following specific aims.

Specific aim 1: To assess the ROS release when neutrophils are stimulated with either nicotine alone, *P. gingivalis* alone, and a combination of both.

The relationship between tobacco smoking and periodontal disease has been well documented (Bergstrom and Floderus-Myrhed 1983; Feldman, Bravacos et al. 1983; Ismail, Burt et al. 1983; Bergstrom, Eliasson et al. 1991). Currently, there is a large body of scientific evidence that smokers have an increased risk, incidence, and severity of periodontal disease as evident by increased gingival recession, tooth loss, and periodontal destruction. Smokers have deeper probing depths (Feldman, Bravacos et al. 1983), deeper pockets,

and more attachment loss (Haffajee and Socransky 2001), as well as more gingival recession (Haffajee and Socransky 2001). They also have more alveolar bone loss (Bergstrom, Eliasson et al. 2000), more teeth with furcation involvement (Mullally and Linden 1996; Craig, Boylan et al. 2001), and suffer more tooth loss than non-smokers (Daniell 1983; McGuire and Nunn 1999).

Tobacco contains about 6700 compounds and almost 4000 compounds of these have been identified in tobacco smoke (Baker, Ainsworth et al. 2000). These compounds include known carcinogens, toxic heavy metals, and many untested chemicals (Rogers 2009). The major additive ingredient in tobacco is nicotine. Many studies in the literature have focused on the adverse effects of nicotine on both cell-mediated and humoral immune responses (Palmer, Wilson et al. 2005), as well as on its effects on various cell types including neutrophils, epithelial cells, and fibroblasts. Nicotine has been reported to have two effects on the release of ROS. First, it inhibits the synthesis of superoxide. Second, it directly absorbs any superoxide that is produced (Pabst, Pabst et al. 1995). These two effects lead to reduced oxidative burst. This is in agreement with Sorensen et al. (Sorensen, Nielsen et al. 2004), who studied the effects of smoking on neutrophils and monocytes. Smoking reduced the neutrophil and monocyte oxidative burst by half, which may translate into impaired oxidative killing and bactericidal activity.

Specific aim 2: To examine the cytotoxicity, ROS production, and MMP-9 release from cigarette smoke condensate (CSC) treated human neutrophils.

CSC is the particulate matter of smoke. It is compromised of more than 4000 compounds and is believed to be a powerful inducer of inflammatory responses (Gao, Chen et al. 2005). It has been intensively investigated during the past twenty years to identify the mechanisms by which it causes a variety of cancers including lung, oral cavity, larynx, paranasal sinuses, urinary bladder, and pancreas (Cancer 2004). The mechanisms involve mutagenic activity and genotoxicity (DeMarini 1983) of tobacco smoke, as well as smoking-related DNA and protein adducts in human tissues (Phillips 2002).

Specific aim 3: To determine the cytotoxicity levels of 4 different tobacco smoke components (2-naphthylamine, hydroquinone, acrolein, and acetaldehyde) on human neutrophils and to investigate the effects of these chemicals on the production of ROS from neutrophils and their release of MMP-9.

2-Naphthylamine is known to be a human carcinogen. In 1969, the International Agency for Research on Cancer classified 2-naphthylamine as a Group 1 Carcinogen based on human and animal evidence of its carcinogenic potency (Smith, Livingston et al. 1997). There is sufficient evidence that chemical workers exposed to 2-naphthylamine are at an increased risk of bladder cancer (IARC 1987). It has been reported that cigarette smoking accounts for about two-thirds of the bladder cancer cases in men in industrialized countries (Brennan, Bogillot et al. 2000). Many carcinogens, such as 2-naphthylamine, can bind to DNA to form adducts which if not repaired can lead to mutations and ultimately cancer (Benhamou, Laplanche et al. 2003).

Hydroquinone is a reactive metabolite from benzene biotransformation (Snyder and Hong 2004). After inhalation of hydroquinone, it is oxidized in the liver by cytochrome P450 to form benzene epoxide (Snyder 2002). This easily penetrates the bone marrow compartment and can be locally metabolized. It accumulates in the bone marrow leading to toxicity (Henderson 1996). A study by Macedo et al. demonstrated that hydroquinone exposure alters neutrophil mobilization, which results in an exacerbated response after an injury (Macedo, Lourenco et al. 2006).

In a study by Poggi et al. (Poggi, Rota et al. 2002), acrolein and acetaldehyde produced similar changes in human gingival fibroblasts. These changes included a decrease in cell viability and adhesion, disruption of micromolecules, decrease in intermediate filaments and actin filaments, changes in cell shape and a decrease in cell size, the presence of vacuoles, and non-specific immunofluorescence patterns (Poggi, Rota et al. 2002). Poggi et al. (Poggi, Rota et al. 2002) used concentrations between 10^{-5} M and 10^{-4} M for acrolein and 10^{-3} M and 10^{-2} M for acetaldehyde. At the highest doses of acrolein (10^{-4} M) and acetaldehyde (10^{-2} M), severe disorganization of the microtubules was noted and the network morphology was no longer visible (Poggi, Rota et al. 2002). These findings were also supported by Cattaneo et al. (Cattaneo, Cetta et al. 2000), who showed that acrolein and acetaldehyde produced dose-dependent inhibition of the attachment and proliferation of HGFs.

Vrsalovic et al. (Vrsalovic, Vrsalovic et al. 2007) investigated the influence of acetaldehyde on neutrophil phagocytic functions. The ability of neutrophils to phagocytose was attenuated significantly in a dose-dependent fashion at concentrations of 0.0625, 0.125, and 0.5 mM. Acetaldehyde had no influence on the neutrophil phagocytic activity (Vrsalovic, Vrsalovic et al. 2007). Cytogenetic effects reported from acetaldehyde treatment included several types of DNA damage (Grafstrom, Dypbukt et al. 1994).

Dissertation Outline

This dissertation is divided into five chapters. The current chapter (Chapter One) is an introduction to periodontal disease and one of its major risk factors (i.e., tobacco usage), as well as MMPs, human neutrophils and the importance of the production of ROS. Chapter Two describes the materials and methods of the three projects which investigated the non-toxic concentrations of the different chemicals and their effects on the ROS production and MMP-9 release with and without *P. gingivalis* from neutrophils. Project one examines nicotine, project two examines CSC, and project three examining four different components of tobacco smoke (2-naohthylamine, hydroquinone, acrolein, and acetaldehyde). Chapter Three describes the results of the three projects. Chapter Four discusses the results. Chapter Five concludes this dissertation and summarizes the results, as well as the significance of the data from these studies. It also describes future directions for continuing the research.

Table 1.1. Classification of matrix metalloproteinases

SUBGROUPS	
Collagenases	MMP-1, MMP-8, MMP-13, and MMP-18
Gelatinases	MMP-2 and MMP-9
Stromelysins	MMP-3, MMP-10, and MMP-11
Membrane-type MMPs	MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25
Others	MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, and MMP-28

CHAPTER TWO

Materials and Methods

Neutrophil Separation

Buffy coats were purchased from the Central Indiana Regional Blood Center in Indianapolis, Indiana following separation from freshly collected whole blood from healthy adult donors (Institutional Review Board approval number NS0806-02). Once in the laboratory, the buffy coats were diluted 1:1 with Roswell Park Memorial Institute (RPMI) cell media (Sigma Aldrich, St. Louis, MO) to maximize the efficiency of separation. The neutrophils were separated by the Double Dextran Gradient Method (Boyum 1968) as follows:

1. Three milliliters of room temperature HISTOPAQUE-1119 (Sigma Aldrich, St. Louis, MO) was added to a 15 mL tube.
2. Three milliliters HISTOPAQUE-1077 (Sigma Aldrich, St. Louis, MO) was carefully layered on the HISTOPAQUE-1119.
3. Six milliliters of the buffy coat/RPMI-1640 mixture was carefully layered on the HISTOPAQUE-1077.
4. The samples were centrifuged at 1700 RPM for 35 minutes at room temperature.
5. The mononuclear layer was drawn off with a pipette.
6. This layer was washed with 10 mL of phosphate buffer saline (PBS) at room temperature and then centrifuged at 950 RPM for 10 minutes.

7. Step 6 was repeated.

8. The samples were washed with 10 mL RPMI-1640.

9. The neutrophils were resuspended in RPMI-1640 to a concentration of 1.0 x 10^6 cells/mL. The neutrophils were counted under a hematocytometer using the Trypan Blue Exclusion Test (Wahaidi 2010). The viability in all of the samples was > 99%.

Bacterial Supernatant

Porphyromonas gingivalis ATCC 33277 was maintained on enriched trypticase soy agar plates containing 3% sheep blood (Bodet, Chandad et al. 2006). Cultures were grown in Todd Hewitt Broth with 0.001% hemin and 0.0001% Vitamin K or menadione. Cultures were incubated in an anaerobic chamber with an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. When the bacterial growth yielded an OD_{600} of 1.0 (1.15 X 10^9 cells/mL), the cultures were stopped. The bacterial supernatants were harvested by centrifugation at 13,000 g for 20 min at 4°C. The collected supernatants were filtered twice through 0.2 µm membranes and then stored at 20°C.

Chemicals

The nicotine, 2-naphthylamine, hydroquinone, acrolein, and acetaldehyde were obtained commercially from Sigma Aldrich (St. Louis, MO), and stored away from light. The CSC was obtained from Murty Pharmaceutical Incorporated (Lexington, KY).

Measurement of Cytotoxicity Using Lactate Dehydrogenase Assays (LDH)

Cell membrane integrity was monitored using the permeability assay based on the determination of the release of lactate dehydrogenase into the media. The conversion of tetrazolium salt into a red formazan product was measured by the Cytotoxicity Detecting Kit (Roche Applied Science, Mannheim, Germany). Neutrophils (1×10^6 cell/mL) were treated with different concentrations of the chemicals for 4 hours. The concentrations of the chemicals/agents used are shown in Tables 2.1, 2.2, and 2.3. The low control consisted of RPMI media from untreated control neutrophils after 4 hours and gave the minimal release of lactate dehydrogenase, while the high control consisted of 1.9 mL of RPMI media with 100 µL of lysis solution added to the control cells as provided by the manufacturer to generate the total cell death of the neutrophils. The experiments were repeated four times and the mean values were calculated. Calculation of the cytotoxicity was determined using the following equation:

Cytotoxicity (%) = (experiment value - low control) / (high control – low control) × 100%.

Chemiluminescence (CL) Assays

The CL assays were performed according to established protocols for neutrophils (Permpanich, Kowolik et al. 2006) in a luminometer. For each run of the experiment, there were 16 reaction cuvettes. To each reaction cuvette, the following was added: 500 µl of neutrophil suspension (1×10^6 cell/mL) in RPMI,

300 µl of PBS and 100 µl luminol (5 amino-2,3-dihydro-1,4-phthalazindione) that was used as a CL probe for signal augmentation and dispensed at baseline. Luminol is an activity amplifier and has been frequently used in the free radical research field (Dahlgren, Karlsson et al. 2007). After 30 minutes, different concentrations of the chemicals with and with *P. gingivalis* were added (Tables 2.4, 2.5, and 2.6). The study groups also included a negative control blank that contained no neutrophils and a 10^{-11} M N-formyl-methionyl-leucyl-phenylalanine (fMLP) treated neutrophil sample that served as a positive control. The reactions were followed for 90 minutes and this represented the neutrophil activation phase. Neutrophil activation was recorded in millivolts, the integrals were calculated, and data analysis was performed on the mean values of triplicate experiments.

Western Blot Analysis

Western blots were performed to examine the release of MMP-9 in the conditioned media. After the incubation of the different chemicals with the neutrophils for 2 hours at the concentrations shown in Tables 2.4, 2.5, and 2.6, and after protein concentrations were equalized, the conditioned media from the treated cells and untreated cells were resolved in 10% SDS-PAGE gels at 200 V. The proteins on the gels were transferred to polyvinylidene fluoride (PVDF) membranes at 0.3 A for 80 minutes using blotting buffer (25 mM Tris-HCL, pH 8.3, 192 mM glycine, and 10% methanol). The membranes were then incubated in 5% milk in PBS solution with 0.1% Tween-20 (pH 7.4) for 1 hour to block non-

specific binding. The membranes were incubated with primary antibody against MMP-9 (pAb-4542-92K-GL) (Pickett, Harber et al. 1999) at 4°C overnight. The membranes were washed three times with phosphate-buffered saline with Tween-20 and incubated with anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) for 1 hour at room temperature. The membranes were then developed with the Amersham ECL^{TM} western blotting detection kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer. Finally, the National Institution of Health (NIH) Image software 1.46 was used to scan the blot exposed x-ray films and for measuring the bands.

Gelatin Zymography

After the incubation of the different chemicals with the neutrophils for 2 hours at the concentrations shown in Tables 2.4, 2.5, and 2.6, the conditioned media from the chemically treated cells and untreated cells (no chemicals), as well as the positive control (neutrophils incubated with fMLP), were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 1 mg/mL of gelatin at 200 V. The gels were then stepwise washed with solution 1 (2.5% (v/v) Triton-X 100 and 2 mM NaN_3), solution 2 (2.5% (v/v) Triton-X 100, 50 mM Tris, pH 7.4, and 3 mM NaN_3), solution 3 (2.5% (v/v) Triton-X 100, 50 mM Tris, pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 3 mM NaN_3), and solution 4 (50 mM Tris, pH 7.4, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 3 mM NaN_3) for 20 minutes each. The gels were then incubated in solution 4 at 37°C overnight. After staining with coomassie blue, the proteinases capable of

digesting the gelatin were visualized as lytic bands against the blue background of the gel. The molecular weights of the proteinases on the zymograms were estimated by measuring the relative migrations of the molecular weight standards.

Statistical Analysis

For the CL assays, the controls were averaged within each experimental run and the results for the other groups were divided by the control mean. Comparisons between the groups were performed using analysis of variance (ANOVA) with a term for group and a random effect for experimental run. Comparisons are presented after adjustment for multiple comparisons using Tukey's method to control the overall significance level. To satisfy the ANOVA assumptions, the analysis were performed on the log-transformed data.

For the cytotoxicity and MMP-9 release, the data was presented a mean and standard deviation. ANOVA was performed with Tukey’s test in the Statistical Package for Social Science (SPSS) 11.5 (SPSS Inc., Chicago, IL). The level of significance was set at $p < 0.05$.

Table 2.1. Project I. Nicotine concentrations

800 µg/ml nicotine
400 µg/ml nicotine
200 µg/ml nicotine
100 µg/ml nicotine
50 µg/ml nicotine
25 µg/ml nicotine
0 µg/ml nicotine

Table 2.2. Project II. CSC concentrations

800 µg/ml CSC
400 µg/ml CSC
200 µg/ml CSC
100 µg/ml CSC
50 µg/ml CSC
25 µg/ml CSC
0 µg/ml CSC

Table 2.3. Project III: 2-Naphthylamine, hydroquinone, acrolein, and acetaldehyde concentrations

2-Naphylamine	**Hydroquinone**	**Acrolein**	**Acetaldehyde**
40 mM	40 mM	100 mM	100 mM
20 mM	20 mM	50 mM	50 mM
10 mM	10 mM	25 mM	25 mM
5 mM	5 mM	12.5 mM	12.5 mM
2.5 mM	2.5 mM	6.25 mM	6.25 mM
1.25 mM	1.25 mM	3.125 mM	3.125 mM
0.6 mM	0.6 mM	1.56 mM	1.56 mM
0.3 mM	0.3 mM	0.78 mM	0.78 mM

Table 2.4 Project I: Chemiluminescence / Nicotine

Blank (RPMI media)
Negative control (neutrophils alone)
Primed neutrophils + 80 µg/ml nicotine
Primed neutrophils + 80 µg/ml nicotine + 10% *P. gingivalis*
Primed neutrophils + 10% *P. gingivalis*
Positive control

Table 2.5. Project II: Chemiluminescence / CSC

Blank (RPMI media)
Negative control (neutrophils alone)
Primed neutrophils + 10 µg/ml CSC
Primed neutrophils + 25 µg/ml CSC
Primed neutrophils + 50µg/ml CSC
Primed neutrophils + 50 µg/ml CSC + 10% *P. gingivalis*
Primed neutrophils + 10% *P. gingivalis*
Positive control

Table 2.6. Project III: Chemiluminescence / 2-Naphthylamine, hydroquinone, acrolein, and acetaldehyde

2-Naphthylamine	**Hydroquinone**	**Acrolein**	**Acetaldehyde**
Blank (RPMI)	Blank (RPMI)	Blank (RPMI)	Blank (RPMI)
Negative control	Negative control	Negative control	Negative control
0.0004 mM	0.03 mM	0.008 mM	0.046 mM
0.004 mM	0.3 mM	0.08 mM	0.46 mM
0.008 mM	0.6 mM	0.16 mM	0.92 mM
0.008 mM + 10% *P. gingivalis*	0.6 mM + 10% *P. gingivalis*	0.16 mM + 10% *P. gingivalis*	0.92 mM + 10% *P. gingivalis*
P. gingivalis alone	*P. gingivalis* alone	*P. gingivalis* alone	*P. gingivalis* alone

CHAPTER THREE

Results

Project One: Responses of Human Neutrophils to Nicotine with/without *P. gingivalis*

Cytotoxicity of Nicotine on Neutrophils by LDH

The cytotoxicity values were calculated after incubating the neutrophils for 4 hrs with different concentrations of nicotine and then compared with the controls. The cytotoxicity was statistically not significant from the low control for 25 µg/mL (0.9 ± 0.5) with a p-value = 0.1000, 50 µg/mL (1.5 ± 0.665) with a p-value = 0.194, and 100 µg/mL of nicotine (2.385 ± 0.74) with a p-value = 0.098, but was statistically significant for 200 (4.65 ± 0.52), 400 (7.341 ± 0.665), and 800 µg/mL (14.33 ± 0.77) with p-values of 0.037, 0.03, and 0.012, respectively (Figure 3.1.1 and Table 3.1.1).

Chemiluminescence Assays

The positive control, *P. gingivalis*, and *P. gingivalis* plus nicotine groups had significantly higher mean, active, and peak CL than the nicotine group, all with p-value < 0.0001 (Figure 3.1.2, Table 3.1.2, and Table 3.1.3). The active CL represents the total energy output under the curve, while the peak CL measures the intensity of the reaction. The active CL of *P. gingivalis* (37806 ± 3915) and *P. gingivalis* plus nicotine (34298 ± 3726) groups were not significantly different

from each other (p-value = 0.98). Similarly, the peak CL of *P. gingivalis* (9.80 ± 1.03) and *P. gingivalis* plus nicotine (8.60 ± 0.83) groups were also not significantly different from each other (p-value = 0.99). The positive control group had significantly higher peak CL (27.31 ± 5.83) than *P. gingivalis* (9.80 ± 1.03) with a p-value = 0.0224, but did not have significantly different active CL (32962 ± 4907) from *P. gingivalis* (37806 ± 3915) with a p-value = 0.10. The positive control group had significantly higher peak CL (27.31 ± 5.83) than *P. gingivalis* plus nicotine (8.60 ± 0.83) with a p-value = 0.0457 and a significantly lower active CL (32962 ± 4907) than *P. gingivalis* plus nicotine (34298 ± 3726) with a p-value = 0.0490.

MMP-9 Western Blot

MMP-9 release was statistically increased in all groups from the control with p-values of 0.032, 0.001, or 0.001 for nicotine alone, nicotine plus *P. gingivalis*, and *P. gingivalis* alone, respectively. (Figure 3.1.3 panel A and Table 3.1.4).

Gelatin Zymography

MMP-9 levels in the zymograms (92 kDa bands) was increased and statistically significant in all the groups compared to the control with p-values of 0.03, 0.03, or 0.01 for nicotine alone, nicotine plus *P. gingivalis*, and *P. gingivalis* alone, respectively. The *P. gingivalis* alone group was also significantly higher than the control with a p-value of 0.001 (Figure 3.1.3 panel B and Table 3.1.4).

Figure 3.1.1. Project I: Cytotoxicity (%) of nicotine on neutrophils. The lysis (high control) was the maximal cytotoxicity (total cell death). Results were presented as mean with standard deviation (SD). Error bars represent SD. *denotes significant difference compared with the control (p-value < 0.05).

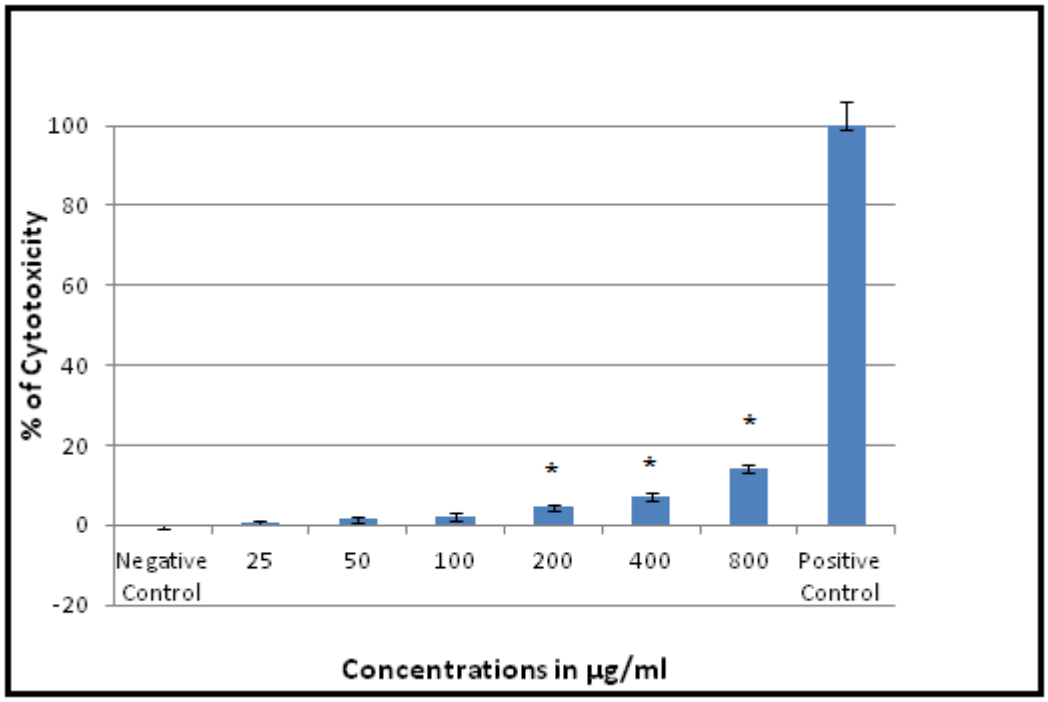

Table 3.1.1. Project I: Cytotoxocity of nicotine on neutrophils

Nicotine Concentration	Value ± SD	p-value
25 µg/ml	0.9 ± 0.5	0.1
50 µg/ml	1.5 ± 0.665	0.194
100 µg/ml	2.385 ± 0.74	0.098
200 µg/ml	4.65 ± 0.52	0.037*
400 µg/ml	7.341 ± 0.66	0.03*
800 µg/ml	14.33 ± 0.77	0.012*

*denotes significant difference compared with the control (p-value < 0.05)

Figure 3.1.2. Project I: Active, mean, and peak chemiluminescence after stimulation the neutrophils with nicotine with/without *P. gingivalis.* *denotes statistical significance p-value < 0.05 when compared to nicotine.

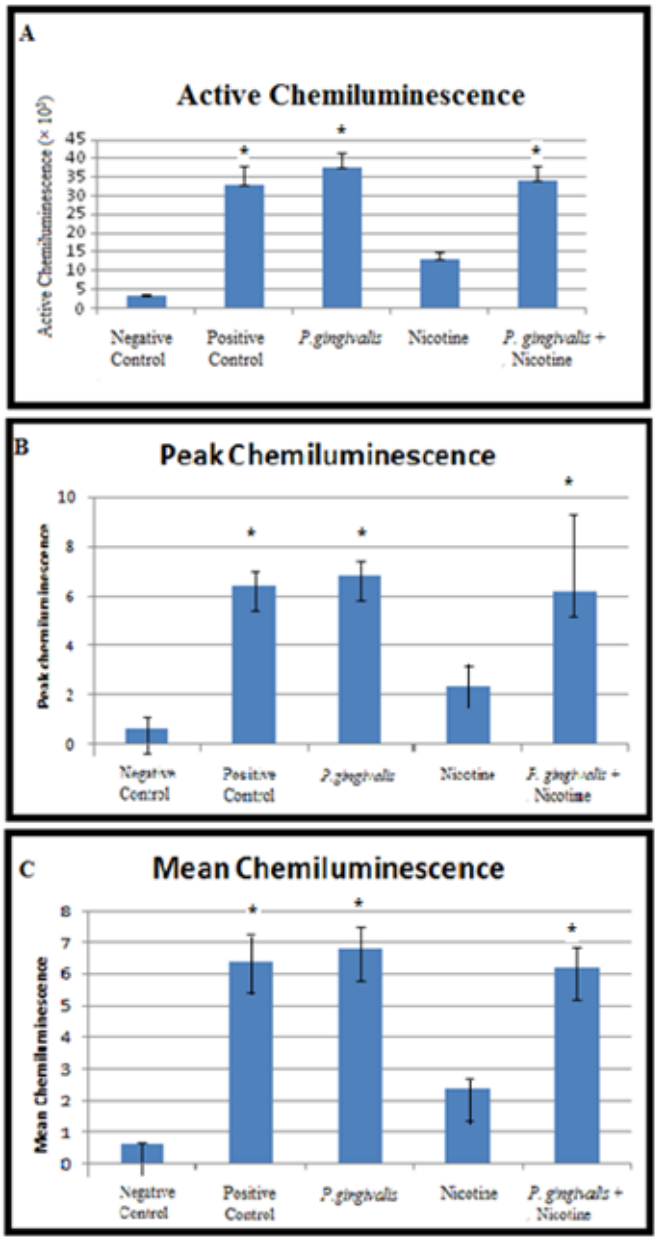

Table 3.1.2. Project I: Active, mean, and peak chemiluminescence after stimulation the neutrophils with nicotine with/without *P. gingivalis*. SE: Standard Error. Min: Minumum, Max: Maximum.

Outcome	Group	Mean	SE	Min	Max
Active	Negative Control	3486	196	2310	5486
	Positive Control	32962	4907	3111	68046
	P. gingivalis	37806	3915	3064	75011
	Nicotine	13041	1893	3644	35040
	P. gingivalis + Nicotine	34298	3726	11779	84847
Mean	Negative Control	0.64	0.04	0.42	0.99
	Positive Control	6.42	0.87	0.57	12.34
	P. gingivalis	6.8	0.7	0.56	13.41
	Nicotine	2.36	0.34	0.67	6.28
	P. gingivalis + Nicotine	6.18	0.67	2.12	15.39
Peak	Negative Control	0.89	0.09	0.48	1.69
	Positive Control	27.31	5.83	0.61	93.23
	P. gingivalis	9.8	1.03	0.6	19.16
	Nicotine	2.99	0.45	0.77	8.27
	P. gingivalis + Nicotine	8.6	0.83	3.1	18.5

Table 3.1.3. Project I: Active and peak chemiluminescence. A: Denotes statistically significant value when compared to nicotine. B: Denotes statistically significant value when compared to the negative control.

	Active		**Peak**	
Group	Mean ± SE	p-value	Mean ± SE	p-value
Negative control	3486 ± 196		0.89 ±0.09	
Positive control	32962 ± 4907	< 0.0001 [A] < 0.0001 [B]	27.31 ± 5.83	< 0.0001 [A] < 0.0001 [B]
P. gingivalis	37806 ± 3915	< 0.0001 [A] < 0.0001 [B]	9.8 ± 1.03	< 0.0001 [A] < 0.0001 [B]
nicotine	13041 ± 1893	< 0.05 [B]	2.99 ± 0.45	< 0.05 [B]
***P. gingivalis* + nicotine**	34298 ± 3726	< 0.0001 [A] < 0.0001 [B]	8.6 ± 0.83	< 0.0001 [A] < 0.0001 [B]

Figure 3.1.3. Project I: A: MMP-9 (92 kDa) western blot. B: MMP-9 gelatin zymography

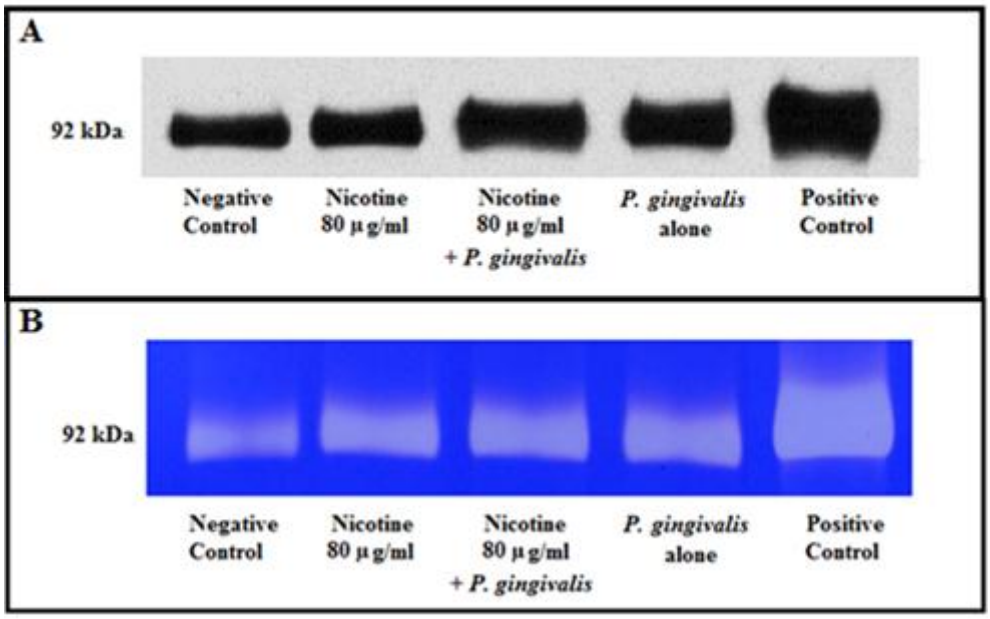

Table 3.1.4. Project I: MMP-9 release by western blot after stimulation of neutrophils with nicotine

	Control	**80 µg/ml nicotine**	**80 µg/ml nicotine + *P. gingivalis***	***P. gingivalis***	**Positive control**
Western Blot	76 ± 9.5	111.3 ± 13.4	135.7 ± 19.4	122.5 ± 20.4	165.2 ± 13.6
Gelatin Zymography	88.25 ± 6.5	83.3 ± 9.5	101.2 ± 3.6	124.9 ± 11.9	188.9 ± 7.1

*denotes significant difference compared with the control (p-value < 0.05)

Project Two: The Responses of Human Neutrophils to CSC with/without *P. gingivalis*

Cytotoxicity of CSC on Neutrophils by LDH

The cytotoxicity values were calculated after incubating the neutrophils for 4 hrs with the different concentrations of CSC and then compared with the controls. The cytotoxicity was not statistically significant for 25 µg/mL (1.96 ± 0.21) with a p-value = 0.103 and 50 µg/mL (4.08 ± 0.88) with a p-value = 0.071, while CSC was cytotoxic at 100 µg/mL (6.3 ± 0.17), 200 µg/mL (8.55 ± 1.67), 400 µg/mL (15.884 ± 5.4), and 800 µg/mL (55.012 ± 9.5) with a p-value = 0.04, p-value = 0.0011, p-value = 0.0011, and p-value = 0.0001 respectively (Figure 3.2.1 and Table 3.2.1).

Chemiluminescence Assays

The amount of ROS produced after the stimulation with 10 µg/mL, 25 µg/mL, or 50 µg/mL CSC was not statistically significantly different from the control with p-values of 0.25, 0.8, and 0.33, respectively. Increasing the concentration of CSC from 10 µg/mL to 50 µg/mL resulted in a non significant dose dependent decrease in the ROS production (p-value = 0.65) compared to the control. *P. gingivalis* plus CSC decreased the production of ROS significantly (p-value = 0.0001), while *P. gingivalis* alone increased ROS production (p-value = 0.0001) (Figure 3.2.2. and Table 3.2.2).

MMP-9 Western Blot

MMP-9 release was increased and statistically significant in all the groups compared to the control with p-values of 0.0001, 0.001, or 0.005 for the concentrations of 10 µg/mL, 25 µg/mL, or 50 µg/mL CSC, respectively. *P. gingivalis* plus CSC caused a significant increase in the release of MMP-9 (p-value = 0.0001) as did *P. gingivalis* alone (p-value = 0.0001) (Figure 3.2.3 panel A and Table 3.2.3).

Gelatin Zymography

MMP-9 in the zymograms (92 kDal bands) showed no significant increase in the 10 µg/mL CSC compared to the control (p-value = 0.15), but concentrations of 25 or 50 µg/mL showed increased release of MMP-9 with p-values of 0.005 and 0.003, respectively. CSC plus *P. gingivalis* and *P. gingivalis* alone groups were also significantly higher than the control with a p-value of 0.0001 and 0.006, respectively (Figure 3.2.3 panel B and Table 3.2.3).

Figure 3.2.1. Project II: Cytotoxicity (%) of CSC on neutrophils. The lysis (high control) was total cell death. Results were presented as mean and standard deviation (SD) Error bars represent SD. *denotes significant difference compared with the control (p-value < 0.05).

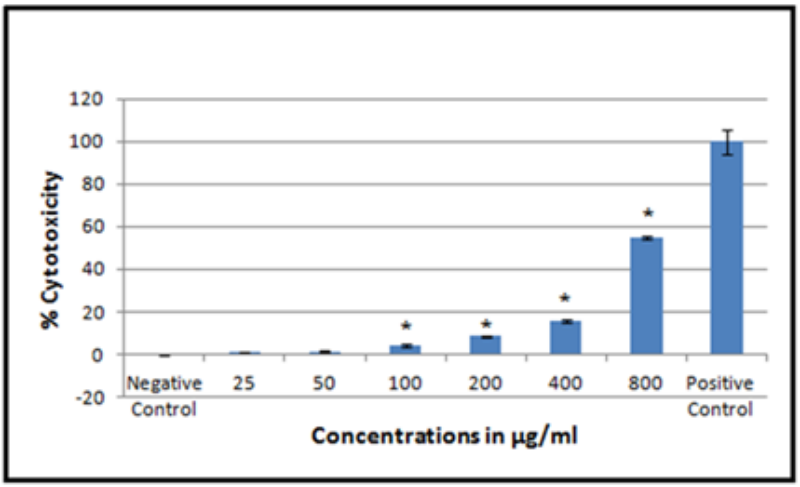

Table 3.2.1. Project II: Cytotoxicity of CSC on neutrophils with p-values.

CSC Concentration	**Value ± SD**	**p-value**
25 µg/ml	1.96 ± 0.21	0.103
50 µg/ml	4.08 ± 0.88	0.071
100 µg/ml	6.3 ± 0.17	0.04*
200 µg/ml	8.55 ± 1.67	0.0011*
400 µg/ml	15.884 ± 5.4	0.0011*
800 µg/ml	55.012 ± 9.5	0.0001*

*denotes significant difference compared with the control (p-value < 0.05).

Figure 3.2.2. Project II: Active, mean, and peak chemiluminescence after stimulation the neutrophils with CSC with/without *P. gingivalis.*

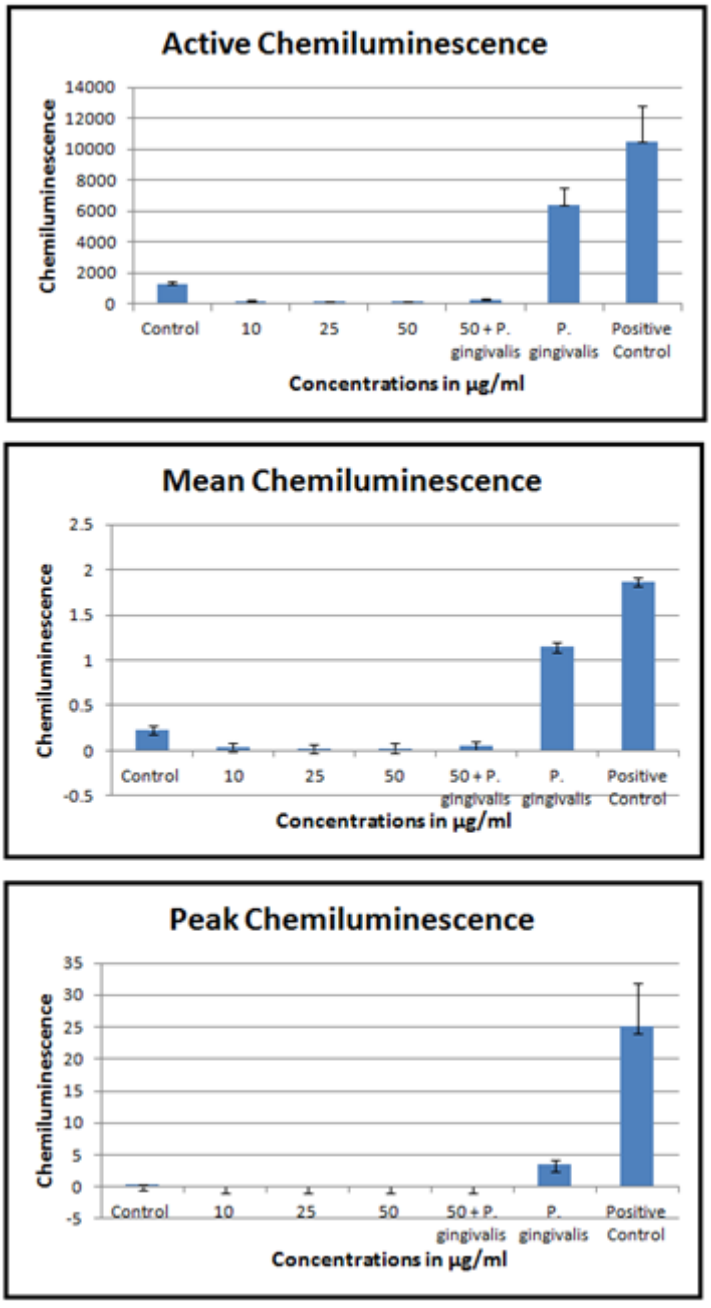

Table 3.2.2. Project II: Active chemiluminescence after the stimulation with different concentrations of CSC. SE. Standard Error.

	Active	**SE**	**p-value**
Control	1250	242	
10 µg/ml CSC	188	55	0.67
25 µg/ml CSC	149	41	0.92
50 µg/ml CSC	120	30	0.95
50 µg/ml CSC + *P. gingivalis*	303	20	0.0001*
P. gingivalis	6412	1062	0.0001*

*denotes significant difference compared with the control (p-value < 0.05)

Figure 3.2.3. Project II: A: MMP-9 (92 kDa) western blot. B: MMP-9 gelatin zymography.

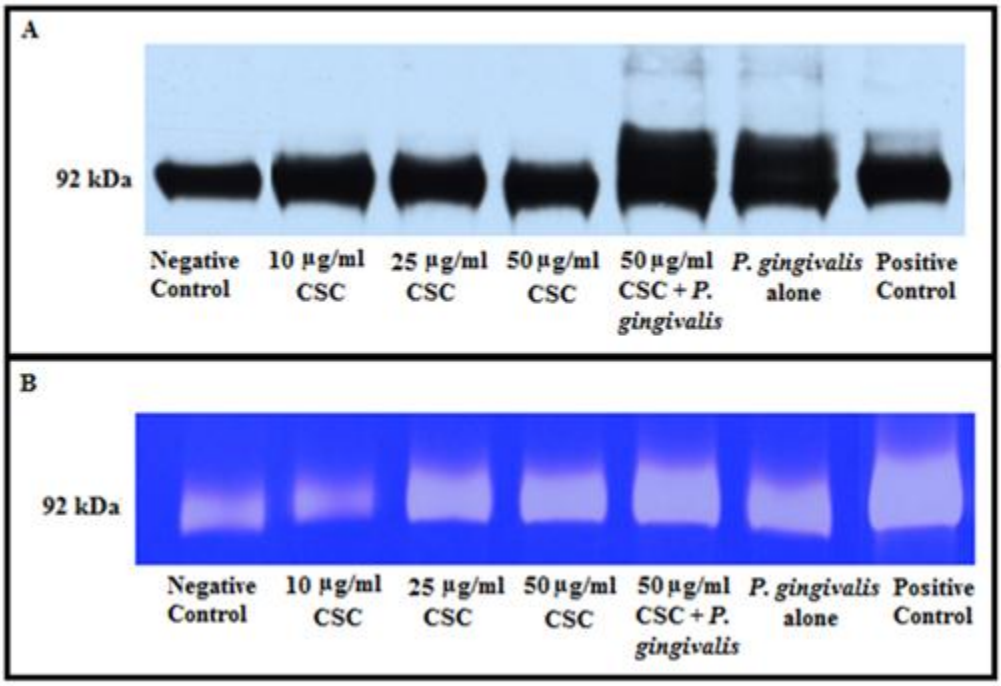

Table 3.2.3. Project II: Release of MMP-9 from neutrophils into the media at the different CSC concentrations compared to the control by western blots and gelatin zymography.

	Control	**10 µg/ml CSC**	**25 µg/ml CSC**	**50 µg/ml CSC**	**50 µg/ml CSC + *P. gingivalis***	***P. gingivalis***
Western Blot	89.22 ± 1.3*	108.2 ± 2.45*	101.5 ± 2.1*	98.9 ± 2.14*	149 ± 3.2*	142.2 ± 3.27*
Gelatin Zymography	136.6 ± 1.39	132.37 ± 1.01*	144.1 ± 1.9*	144.4 ± 1.9*	150.5 ± 2.2*	143.8 ± 1.8*

*denotes significant difference compared with the control (p-value < 0.05).

.

Project Three: The Responses of Human Neutrophils to Four Different Smoke Components with/without *P. gingivalis*

Cytotoxicity of 2-Naphthylamine, Hydroquinone, Acrolein, and Acetaldehyde on Neutrophils by LDH

2-Naphthylamine: The cytotoxicity values were not significant at 0.3 mM (p-value = 0.87), 0.6 mM (p-value = 0.8), 1.25 mM (p-value = 0.42), or 2.5 mM (p-value = 0.1), but were statistically significant at 5 mM (p-value = 0.04). The high dimethyl sulfoxide (HDMSO) group contained 8% DMSO and represented the same concentration of DMSO found in the 40 mM group, while the low DMSO (LDMSO) group contained 0.06% DMSO and represented the same concentration of DMSO found in the 0.3 mM group. The median DMSO (MDMSO) represented the concentration of DMSO found in the 1.25 mM group (Figure 3.3.1 and Table 3.3.1).

Hydroquinone: The cytotoxicity values were not significant at 0.3 mM (p-value = 0.89), 0.6 mM (p-value = 0.414) or 1.25 mM (p-value = 0.109), but were statistically significant at 2.5 mM with a p-value < 0.001 (Figure 3.3.1 and Table 3.3.1).

Acrolein and Acetaldehyde: Both acrolein and acetaldehyde had similar results. They were not toxic to the neutrophils at any of the concentrations examined (Figure 3.3.1 and Table 3.3.1).

Chemiluminescence

2-Naphthylamine: The amount of ROS produced after the stimulation with 2-naphthylamine from one cigarette was not statistically significant from the control (p-value = 0.35). The ROS production from neutrophils treated with 2-naphthylamine concentrations in 10 cigarettes and 20 cigarettes was statistically significantly higher than the control with p-values < 0.001 for both. The 20 cigarettes treatment resulted in more ROS production than any of the other chemicals at any concentration (49036 ± 21590). *P. gingivalis* alone and *P. gingivalis* plus 2-naphthylamine were statistically significant from the control with p-values < 0.001 for both (Figure 3.3.2. and Table 3.3.2).

Hydroquinone: The amount of ROS produced by neutrophils after the stimulation with the different concentrations of hydroquinone was statistically significantly lower than the control with p-values < 0.001 for all the concentrations. Increasing the concentration of hydroquinone to that found in 1 cigarette to 20 cigarettes decreased the ROS production compared to the control. *P. gingivalis* plus hydroquinone also decreased the production of ROS significantly (p-value < 0.001), while *P. gingivalis* alone increased ROS production (p-value = 0.001) (Figure 3.3.2. and Table 3.3.2).

Acrolein: Similar to hydroquinone, the amount of ROS produced after the stimulation of neutrophils with all the concentrations of acrolein (10, 25, and 50 µg/mL) were statistically significant from the control with p-values of 0.0206, 0.00001, and 0.00001, respectively. *P. gingivalis* plus hydroquinone also

decreased the production of ROS significantly while *P. gingivalis* alone increased ROS production (all with p-values of 0.001) (Figure 3.3.2. and Table 3.3.2).

Acetaldehyde: The stimulation of neutrophils with acetaldehyde resulted in statistical significant increases in the ROS production at the concentrations found in 1 cigarette, 10 cigarettes, and 20 cigarettes with p-values of 0.0018, 0.0195, and 0.0002, respectively. Acetaldehyde plus *P. gingivalis* and *P. gingivalis* alone also increased the ROS production with p-values of 0.0002 and 0.0001, respectively (Figure 3.3.2. and Table 3.3.2).

MMP-9 Western Blotting

2-Naphthylamine: MMP-9 release was increased and statistically significant in all the groups from the control with p-values of 0.002, 0.0001, and 0.0001 for the concentrations found in 1 cigarette, 10 cigarettes, and 20 cigarettes, respectively. *P. gingivalis* plus 2-naphthylamine and *P. gingivalis* alone were significantly different from the control with p-values of 0.0001 for both (Figure 3.3.3 panel A and Table 3.3.3).

Hydroquinone and Acrolein: MMP-9 release was significantly decreased when hydroquinone or acrolein were added with *P. gingivalis* with p-values of 0.006 and 0.0001, respectively (Table 3). There was also a non significant decrease in MMP-9 release when the concentrations of these tobacco components were increased from 1 cigarette to 20 cigarettes with all p-values > 0.05 (Figure 3.3.3 panel A and Table 3.3.3).

Acetaldehyde: No detectable changes were detected in MMP-9 release when acetaldehyde was added alone at any concentration. When combined with *P. gingivalis*, MMP-9 release was significantly increased compared to the control (p-value = 0.0001) (Figure 3.3.3 panel A and Table 3.3.3). *P. gingivalis* alone was increased significantly compared to the compared with a p-value = 0.0001.

Gelatin Zymography

2-Naphthylamine: MMP-9 release was increased from the control but was only statistically significant in the groups for the concentrations found in 10 cigarettes and 20 cigarettes with p-values of < 0.001 for both. *P. gingivalis* plus 2-naphthylamine and *P. gingivalis* alone were significantly different from the control with p-values < 0.0001 for both (Figure 3.3.3 panel B).

Hydroquinone and Acrolein: MMP-9 release was significantly decreased when hydroquinone or acrolein were added with *P. gingivalis* with p-values of 0 0.0001 for both (Figure 3.3.3 panel B). There was also a significant decrease in MMP-9 release compared to the control when the concentrations of hydroquinone were increased from 10 cigarettes to 20 cigarettes with p-values < 0.0001 for both. In regard to acrolein, there was a statistically significant decrease when the cells were treated with concentrations of 1 cigarette,10 cigarettes, or 20 cigarettes compared to the control with p-values of 0.005, 0.001, and 0.001, respectively (Figure 3.3.3 panel B).

Acetaldehyde: MMP-9 release was statistically significantly increased when acetaldehyde was added alone at all the concentrations tested with p-

values < 0.001 for all. *P. gingivalis* alone or when combined with acetaldehyde showed significant increase compared to the control (all with p-values < 0.0001) (Figure 3.3.3 panel B).

Figure 3.3.1. Project III: Cytotoxicity (%) of A: 2-Naphthylamine, B: Hydroquinone, C: Acrolein, and D: Acetaldehyde. The lysis (high control) was the maximal cytotoxicity to the neutrophils (total cell death). Results were presented as mean and standard deviation (SD). Error bars represent SD. *denotes significant difference compared with the control (p-value < 0.05).

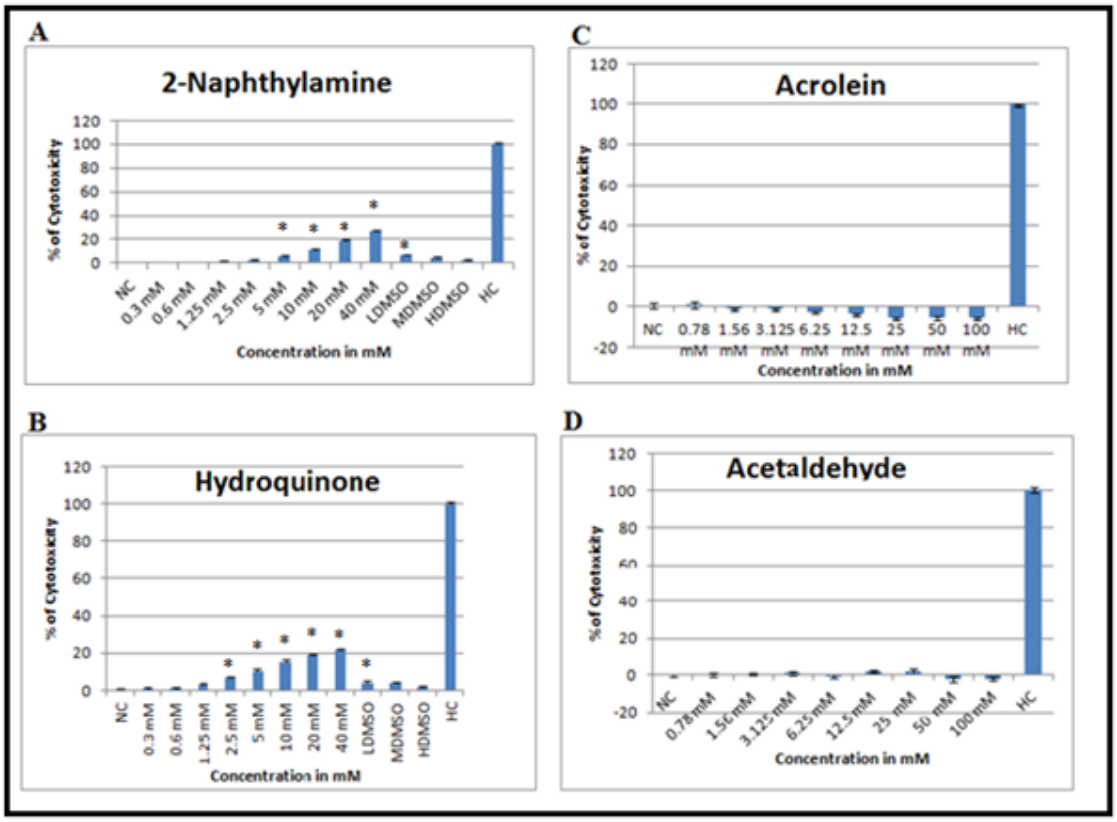

Table 3.3.1. Project III: Cytotoxicity results with p-values

Chemical	Concentration	Value ± SD	p-value
2-Naphthylamine	0.3 mM	0.39 ± 4.3	0.87
	0.6 mM	0.24 ± 0.36	0.8
	1.25 mM	0.64 ± 0.52	0.42
	2.5 mM	1.7 ± 0.32	0.1
	5 mM	5.11 ± 0.75	0.04
	10 mM	11.14 ± 0.79	< 0.001
	20 mM	18.5 ± 0.87	<0.001
	40 mM	26.5 ± 1.27	<0.001
Hydroquinone	0.3 mM	0.54 ± 2.1	0.89
	0.6 mM	0.49 ± 0.25	0.414
	1.25 mM	2.98 ±0.5	0.109
	2.5 mM	6.4 ± 0.43	<0.001
	5 mM	10.2 ± 1.33	<0.001
	10 mM	15.2 ± 0.6	<0.001
	20 mM	18.6 ± 1.9	<0.001
	40 mM	21.3 ± 0.8	<0.001
Acrolein	0.78 mM	0.22 ± 1.8	1
	1.56 mM	-1.15 ± 0.25	1.00
	3.125 mM	-1.2 ± 0.52	1.00
	6.25 mM	-2.6 ± 0.41	0.997
	12.5 mM	-3.77 ± 0.05	0.962
	25 mM	-5.5 ± 0.28	0.961
	50 mM	-5.5 ± 0.45	0.951
	100 mM	-5.7 ± 0.45	0.95
Acetaldehyde	0.78 mM	0.41 ± 0.9	1
	1.56 mM	0.5 ± 0.6	1
	3.125 mM	0.86 ± 0.98	1
	6.25 mM	-0.73 ± 0.98	1.00
	12.5 mM	2.0 ± 0.25	0.993
	25 mM	1.5 ± 1.57	0.999
	50 mM	-2.24 ± 1.56	0.986
	100 mM	-2.09± 1	0.992

Figure 3.3.2. Project III: Active chemiluminescence after stimulation of the neutrophils with 2-naphthylamine, hydroquinone, acrolein or acetaldehyde.

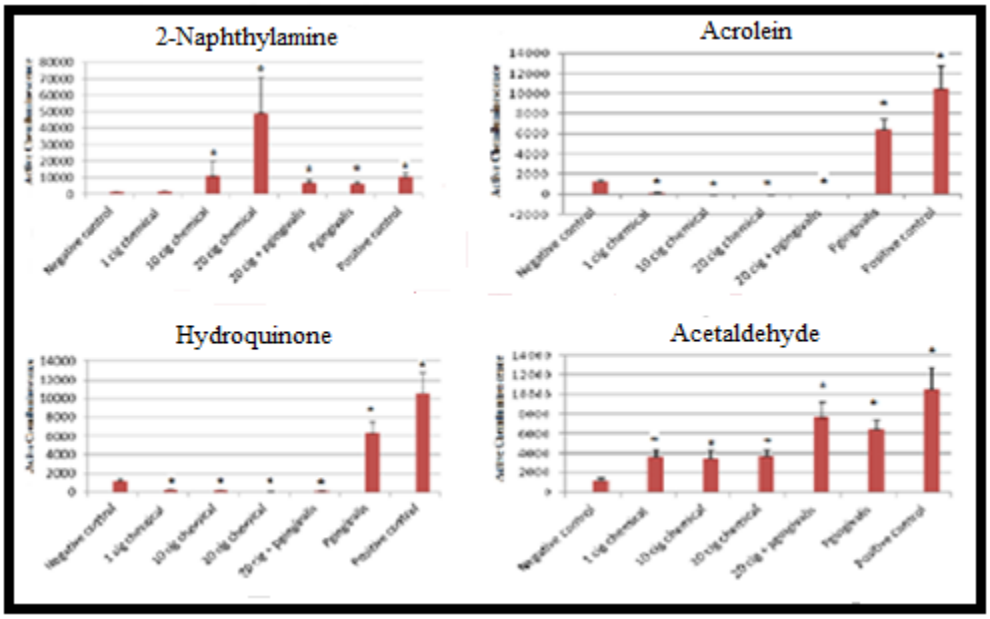

Table 3.3.2. Project III: Active chemiluminescence (CL) after stimulation of neutrophils with 2-naphthylamine, hydroquinone, acrolein or acetaldehyde. Conc 1: concentration found in 1 cigarette, Conc 2: concentration found in 10 cigarettes, Conc 3: Concentration found in 20 cigarettes, Conc 4: Concentration found in 20 cigarettes + 10% *P. gingivalis*, and Conc 5: 10% *P. gingivalis* alone.

	2-Naphthylamine		**Hydroquinone**		**Acrolein**		**Acetaldehyde**	
	CL	**SD**	**CL**	**SD**	**CL**	**SD**	**CL**	**SD**
Negative control	1250	242	1250	242	1250	242	1250	242
Conc 1	1652	245	253*	27	128*	68	3605*	769
Conc 2	10768*	9033	175*	24	-54*	39	3428*	827
Conc 3	49036*	21590	45*	11	-85*	25	3785*	559
Conc 4	6770*	1892	115*	31	-1*	39	7623	1701
Conc 5	6412*	1062	6412*	1062	6412*	1062	6412*	1062
Positive control	10525*	2261	10525*	2261	10525*	2261	10525*	2261

*denotes statistical significance difference of p-value < 0.05.

Figure 3.3.3. Project III: MMP-9 (92 kDa) western blot and gelatin zymography after stimulation of neutrophils with 2-naphthylamine, hydroquinone, acrolein or acetaldehyde. Conc 1: concentration found in 1 cigarette, Conc 2: concentration found in 10 cigarettes, Conc 3: concentration found in 20 cigarettes, Conc 4: concentration found in 29 cigarettes + 10% *P. gingivalis*, Conc 5: 10 % *P. gingivalis* alone. *denotes statistical significance difference (p-value < 0.05). 1: Naphthylamine, 2: Hydroquinone, 3: Acrolein, and 4: Acetaldehyde.

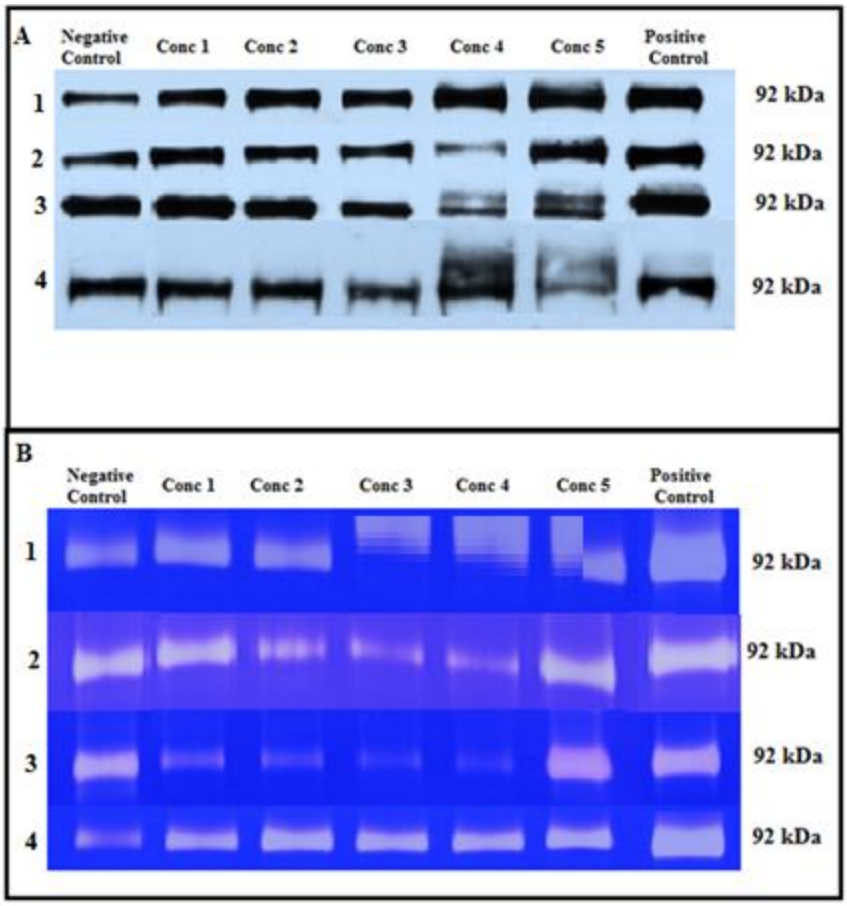

Table 3.3.3. Project III: MMP-9 (92 kDa) western blot release after stimulation of neutrophils with 2-naphthylamine, hydroquinone, acrolein, and acetaldehyde. Concentration (Conc 1): concentration found in 1 cigarette, Conc 2: concentration found in 10 cigarettes, Conc 3: Concentration found in 20 cigarettes, Conc 4: Concentration found in 20 cigarettes + 10% *P. gingivalis*, Conc 5: 10% *P. gingivalis* alone.

	2-Naphthylamine	**Hydroquinone**	**Acrolein**	**Acetaldehyde**
Negative control	53.51± 5.43	78.28 ± 11.5	128.47 ± 8.37	128.5 ± 8.4
Conc 1	79.18 ±3.4*	123.07 ± 16.09	158 ± 6.7	130.99 ± 8.66
Conc 2	104.46 ± 4.08*	105.58 ± 14.3	147.93 ± 8.61	120.3 ± 7.5
Conc 3	107.91 ±3.9*	94.41 ± 12.57	101.32 ± 7.54	115.47 ± 5.9
Conc 4	126.29 ± 7.22*	40.60 ± 5.325*	102.4 ± 5.855*	143.2 ± 4.2*
Conc 5	141.84 ± 10.5*	149.20 ± 7.7*	113.172 ± 4.1*	130.6 ± 6.2*
Positive control	130.22 ± 4.82*	143.53 ±16.98*	150.59 ± 7.1*	148.071 ± 6.7*

*denotes statistical significance difference < 0.05.

CHAPTER FOUR

Discussion

Tobacco smoking is considered a major modifiable risk factor for periodontal disease (Johnson and Guthmiller 2007). It affects the ecology of the oral environment, the gingival tissues, and the vasculature, as well as the host immune response (Palmer, Wilson et al. 2005). Tobacco smoking is associated with a two- to seven-fold increased risk for periodontal attachment loss and bone loss (Bergstrom, Eliasson et al. 1991; Gelskey, Young et al. 1998; Calsina, Ramon et al. 2002). Tobacco contains about 6700 compounds and almost 4000 of them have been identified in tobacco smoke (Baker, Ainsworth et al. 2000). These components include known carcinogens, toxic heavy metals, and many unidentified chemicals (Rogers 2009).

Nicotine is the major addictive agent in tobacco. Many studies in the literature have focused on the adverse effects of nicotine on both cell-mediated and humoral immune responses (Palmer, Wilson et al. 2005), as well as on its effects on various cell types in the body such as neutrophils, epithelial cells, and fibroblasts (Drost, Selby et al. 1992; Theilig, Bernd et al. 1994; Giannopoulou, Geinoz et al. 1999; Giannopoulou, Roehrich et al. 2001). Nicotine is an alkaloid and a tertiary amine consisting of a pyridine and pyrrolidine ring (Dani, Jenson et al. 2011). It has been reported that the unprotonated form of nicotine is absorbed through the mucous membrane during cigarette smoking, while the protonated

form is deposited in the lung and later absorbed (Benowitz 1988; Dani J.A. 2009).

Neutrophils are the first line of defense in the body and are critical cells in the maintenance of periodontal health for their role in the control of bacteria (Battino, Bullon et al. 1999), but they can also contribute to the progression of periodontal disease through the production of reactive oxygen species (ROS) (Canakci, Cicek et al. 2005). These ROS are primarily released to kill the bacteria, but the extracellular release of ROS also results in collateral damage of the surrounding tissues. The ROS include oxygen derived free radicals such as the superoxide radical, hydroxyl radical, and nitric oxide radical species, as well as non-radical derivatives of oxygen such as hydrogen peroxide and hypochlorous acid (Waddington, Moseley et al. 2000). It is known that ROS, particularly the active hydroxyl radicals, can degrade a number of structurally and metabolically functional macromolecules in an effort to balance its unpaired electronic state and thus result in cellular damage (Waddington, Moseley et al. 2000).

A study by Guentsch et al. (Guentsch, Puklo et al. 2009) showed that the extracellular release of ROS by neutrophils was detected when they were exposed to *P. gingivalis*, thus resulting probably in not only the killing of the bacteria but also in damaging of the surrounding periodontal tissues. The ROS have also been shown to be capable of degrading a number of extracellular matrix components (e.g., proteoglycans and glycosaminoglycans) (Fujisawa and Kuboki 1991; Limeback 1991). They have been reported to play important roles

in attacking collagen and making it more susceptible to breakdown by the collagenases, which can be activated by ROS (Madison, McCallum et al. 2002). ROS is also believed to activate osteoclasts (Bax, Alam et al. 1992) and increase their numbers (Garrett, Boyce et al. 1990).

Activated neutrophils release ROS, which can be assessed by luminol-enhanced chemiluminescence (CL). Luminol-enhanced CL is produced by the neutrophil-released ROS during host/bacteria interactions and has been demonstrated to correlate well with antibacterial integrity (Dahlgren and Karlsson 1999).

Project one investigated the effects of nicotine with/without *P. gingivalis* supernatant on the ability of neutrophils to release ROS. Various studies have used nicotine concentrations ranging between 50 µg/mL to 100 µg/mL (Theilig, Bernd et al. 1994; Payne, Johnson et al. 1996; Qui, Mei et al. 2004; Roman, Ritzenthaler et al. 2004), but none of these studies examined ROS release. Therefore, an intermediate concentration of 80 µg/mL was utilized for the current study. Ten percent *P. gingivalis* supernatant was utilized based on previous studies by Zhou and Windsor (Zhou, Olson et al. 2007; Zhou and Windsor 2007).

Priming of the neutrophils with 10^{-11} M fMLP was performed before adding the stimulants. This increases the number of receptors and surface proteins on the neutrophils and results in the potential for full scale neutrophil activation (Gasmi, McLennan et al. 1994). Priming is considered an intermediate state between resting and activation, and it enables the neutrophils to respond to the stimulant (e.g., bacteria) in a more powerful way (Smith 1994). After adding the

stimulants, the CL was measured. CL is the light energy generated from the neutrophils interaction with any stimulant. It quantifies the level of peripheral neutrophils metabolic oxidative activity. It has been confirmed in the literature that CL is a very sensitive and non-invasive technique to measure the ROS that are produced as a consequence of NADPH oxidase activation (Gasmi, McLennan et al. 1994).

NADPH oxidase in neutrophils produces superoxide ($O_2^{\cdot -}$), which initiates generation of the respiratory burst that is crucial for the bacterial destruction. The NADPH enzyme complex consists of two membrane-bound components $P22^{phox}$ and $gp91^{phox}$. These components comprise the enzymatic center of the NADPH complex (Valko, Leibfritz et al. 2007). Superoxide anion is considered the primary ROS and can further interact with other molecules to produce secondary ROS.

It has been reported that nicotine has two effects on the release of ROS: (1) inhibition of synthesis of superoxide and (2) the direct absorption of any superoxide that is produced (Pabst, Pabst et al. 1995). Sorensen et al. (Sorensen, Nielsen et al. 2004) studied the effects of smoking on the oxidative burst and the reactivity of neutrophils. They found that smoking reduced the oxidative burst of the neutrophils by half when compared to neutrophils stimulated with fMLP, which may translate into impaired oxidative killing and bactericidal activity.

Interestingly, in the current study, the *P. gingivalis* supernatant plus nicotine group did not differ significantly from the *P. gingivalis* group. Nicotine has been reported to compromise ROS production when compared with the

maximum release of ROS produced by the neutrophils when treated with a stimulant such as fMLP (Sorensen, Nielsen et al. 2004), which may reduce the capacity of the neutrophils to destroy the bacterial plaque. In the presence of *P. gingivalis*, the neutrophils produced significant higher amounts of than the positive control. The *P. gingivalis* group (37806 ± 3915) and the *P. gingivalis* plus nicotine group (34298 ± 3726) had a consistently higher ROS production than the nicotine group alone (13041 ± 1893) (Table 2.1). Thus, it appears that any effects of nicotine on neutrophil ROS production may be masked by the effects of the *P. gingivalis*. The negative control did have low level CL that could have been due to either handling of the neutrophils in vitro or minor contaminants found in the air during the CL assays.

P. gingivalis supernatant has been used in multiple investigations in the literature (Pattamapun, Tiranathanagul et al. 2003; Zhang, Song et al. 2010; Zhou, Zhang et al. 2012), but this could be a limitation of this study. It is unknown if live bacteria could affect the priming with/without activation of the neutrophils in a different manner. If so, the effects that *P. gingivalis* exerted on the neutrophils may not fully reflect the situation with the live bacteria. Another point to remember is that this study was limited to studying ROS, which is the most important aspect for activation of the neutrophils. But other neutrophil activation components should also be considered (Wahaidi 2010) such as elastase activity as it has been reported to be significant during the activation of the neutrophils with other bacteria such as *Fusobacterium nucleatum* (Sheikhi, Gustafsson et al. 2000).

The level of ROS release induced by nicotine was not at the same level as seen with the bacteria supernatant, but still could contribute to the damage that occurs in the extracellular matrix during periodontal disease. The nicotine did not further enhance the ROS release by the neutrophils in the presence of bacterial supernatant, thus suggesting that *P. gingivalis* induced the maximum ROS release. Nicotine only induced 34% of the ROS level as that induced by *P. gingivalis*. However, it should be emphasized that ROS is only one mechanism by which neutrophils contribute to tissue damage. They also express matrix metalloproteinases that are released from granules when stimulated with bacteria supernatant or nicotine. In addition, it has been reported that ROS can disrupt the protective function of the tissue inhibitors of matrix metalloproteinases (TIMPs) by either preventing their expression or by modifying them (Hadjigogos 2003).

Iho et al. (Iho, Tanaka et al. 2003) demonstrated that nicotine stimulates neutrophils to produce interleukin-8 in vitro. This process would be expected to recruit more neutrophils in vivo and thus enhancing the production of the ROS (Palmer, Wilson et al. 2005)**.** It may be that nicotine increases ROS not by maximizing its production by neutrophils, but by increasing the migration of more neutrophils to the area whereas bacteria maximize ROS production. This study demonstrated that *P. gingivalis* supernatant plus nicotine did not have an additive effect on ROS production when compared to the stimulation with *P. gingivalis* supernatant alone. Nicotine is only one compound in a mixture of 4000 in tobacco smoke that might have different effects on ROS production.

Project two investigated the effects of CSC on neutrophil functions. Cigarette smoke is a complex of more than 4000 compounds with more than 100 that are known carcinogens, mutagens and/or tumor promoters (Barbour, Nakashima et al. 1997). Cigarette smoke can be separated into two phases, a particulate tar phase that can be trapped and collected as a condensate and a gas phase that can be divided into mainstream and sidestream smoke. The mainstream smoke is drawn through the burning tobacco column and filter tip, and runs through the mouthpiece of the cigarette while the sidestream smoke is diluted into the surrounding air from the end of the smoldering cigarette and is mostly inhaled by nonsmokers. Cigarette smoking has long been known as a risk factor for periodontal disease and the relationship between tobacco smoking and periodontal disease has been well documented (Bergstrom and Floderus-Myrhed 1983; Feldman, Bravacos et al. 1983; Ismail, Burt et al. 1983; Bergstrom, Eliasson et al. 1991). Smokers have an increased risk, incidence, and severity of periodontal disease as evident by increased gingival recession, tooth loss, and periodontal destruction.

Cigarette smoke condensate (CSC) contains thousands of chemicals including nicotine, cadmium, heavy metals, and chemical carcinogens. Many studies have demonstrated that CSC can induce DNA strand breaks in mammalian cells in either culture or in vitro (Nakayama, Kaneko et al. 1985; Fielding, Short et al. 1989). Using human gingival fibroblasts seeded on collagen plates, Zhang et al. (Zhang, Song et al. 2010) demonstrated that adding CSC and *P. gingivalis* to the cells would lead to more collagen degradation by

destroying the balance between the MMPs and their inhibitors, as well as by increasing MMP activation. The MMPs are a group of zinc dependent endopeptidases that play major roles in physiological and pathological conditions (Chaussain-Miller, Fioretti et al. 2006). It is believed that during periodontal disease, several members of the MMPs are involved in the process such as MMP-1, MMP-2, and MMP-3 (Sorsa, Tjaderhane et al. 2004).

The neutrophils participate in the inflammatory process by producing ROS through the multi-component enzyme, NADPH oxidase. In addition to the membrane components previously mentioned, NAPDH oxidase is comprised of several cytosolic components, p67phox, p47phox, p40phox, and Rac2 (Quinn and Gauss 2004). Before activation, p47phox is phosphorylated and then translocated to the membrane. This is an essential conformational change that is needed to activate the microbicidal process. Upon translocation, p47phox, p67phox, and Rac 2 assemble themselves at the membrane, and the enzyme complex converts molecular oxygen to superoxide anion through a one electron transfer (Sigal, Gorzalczany et al. 2003).

Studying the cytotoxicity of chemicals and other agents on different human cells allows for better understanding of the mechanisms of action of these chemicals on cells and tissues. It is believed that cytotoxicity plays an important role in some pathological conditions such as carcinogenesis and inflammation (Zhang, Song et al. 2009).

Concentrations ranging from 0-800 µg/mL of CSC were used to treat the neutrophils in the current study. According to Hellerman et al. (Hellermann, Nagy et al. 2002), the average yield of CSC is 26.1 mg/cigarette or in other words 522 mg/pack. With salivary secretion around 1L/day, the dilution of the CSC will range around 522 µg/mL. The maximum amount of CSC used on human neutrophils without significant cytotoxicity was 50 µg/mL, which is much lower than what a smoker's saliva might yield, whereas Zhang et al. reported a higher cytotoxic level for human gingival fibroblasts when treated with CSC up to 200 µg/mL (Zhang, Fang et al. 2011).

Regarding the ROS experiments, the concentrations of CSC used were 10, 25, and 50 µg/mL. They all inhibited the ROS production from the neutrophils in a dose dependent response manner. The active CL for 10, 25, and 50 µg/mL was 188 ± 55, 149 ± 41, and 130 ± 30 respectively. The *P. gingivalis* group produced a high level of ROS (6412 ± 1062) comparable to the positive control (10525 ± 2261).

Project two showed that *P. gingivalis* increases the ROS production from neutrophils, but the addition of CSC inhibited it. This inhibition may lead to the survival of the bacteria in the periodontal tissues and secretion of the toxins and enzymes leading to periodontal destruction, as well as activation of host responses. It is unclear how CSC inhibits the ROS production, but assumptions can made similar to that of the ROS inhibition by nicotine. Pabst et al. (Pabst, Pabst et al. 1995) assumed that nicotine might inhibit the ROS production either by the inhibition of the synthesis of superoxide and/or the direct absorption of

any superoxide that is produced. This is inconsistent with one study (Al-Shibani, Labban et al. 2011) where nicotine increased the ROS production from neutrophils by 34% of that induced by *P. gingivalis*.

In western blot analysis and gelatin zymography, MMP-9 showed increased release with 10, 25, or 50 µg/mL CSC compared to the control. *P. gingivalis* plus CSC group had the most MMP-9 release in both the western blot and gelatin zymography assays. MMP-9 has been associated with periodontal disease and numerous studies have shown it has increased circulation levels in periodontal disease patients (Marcaccini, Novaes et al. 2009; Ozcaka, Bicakci et al. 2011). A study by Ozcaka et al. (Ozcaka, Bicakci et al. 2011) found a significant increase in the serum concentration of MMP-9 in smokers with chronic periodontal disease. Their results support those of this study that found significant increases of MMP-9 release in the *P. gingivalis* and CSC group. Persistent smoking and the presence of periodontal bacteria can enhance the release of MMP-9.

Other studies have also demonstrated a relationship between CSC exposure and the production of ROS. Jaimes et al. (Jaimes, DeMaster et al. 2004) reported that CSC treated endothelial cells resulted in an increase in ROS production, while Dunn et al. (Dunn, Freed et al. 2005) demonstrated that CSC alone did not prompt ROS production from the neutrophils. This could be due to lower concentrations used in their study. In their study, they prepared fresh CSC extract from a commercial brand, then serial dilutions were made to 1:25, 1:250, 1:2500, and 1:25,000.

This current study showed that *P. gingivalis* increases the ROS production from neutrophils, but the addition of CSC inhibited the ROS production. This inhibition may lead to the survival of the bacteria in the periodontal tissue and their secretion of the toxins and enzymes leading to periodontal destruction. MMP-9 release was increased in all the concentrations of the CSC groups and even more in the CSC plus *P. gingivalis* group, demonstrating the detrimental effects that CSC has on neutrophil normal functions. Interestingly, smokers are known to have increased risk of *P. gingivalis* infections (Haffajee and Socransky 2001), and other reports have confirmed that the number of *P. gingivalis* cells are greater in patients who smoke than non-smokers (Zambon, Grossi et al. 1996).

Project three investigated four different components of smoke. 2-Naphthylamine is an arylamine and has a molecular weight of 143.2 g/mL (Stabbert, Schafer et al. 2003). It has the characteristics of primary aromatic amines and is also a weak base. The concentration of 2-naphthylamine in one cigarette has been determined to be between 0.0002 mM to 0.0004 mM (Hoffmann, Djordjevic et al. 1997). 2-Naphthylamine is known to be a human carcinogen and has been reported to cause bladder cancer (Lyon 1987). Hydroquinone is another major compound found in smoke at concentrations ranging between 0.001 mM and 0.03 mM in one cigarette (Hoffmann D 1986). It has a molecular weight of 110.06 g/mL. It is a white crystalline solid and is soluble in water (Regev, Wu et al. 2012). Joseph et al. (Joseph, Klein-Szanto et al. 1998) demonstrated that hydroquinones are mutagenic compounds. Their experiments showed that hydroquinones can cause specific deletion of a

cytosine from a group of five cytosines or a single guanosine from a group of five guanosines in the complementary strand, which results in a frameshift mutation (Joseph, Klein-Szanto et al. 1998). It was also concluded that hydroquinones also function as initiators of carcinogenesis (Joseph, Klein-Szanto et al. 1998). Acrolein and acetaldehyde are two of the most active aldehydes in tobacco smoke. Acrolein is a volatile flammable liquid with a molecular weight of 56.06 g/mL (Anand, Emmadi et al. 2011) and acetaldehyde has a molecular weight of 44.05 g/mL. A study by Anand demonstrated that acrolein has a dose-dependent cytotoxic effect on human gingival fibroblasts with complete inhibition of attachment and proliferation at 10^{-4} M (Anand, Emmadi et al. 2011). A study by Wang reported that only acrolein and not acetaldehyde inhibited chemotaxis of human bronchial epithelial cells and altered their functions (Wang, Liu et al. 2001). The concentrations of acrolein and acetaldehyde in one cigarette have been determined to be between 0.003 mM to 0.008 mM and 0.02 mM and 0.046 mM, respectively (Fujioka and Shibamoto 2006).

ROS production is considered a powerful method of bacterial elimination by the neutrophil defense system. When the neutrophils are activated, the NADPH oxidase produces superoxide by transferring electrons from NAPDH across the membrane and these electrons couple to oxygen, so generating a superoxide anion. The superoxide anion undergoes secondary reactions to produce more ROS that are powerful agents that kill microbes.

The tobacco components tested are only four of thousands of components found in tobacco smoke, but were chosen for specific reasons. 2-Naphthylamine

and hydroquinone were chosen because of their high concentrations in tobacco smoke, while acrolein and acetaldehyde were chosen because they are two of the most active aldehydes found in smoke. This should not undermine the importance of the other components in smoke that might be present in minute concentrations, but have potent effects on neutrophils and other oral cavity cells.

2-Naphthylamine and hydroquinone were toxic to the neutrophils at very high concentrations (5 mM and 2.5 mM, respectively). Although both components can be dissolved in water, they were not completely soluble in water at the high concentration (40 mM) used in the cytotoxicity study. DMSO was used as a solvent for both components in these experiments.

2-Naphthylamine is an aromatic amine that is present in mainstream smoke (which is inhaled by the smoker) and also found in sidestream smoke (which is released in the air). It has been reported that active smokers are exposed to 2-naphthylamine greater than passive smokers due to the inhaled toxic agents in addition to the polluted atmosphere (Goniewicz and Czogala 2005). Hydroquinone is another major component of smoke and it has been reported to pollute the atmosphere indoors and outdoors (McGregor 2007). In a mice study (Shimada, Ribeiro et al. 2012) after exposure to hydroquinone, the mice had reduced secretion of monocyte chemoattractant protein-1 (MCP-1). The reduction of MCP-1 secretion by hydroquinone treated cells could impair the onset and the resolution of any inflammatory process, which may contribute to the higher incidence of lung infections in smokers.

Acrolein and acetaldehyde were not toxic to the neutrophils at any of the concentrations tested. In addition, increasing the concentrations of acrolein seemed to protect cells from necrosis. In addition to the effects that acrolein has on fibroblasts as discussed in Chapter One, acrolein was reported to suppress macrophage activation (Li and Holian 1998) and reduce the innate immune responses to LPS (Kasahara, Poynter et al. 2008).

The cytotoxicity and the effects on proliferation of these components on human gingival fibroblasts were previously determined (Allam 2011). The cytotoxic concentrations detected were lower than those determined for the neutrophils. This could be due to the fact that the fibroblasts were incubated with the tobacco components for three days while the neutrophils were incubated only for 2 hours. Secondly, neutrophils are phagocytic and defensive cells, so it would be logical for them to have a higher threshold for stimulants compared to other cells.

It is imperative to note that although 2-naphthylamine is toxic to the neutrophils at 5 mM, it has been reported to be toxic to the fibroblasts at concentrations as low as 5 µM (Allam 2011). The concentrations of 2-naphthylamine in one cigarette were reported to be between 0.2 and 0.4 µM. If the higher end of the scale was used, the concentration of 2-naphthylamine in 20 cigarettes (smokers who smoke one pack a day) will be 8 µM, which is toxic to fibroblasts. It would be interesting to determine the cytotoxicity of these components on human epithelial cells as they are the first cells to be exposed to the tobacco smoke in the oral cavity (Table 5.1).

2-Naphthylamine increased ROS production with 20 cigarettes having the most impact in comparison to all the other chemicals tested. Interestingly, hydroquinone and acrolein inhibited ROS production at all the concentrations tested. When *P. gingivalis* was also added to them, they inhibited the ROS production and masked the effects of *P. gingivalis*. A previous study by Al-Shibani et al. (Al-Shibani, Labban et al. 2011) reported that *P. gingivalis* alone increased ROS production to a level similar to the positive control (fMLP). Acetaldehyde showed increased ROS production to a lesser degree than 2-naphthylamine.

In the western blots, 2-naphthylamine showed a dose dependent increase in the MMP-9 release that was significant at all the concentrations tested (all p-values < 0.05) (Table 1). The concentration in 20 cigarettes of 2-naphthylamine plus *P. gingivalis* and *P. gingivalis* alone groups showed the highest release of MMP-9. Hydroquinone and acrolein showed decreased MMP-9 release especially at higher concentrations of the tobacco components. There was a significant decrease noticed when concentrations of hydroquinone or acrolein found in 20 cigarettes and *P. gingivalis* were added (Figure 3). It appears that these components decrease or weakens the normal physiological function of the neutrophils. Acetaldehyde did not significantly alter the MMP-9 release, but there was a significant increase in MMP-9 with the combination of acetaldehyde and *P. gingivalis*.

These are only 4 tobacco smoke components in a mixture of more than 6700 other components, but it is crucial to know how each tobacco component affects host cells and alters host defense mechanisms. This study showed that *P. gingivalis* increases the ROS production from neutrophils, but the addition of hydroquinone and acrolein inhibits this ROS production. This inhibition may lead to the survival of the bacteria in the periodontal tissue with the secretion of toxins and enzymes, as well as activation of host responses, leading to periodontal tissue destruction. MMP-9 release showed gradual decrease in both hydroquinone and acrolein groups suggesting the detrimental effects that these chemicals have on normal neutrophil functions.

CHAPTER FIVE

Summary and Conclusion

This chapter provides a general summary and conclusions from the 3 studies that were described in Chapters Two through Four of this dissertation. This chapter also discusses the significance of these results in general terms and suggests future directions for continuing the research.

The broad objective of these investigations was to increase the knowledge of the causal association between tobacco and periodontal disease. With a better understanding of the mechanisms of smoke-related periodontal disease, its etiology can be defined more clearly.

These studies aimed at exploring the biological mechanisms underlying the possible etiological links between tobacco and periodontal disease. In general, it was hypothesized that tobacco smoke components will increase the ROS production from the neutrophils to aid in killing the foreign objects (bacteria) in the tissues and with the additional stimulation by *P. gingivalis* that the ROS would increase even more. MMP-9 release from the neutrophils, after their stimulation with the tobacco smoke components, was also examined.

In the first study, the ROS production was examined when neutrophils were stimulated with either 80 µg/mL nicotine and/or 10% *P. gingivalis*. The results showed that nicotine increased the ROS production but this increase was significantly less than the increase caused by stimulation with *P. gingivalis* alone. The stimulation of neutrophils with both nicotine and *P. gingivalis* did not have an

additive effect on the production of ROS. These results were inconsistent with other studies that showed that nicotine inhibited the ROS produced from neutrophils. Pabst et al. (Pabst, Pabst et al. 1995) reported inhibition of ROS production from neutrophils when exposed to nicotine. The concentrations they used were higher than what was used in the current studies ranging between 10^{-6} and 10^{-4} M of nicotine.

The second study investigated the effects of CSC on the ROS production and MMP-9 release from the neutrophils. Both CSC and the combination of CSC with P. gingivalis inhibited the ROS production. In addition, MMP-9 release increased as the concentrations of CSC increased.

The third study investigated the effects of four components of tobacco smoke on ROS production and MMP-9 release from neutrophils. 2-Naphthylamine was incubated with neutrophils at different concentrations and was found to increase ROS production significantly. The MMP-9 release was also increased. Adding *P. gingivalis* and 2-naphthylamine together increased ROS production and MMP-9 release from the neutrophils. Hydroquinone and acrolein inhibited the ROS production of the neutrophils. Interestingly, they also inhibited the effects of *P. gingivalis* enhanced ROS production when either of these chemicals were simultaneously added with *P. gingivalis*. The MMP-9 release was also decreased with these two chemicals. The MMP-9 release from the *P. gingivalis*-hydroquinone or *P. gingivalis*-acrolein groups was decreased significantly. The fourth component investigated was acetaldehyde, which showed increased ROS production. MMP-9 did not show any significant change

when the neutrophils were stimulated with acetaldehyde. But the addition of acetaldehyde and *P. gingivalis* resulted in a significant release of MMP-9.

The studies completed are considered preliminary investigations to develop more specific and focused investigations. Cigarette smoking related health issues is a dominant health burden for our society. Understanding the mechanisms for smoking-induced pathological changes such as ROS production and MMP release facilitate effective preventive treatment for various smoking associated diseases.

In the first study, nicotine increased the ROS production from neutrophils but not as much as *P. gingivalis*-treated neutrophils. The increase of ROS for nicotine-treated neutrophils will open new research related to the nicotine replacement therapies including nicotine gums. Nicotine gums are used to deliver nicotine to the body because it is the addictive component in tobacco. This has been widely used for the therapeutic purposes of smoking cessation in clinic and daily life, but this continuous intake of nicotine could still interrupt the functional recovery of neutrophils in smokers who are trying to quit cigarettes. Future investigations are needed to determine if the nicotine gums can induce ROS in the oral cavity at concentrations that can lead to extracellular matrix degradation and periodontal disease.

The second study investigated ROS production and MMP-9 release from the neutrophils when stimulated with CSC. The mechanism of how CSC inhibits the ROS is still not clear. Further studies are needed to evaluate if this inhibition is due to CSC's interference with the NADPH proteins translocation or

interference in the signaling pathways of the NADPH oxidase. In addition, although nicotine is the major addictive ingredient in CSC, it did not influence the ROS production as much as expected. It seems that other components in CSC have a bigger influence on inhibiting ROS, including acrolein and hydroquinone. If all the components in CSC can be identified and categorized according to their effects on the ROS, then it would be possible to remove some of these components to a level that would not inhibit the ROS when *P. gingivalis* is present, or add other components that would inhibit this effect. Understanding these mechanisms, based on these preliminary data, could lead to modifying the effects that CSC has on the effects of *P. gingivalis* or ROS, which will lead to the survival of bacteria in the tissue and eventually periodontal disease.

The third study investigated the ROS production of four different components of smoke. 2-Naphthylamine has been classified by the International Agency of Research on Cancer (IARC) as IARC Group I human carcinogen and has been reported to be present in the mainstream smoke. Hydroquinone is an aromatic amine that has been reported to a cardiovascular toxin and has shown genotoxic activity. Acrolein and acetaldehyde are the most active aldehydes in tobacco smoke. The exposure of neutrophils to these different chemicals resulted in different responses with the ROS production. These biological mechanisms could explain part of the etiological relationship between tobacco and the tissue destruction found in periodontal disease. These are only four compounds in a complex of 4000 other compounds in tobacco smoke and more research is

needed to successfully put the pieces of the puzzle together to have an overview of all the significant compounds and their effects on the oral cavity.

In addition to exploring and understanding the mechanisms by which tobacco smoke acts on ROS production from neutrophils, it is also important to standardize in vitro and in vivo models to investigate additional active components in tobacco smoke that cause damage. The studies accomplished here were all conducted in vitro. It would be interesting to translate these studies into animal models to investigate the role of tobacco and it's mechanisms in periodontal disease. The neutrophils were exposed to tobacco components for short period of times, whereas patients who are smokers are exposed to tobacco for very long periods of time. Further studies are needed to explore these long term effects in addition to the stimulation of neutrophils to live bacteria and other bacteria relevant to periodontal disease (e.g., *Actinobacillus actinomycetemcomitans).*

Table 5.1. Cytotoxicity of human gingival fibroblasts when exposed to 2-naphthylamine, hydroquinone, acrolein, and acetaldehyde.

Chemical	concentration (um)	Cytotoxicity ± SD
	0	0.00 ± 0.00
	1.25	7.25 ± 1.24
2-Naphthylamine	2.5	10.10 ± 0.26
	5	14.98 ± 1.21*
	10	75.10 ± 3.27*
	0	0.00 ± 0.00
	25	-2.95 ± 3.84
Hydroquinone	50.0000	0.19 ± 0.86
	100	37.64± 7.11*
	200	37.54 ± 21.93*
	0	0.00 ± 0.00
	0.0063	4.34 ± 0.11
Acrolein	0.0125	14.20 ± 0.50
	0.025	9.57 ± 0.18*
	0.05	22.34 ± 0.44*
	0	0.00 ± 0.00
	0.0781	5.80 ± 0.55
Acetaldehyde	0.1563	21.72 ± 0.87
	0.3125	14.96 ± 0.66
	0.625	69.50 ± 2.66*

*denotes statistical significance difference (p-value < 0.05)

REFERENCES

Al-Shibani, N. and L. J. Windsor (2008). "Effects of *Porphyromonas gingivalis* on human gingival fibroblasts from healthy and inflamed tissues." J Periodontal Res 43(4): 465-470.

Al-Shibani, N. K., N. Y. Labban, et al. (2011). "Responses of human neutrophils to nicotine and/or *Porphyromonas gingivalis*." J Periodontol 82(10): 1504-1508.

Allam, E., W. Zhang, C. Zheng, R.L. Gregory, and L.J. Windsor. (2011). Smoking and oral health. In: Cigarette Smoke Toxicity, Wiley-VCH Verlag GmbH & Co. KGaA.

Alpiste Illueca, F. M., P. Buitrago Vera, et al. (2006). "Periodontal regeneration in clinical practice." Med Oral Patol Oral Cir Bucal 11(4): E382-392.

Anand, N., P. Emmadi, et al. (2011). "Effect of a volatile smoke component (acrolein) on human gingival fibroblasts: An in vitro study." J Indian Soc Periodontol 15(4): 371-375.

Assuma, R., T. Oates, et al. (1998). "IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis." J Immunol 160(1): 403-409.

Bahekar, A. A., S. Singh, et al. (2007). "The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: a meta-analysis." Am Heart J 154(5): 830-837.

Baker, F., S. R. Ainsworth, et al. (2000). "Health risks associated with cigar smoking." JAMA 284(6): 735-740.

Barbour, S. E., K. Nakashima, et al. (1997). "Tobacco and smoking: environmental factors that modify the host response (immune system) and have an impact on periodontal health." Crit Rev Oral Biol Med 8(4): 437-460.

Bascones-Martinez, A., M. Munoz-Corcuera, et al. (2009). "Host defence mechanisms against bacterial aggression in periodontal disease: Basic mechanisms." Med Oral Patol Oral Cir Bucal 14(12): e680-685.

Battino, M., P. Bullon, et al. (1999). "Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species." Crit Rev Oral Biol Med 10(4): 458-476.

Bax, B. E., A. S. Alam, et al. (1992). "Stimulation of osteoclastic bone resorption by hydrogen peroxide." Biochem Biophys Res Commun 183(3): 1153-1158.

Benhamou, S., A. Laplanche, et al. (2003). "DNA adducts in normal bladder tissue and bladder cancer risk." Mutagenesis 18(5): 445-448.

Benowitz, N. L. (1988). "Drug therapy. Pharmacologic aspects of cigarette smoking and nicotine addition." N Engl J Med 319(20): 1318-1330.

Bentwood, B. J. and P. M. Henson (1980). "The sequential release of granule constitutents from human neutrophils." J Immunol 124(2): 855-862.

Bergstrom, J., S. Eliasson, et al. (2000). "A 10-year prospective study of tobacco smoking and periodontal health." J Periodontol 71(8): 1338-1347.

Bergstrom, J., S. Eliasson, et al. (1991). "Cigarette smoking and periodontal bone loss." J Periodontol 62(4): 242-246.

Bergstrom, J. and B. Floderus-Myrhed (1983). "Co-twin control study of the relationship between smoking and some periodontal disease factors." Community Dentistry & Oral Epidemiology 11(2): 113-116.

Birkedal-Hansen, H. (1993). "Role of cytokines and inflammatory mediators in tissue destruction." J Periodontal Res 28(6 Pt 2): 500-510.

Birkedal-Hansen, H. (1993). "Role of matrix metalloproteinases in human periodontal diseases." J Periodontol 64(5 Suppl): 474-484.

Bodet, C., F. Chandad, et al. (2006). "Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia." Microbes Infect 8(1): 27-35.

Boyum, A. (1968). "Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g." Scand J Clin Lab Invest Suppl 97: 77-89.

Brennan, P., O. Bogillot, et al. (2000). "Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case-control studies." Int J Cancer 86(2): 289-294.

Cainciola, L. J., R. J. Genco, et al. (1977). "Defective polymorphonuclear leukocyte function in a human periodontal disease." Nature 265(5593): 445-447.

Calsina, G., J. M. Ramon, et al. (2002). "Effects of smoking on periodontal tissues." J Clin Periodontol 29(8): 771-776.

Canakci, C. F., Y. Cicek, et al. (2005). "Reactive oxygen species and human inflammatory periodontal diseases." Biochemistry (Mosc) 70(6): 619-628.

Cancer, I. A. f. R. o. (2004). Tobacco smoking and tobacco smoke. IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans,. 83.

Carrassi, A., S. Abati, et al. (1989). "Periodontitis in a patient with chronic neutropenia." J Periodontol 60(6): 352-357.

Cattaneo, V., G. Cetta, et al. (2000). "Volatile components of cigarette smoke: effect of acrolein and acetaldehyde on human gingival fibroblasts in vitro." J Periodontol 71(3): 425-432.

Chapple, I. L. and J. B. Matthews (2007). "The role of reactive oxygen and antioxidant species in periodontal tissue destruction." Periodontol 2000 43: 160-232.

Chaussain-Miller, C., F. Fioretti, et al. (2006). "The role of matrix metalloproteinases (MMPs) in human caries." J Dent Res 85(1): 22-32.

Craig, R. G., R. Boylan, et al. (2001). "Prevalence and risk indicators for destructive periodontal diseases in 3 urban American minority populations." J Clin Periodontol 28(6): 524-535.

Curtis, M. A., H. K. Kuramitsu, et al. (1999). "Molecular genetics and nomenclature of proteases of Porphyromonas gingivalis." J Periodontal Res 34(8): 464-472.

Curtis, M. A., A. Thickett, et al. (1999). "Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of Porphyromonas gingivalis W50." Infect Immun 67(8): 3816-3823.

Cutler, C. W., J. R. Kalmar, et al. (1995). "Pathogenic strategies of the oral anaerobe, Porphyromonas gingivalis." Trends Microbiol 3(2): 45-51.

Dahlgren, C. and A. Karlsson (1999). "Respiratory burst in human neutrophils." J Immunol Methods 232(1-2): 3-14.

Dahlgren, C., A. Karlsson, et al. (2007). "Measurement of respiratory burst products generated by professional phagocytes." Methods Mol Biol 412: 349-363.

Dani J.A., K. T. R., Benowitz N.L. (2009). The pharmacology of nicotine and tobacco. Philadelphia, PA, Lippincott Williams & Wilkins, Wolters Kluwer.

Dani, J. A., D. Jenson, et al. (2011). "Neurophysiology of Nicotine Addiction." J Addict Res Ther S1(1).

Daniell, H. W. (1983). "Postmenopausal tooth loss. Contributions to edentulism by osteoporosis and cigarette smoking." Arch Intern Med 143(9): 1678-1682.

Darveau, R. P., T. T. Pham, et al. (2004). "Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4." Infect Immun 72(9): 5041-5051.

DeCarlo, A. A., H. E. Grenett, et al. (1998). "Induction of matrix metalloproteinases and a collagen-degrading phenotype in fibroblasts and epithelial cells by secreted Porphyromonas gingivalis proteinase." Journal of Periodontal Research 33(7): 408-420.

DeCarlo, A. A., Jr., L. J. Windsor, et al. (1997). "Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe Porphyromonas gingivalis." J Dent Res 76(6): 1260-1270.

Delcourt-Debruyne, E. M., H. R. Boutigny, et al. (2000). "Features of severe periodontal disease in a teenager with Chediak-Higashi syndrome." J Periodontol 71(5): 816-824.

DeMarini, D. M. (1983). "Genotoxicity of tobacco smoke and tobacco smoke condensate." Mutat Res 114(1): 59-89.

Dixon, D. R., B. W. Bainbridge, et al. (2004). "Modulation of the innate immune response within the periodontium." Periodontol 2000 35: 53-74.

Drost, E. M., C. Selby, et al. (1992). "Changes in neutrophil deformability following in vitro smoke exposure: mechanism and protection." Am J Respir Cell Mol Biol 6(3): 287-295.

Dunn, J. S., B. M. Freed, et al. (2005). "Inhibition of human neutrophil reactive oxygen species production and p67phox translocation by cigarette smoke extract." Atherosclerosis 179(2): 261-267.

Ejeil, A. L., S. Igondjo-Tchen, et al. (2003). "Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva." J Periodontol 74(2): 188-195.

Feldman, R. S., J. S. Bravacos, et al. (1983). "Association between smoking different tobacco products and periodontal disease indexes." J Periodontol 54(8): 481-487.

Fielding, S., C. Short, et al. (1989). "Studies on the ability of smoke from different types of cigarettes to induce DNA single-strand breaks in cultured human cells." Mutat Res 214(1): 147-151.

Fravalo, P., C. Menard, et al. (1996). "Effect of Porphyromonas gingivalis on epithelial cell MMP-9 type IV collagenase production." Infect Immun 64(12): 4940-4945.

Fujioka, K. and T. Shibamoto (2006). "Determination of toxic carbonyl compounds in cigarette smoke." Environ Toxicol 21(1): 47-54.

Fujisawa, R. and Y. Kuboki (1991). "Preferential adsorption of dentin and bone acidic proteins on the (100) face of hydroxyapatite crystals." Biochim Biophys Acta 1075(1): 56-60.

Gao, S., K. Chen, et al. (2005). "Transcriptional and posttranscriptional inhibition of lysyl oxidase expression by cigarette smoke condensate in cultured rat fetal lung fibroblasts." Toxicol Sci 87(1): 197-203.

Garcia-Garcia, E. (2005). Molecular Mechanisms of Phagocytosis. Georgetown, Texas, Landes Bioscience.

Garcia, R. I., M. E. Nunn, et al. (2001). "Epidemiologic associations between periodontal disease and chronic obstructive pulmonary disease." Ann Periodontol 6(1): 71-77.

Garrett, I. R., B. F. Boyce, et al. (1990). "Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo." J Clin Invest 85(3): 632-639.

Gasmi, L., A. G. McLennan, et al. (1994). "Priming of the respiratory burst of human neutrophils by the diadenosine polyphosphates, AP4A and AP3A: role of intracellular calcium." Biochem Biophys Res Commun 202(1): 218-224.

Gelskey, S. C., T. K. Young, et al. (1998). "Factors associated with adult periodontitis in a dental teaching clinic population." Community Dentistry & Oral Epidemiology 26(4): 226-232.

Giannobile, W. V. (2008). "Host-response therapeutics for periodontal diseases." J Periodontol 79(8 Suppl): 1592-1600.

Giannopoulou, C., A. Geinoz, et al. (1999). "Effects of nicotine on periodontal ligament fibroblasts in vitro." J Clin Periodontol 26(1): 49-55.

Giannopoulou, C., N. Roehrich, et al. (2001). "Effect of nicotine-treated epithelial cells on the proliferation and collagen production of gingival fibroblasts." J Clin Periodontol 28(8): 769-775.

Goniewicz, M. L. and J. Czogala (2005). "Exposure of active and passive smokers to aromatic amines present in tobacco smoke." Toxicol Mech Methods 15(3): 235-245.

Grafstrom, R. C., J. M. Dypbukt, et al. (1994). "Pathobiological effects of acetaldehyde in cultured human epithelial cells and fibroblasts." Carcinogenesis 15(5): 985-990.

Grayson, R., C. W. Douglas, et al. (2003). "Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and Porphyromonas gingivalis." J Clin Periodontol 30(6): 542-550.

Guentsch, A., M. Puklo, et al. (2009). "Neutrophils in chronic and aggressive periodontitis in interaction with Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans." Journal of Periodontal Research 44(3): 368-377.

Hadjigogos, K. (2003). "The role of free radicals in the pathogenesis of rheumatoid arthritis." Panminerva Med 45(1): 7-13.

Haffajee, A. D. and S. S. Socransky (1994). "Microbial etiological agents of destructive periodontal diseases." Periodontol 2000 5: 78-111.

Haffajee, A. D. and S. S. Socransky (2001). "Relationship of cigarette smoking to attachment level profiles." J Clin Periodontol 28(4): 283-295.

Haffajee, A. D. and S. S. Socransky (2001). "Relationship of cigarette smoking to the subgingival microbiota." J Clin Periodontol 28(5): 377-388.

Hellermann, G. R., S. B. Nagy, et al. (2002). "Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells." Respir Res 3: 22.

Henderson, R. F. (1996). "Species differences in the metabolism of benzene." Environ Health Perspect 104 Suppl 6: 1173-1175.

Hoffmann, D., M. V. Djordjevic, et al. (1997). "The changing cigarette." Prev Med 26(4): 427-434.

Hoffmann D, W. E. (1986). Chemical constituents and bioactivity of tobacco smoke. IARC, Sci Publ 314: 145-165.

Holt, S. C. and T. E. Bramanti (1991). "Factors in virulence expression and their role in periodontal disease pathogenesis." Crit Rev Oral Biol Med 2(2): 177-281.

IARC (1987). Overall Evaluations of Carcinogenicity. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. International Agency for Research on Cancer. Supplement 7 : 440.

Iho, S., Y. Tanaka, et al. (2003). "Nicotine induces human neutrophils to produce IL-8 through the generation of peroxynitrite and subsequent activation of NF-kappaB." J Leukoc Biol 74(5): 942-951.

Imamura, T. (2003). "The role of gingipains in the pathogenesis of periodontal disease." J Periodontol 74(1): 111-118.

Imamura, T., S. Tanase, et al. (2001). "Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from Porphyromonas gingivalis." Biochem J 353(Pt 2): 325-331.

Inaloz, H. S., M. Harman, et al. (2001). "Atypical familial Papillon-Lefevre syndrome." J Eur Acad Dermatol Venereol 15(1): 48-50.

Ismail, A. I., B. A. Burt, et al. (1983). "Epidemiologic patterns of smoking and periodontal disease in the United States." J Am Dent Assoc 106(5): 617-621.

Jaimes, E. A., E. G. DeMaster, et al. (2004). "Stable compounds of cigarette smoke induce endothelial superoxide anion production via NADPH oxidase activation." Arterioscler Thromb Vasc Biol 24(6): 1031-1036.

Johnson, G. K. and J. M. Guthmiller (2007). "The impact of cigarette smoking on periodontal disease and treatment." Periodontol 2000 44: 178-194.

Johnson, L. L., R. Dyer, et al. (1998). "Matrix metalloproteinases." Curr Opin Chem Biol 2(4): 466-471.

Joseph, P., A. J. Klein-Szanto, et al. (1998). "Hydroquinones cause specific mutations and lead to cellular transformation and in vivo tumorigenesis." Br J Cancer 78(3): 312-320.

Kasahara, D. I., M. E. Poynter, et al. (2008). "Acrolein inhalation suppresses lipopolysaccharide-induced inflammatory cytokine production but does not affect acute airways neutrophilia." J Immunol 181(1): 736-745.

Kinane, D. F. (2001). "Causation and pathogenesis of periodontal disease." Periodontol 2000 25: 8-20.

Kinane, D. F., I. B. Darby, et al. (2003). "Changes in gingival crevicular fluid matrix metalloproteinase-8 levels during periodontal treatment and maintenance." J Periodontal Res 38(4): 400-404.

Kornman, K. S., R. C. Page, et al. (1997). "The host response to the microbial challenge in periodontitis: assembling the players." Periodontol 2000 14: 33-53.

Lamont, R. J. and H. F. Jenkinson (1998). "Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis." Microbiol Mol Biol Rev 62(4): 1244-1263.

Lepidi, S., R. D. Kenagy, et al. (2001). "MMP9 production by human monocyte-derived macrophages is decreased on polymerized type I collagen." J Vasc Surg 34(6): 1111-1118.

Li, L. and A. Holian (1998). "Acrolein: a respiratory toxin that suppresses pulmonary host defense." Rev Environ Health 13(1-2): 99-108.

Limeback, H. (1991). "Molecular mechanisms in dental hard tissue mineralization." Curr Opin Dent 1(6): 826-835.

Lyon, F. (1987). "Overall Evaluations of Carcinogenicity. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans." International Agency for Research on Cancer Supplement 7: 440

Macedo, S. M., E. L. Lourenco, et al. (2006). "Effect of in vivo phenol or hydroquinone exposure on events related to neutrophil delivery during an inflammatory response." Toxicology 220(2-3): 126-135.

Madison, S. A., J. E. McCallum, et al. (2002). "Hydroperoxide formation in model collagens and collagen type I." Int J Cosmet Sci 24(1): 43-52.

Makela, M., T. Salo, et al. (1994). "Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status." J Dent Res 73(8): 1397-1406.

Marcaccini, A. M., C. A. Meschiari, et al. (2010). "Gingival crevicular fluid levels of MMP-8, MMP-9, TIMP-2, and MPO decrease after periodontal therapy." J Clin Periodontol 37(2): 180-190.

Marcaccini, A. M., A. B. Novaes, Jr., et al. (2009). "Circulating matrix metalloproteinase-8 (MMP-8) and MMP-9 are increased in chronic periodontal disease and decrease after non-surgical periodontal therapy." Clin Chim Acta 409(1-2): 117-122.

McGregor, D. (2007). "Hydroquinone: an evaluation of the human risks from its carcinogenic and mutagenic properties." Crit Rev Toxicol 37(10): 887-914.

McGuire, M. K. and M. E. Nunn (1999). "Prognosis versus actual outcome. IV. The effectiveness of clinical parameters and IL-1 genotype in accurately predicting prognoses and tooth survival." J Periodontol 70(1): 49-56.

Meng, H., L. Xu, et al. (2007). "Determinants of host susceptibility in aggressive periodontitis." Periodontol 2000 43: 133-159.

Mercado, F. B., R. I. Marshall, et al. (2003). "Inter-relationships between rheumatoid arthritis and periodontal disease. A review." J Clin Periodontol 30(9): 761-772.

Mullally, B. H. and G. J. Linden (1996). "Molar furcation involvement associated with cigarette smoking in periodontal referrals." J Clin Periodontol 23(7): 658-661.

Nakayama, K., T. Kadowaki, et al. (1995). "Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of Porphyromonas gingivalis. Evidence for significant contribution of Arg-gingipain to virulence." J Biol Chem 270(40): 23619-23626.

Nakayama, T., M. Kaneko, et al. (1985). "Cigarette smoke induces DNA single-strand breaks in human cells." Nature 314(6010): 462-464.

Nussbaum, G. and L. Shapira (2011). "How has neutrophil research improved our understanding of periodontal pathogenesis?" J Clin Periodontol 38 Suppl 11: 49-59.

Olson, M. W., M. M. Bernardo, et al. (2000). "Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1." J Biol Chem 275(4): 2661-2668.

Ozcaka, O., N. Bicakci, et al. (2011). "Smoking and matrix metalloproteinases, neutrophil elastase and myeloperoxidase in chronic periodontitis." Oral Dis 17(1): 68-76.

Pabst, M. J., K. M. Pabst, et al. (1995). "Inhibition of neutrophil and monocyte defensive functions by nicotine." J Periodontol 66(12): 1047-1055.

Page, R. C. and H. E. Schroeder (1976). "Pathogenesis of inflammatory periodontal disease. A summary of current work." Lab Invest 34(3): 235-249.

Palmer, L. (2010). Neutrophil extracellular traps in periodontitis. Department of Periodontology. Birmingham University of Birmingham. Ph.D.: 322.

Palmer, R. M., R. F. Wilson, et al. (2005). "Mechanisms of action of environmental factors--tobacco smoking." J Clin Periodontol 32 Suppl 6: 180-195.

Pattamapun, K., S. Tiranathanagul, et al. (2003). "Activation of MMP-2 by Porphyromonas gingivalis in human periodontal ligament cells." J Periodontal Res 38(2): 115-121.

Payne, J. B., G. K. Johnson, et al. (1996). "Nicotine effects on PGE2 and IL-1 beta release by LPS-treated human monocytes." Journal of Periodontal Research 31(2): 99-104.

Permpanich, P., M. J. Kowolik, et al. (2006). "Resistance of fluorescent-labelled Actinobacillus actinomycetemcomitans strains to phagocytosis and killing by human neutrophils." Cell Microbiol 8(1): 72-84.

Phillips, D. H. (2002). "Smoking-related DNA and protein adducts in human tissues." Carcinogenesis 23(12): 1979-2004.

Pickett, K. L., G. J. Harber, et al. (1999). "92K-GL (MMP-9) and 72K-GL (MMP-2) are produced in vivo by human oral squamous cell carcinomas and can enhance FIB-CL (MMP-1) activity in vitro." J Dent Res 78(7): 1354-1361.

Poggi, P., M. T. Rota, et al. (2002). "The volatile fraction of cigarette smoke induces alterations in the human gingival fibroblast cytoskeleton." Journal of Periodontal Research 37(3): 230-235.

Potempa, J., A. Sroka, et al. (2003). "Gingipains, the major cysteine proteinases and virulence factors of Porphyromonas gingivalis: structure, function and assembly of multidomain protein complexes." Curr Protein Pept Sci 4(6): 397-407.

Pussinen, P. J., S. Paju, et al. (2007). "Serum microbial- and host-derived markers of periodontal diseases: a review." Curr Med Chem 14(22): 2402-2412.

Qui, B. S., Q. B. Mei, et al. (2004). "Effects of nitric oxide on gastric ulceration induced by nicotine and cold-restraint stress." World J Gastroenterol 10(4): 594-597.

Quinn, M. T. and K. A. Gauss (2004). "Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases." J Leukoc Biol 76(4): 760-781.

Regev, L., M. Wu, et al. (2012). "Hydroquinone, a benzene metabolite, and leukemia: a case report and review of the literature." Toxicol Ind Health 28(1): 64-73.

Rogers, J. M. (2009). "Tobacco and pregnancy." Reprod Toxicol 28(2): 152-160.

Roman, J., J. D. Ritzenthaler, et al. (2004). "Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling." FASEB J 18(12): 1436-1438.

Ryan, M. E. and L. M. Golub (2000). "Modulation of matrix metalloproteinase activities in periodontitis as a treatment strategy." Periodontol 2000 24: 226-238.

Saremi, A., R. G. Nelson, et al. (2005). "Periodontal disease and mortality in type 2 diabetes." Diabetes Care 28(1): 27-32.

Schenkein, H. A. (2006). "Host responses in maintaining periodontal health and determining periodontal disease." Periodontol 2000 40: 77-93.

Scott, D. A. and J. Krauss (2012). "Neutrophils in periodontal inflammation." Front Oral Biol 15: 56-83.

Sheikhi, M., A. Gustafsson, et al. (2000). "Cytokine, elastase and oxygen radical release by Fusobacterium nucleatum-activated leukocytes: a possible pathogenic factor in periodontitis." J Clin Periodontol 27(10): 758-762.

Shimada, A. L., A. L. Ribeiro, et al. (2012). "In vivo hydroquinone exposure impairs MCP-1 secretion and monocyte recruitment into the inflamed lung." Toxicology 296(1-3): 20-26.

Shultis, W. A., E. J. Weil, et al. (2007). "Effect of periodontitis on overt nephropathy and end-stage renal disease in type 2 diabetes." Diabetes Care 30(2): 306-311.

Sigal, N., Y. Gorzalczany, et al. (2003). "Two pathways of activation of the superoxide-generating NADPH oxidase of phagocytes in vitro--distinctive effects of inhibitors." Inflammation 27(3): 147-159.

Slots, J. and M. Ting (1999). "Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment." Periodontol 2000 20: 82-121.

Smith, C. J., S. D. Livingston, et al. (1997). "An international literature survey of "IARC Group I carcinogens" reported in mainstream cigarette smoke." Food Chem Toxicol 35(10-11): 1107-1130.

Smith, J. A. (1994). "Neutrophils, host defense, and inflammation: a double-edged sword." J Leukoc Biol 56(6): 672-686.

Snyder, R. (2002). "Benzene and leukemia." Crit Rev Toxicol 32(3): 155-210.

Snyder, R. and J. Y. Hong (2004). "Metabolic and biochemical issues in the molecular epidemiology of cancer." IARC Sci Publ(157): 51-69.

Sorensen, L. T., H. B. Nielsen, et al. (2004). "Effect of smoking and abstention on oxidative burst and reactivity of neutrophils and monocytes." Surgery 136(5): 1047-1053.

Sorsa, T., Y. L. Ding, et al. (1995). "Cellular source, activation and inhibition of dental plaque collagenase." J Clin Periodontol 22(9): 709-717.

Sorsa, T., L. Tjaderhane, et al. (2004). "Matrix metalloproteinases (MMPs) in oral diseases." Oral Dis 10(6): 311-318.

Sorsa, T., V. J. Uitto, et al. (1988). "Comparison of interstitial collagenases from human gingiva, sulcular fluid and polymorphonuclear leukocytes." J Periodontal Res 23(6): 386-393.

Stabbert, R., K. H. Schafer, et al. (2003). "Analysis of aromatic amines in cigarette smoke." Rapid Commun Mass Spectrom 17(18): 2125-2132.

Tapper, H. (1996). "The secretion of preformed granules by macrophages and neutrophils." J Leukoc Biol 59(5): 613-622.

Teng, Y. T., J. Sodek, et al. (1992). "Gingival crevicular fluid gelatinase and its relationship to periodontal disease in human subjects." J Periodontal Res 27(5): 544-552.

Theilig, C., A. Bernd, et al. (1994). "Reactions of human keratinocytes in vitro after application of nicotine." Skin Pharmacol 7(6): 307-315.

Tokuda, M., M. Duncan, et al. (1996). "Role of Porphyromonas gingivalis protease activity in colonization of oral surfaces." Infect Immun 64(10): 4067-4073.

Travis, J., R. Pike, et al. (1997). "Porphyromonas gingivalis proteinases as virulence factors in the development of periodontitis." Journal of Periodontal Research 32(1 Pt 2): 120-125.

Valko, M., D. Leibfritz, et al. (2007). "Free radicals and antioxidants in normal physiological functions and human disease." Int J Biochem Cell Biol 39(1): 44-84.

Visse, R. and H. Nagase (2003). "Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry." Circ Res 92(8): 827-839.

Vrsalovic, M., M. M. Vrsalovic, et al. (2007). "Modulating role of alcohol and acetaldehyde on neutrophil and monocyte functions in vitro." J Cardiovasc Pharmacol 50(4): 462-465.

Waddington, R. J., R. Moseley, et al. (2000). "Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases." Oral Dis 6(3): 138-151.

Wahaidi, V. (2010). The systemic inflammatory response to dental plaque. School of Dentistry. Indianapolis Indiana University Ph.D.

Wang, H., X. Liu, et al. (2001). "Cigarette smoke inhibits human bronchial epithelial cell repair processes." Am J Respir Cell Mol Biol 25(6): 772-779.

Williams, R. C. (1990). "Periodontal disease." N Engl J Med 322(6): 373-382.

Williams, R. C., A. H. Barnett, et al. (2008). "The potential impact of periodontal disease on general health: a consensus view." Curr Med Res Opin 24(6): 1635-1643.

Zambon, J. J., S. G. Grossi, et al. (1996). "Cigarette smoking increases the risk for subgingival infection with periodontal pathogens." J Periodontol 67(10 Suppl): 1050-1054.

Zhang, W., M. Fang, et al. (2011). "Effects of cigarette smoke condensate and nicotine on human gingival fibroblast-mediated collagen degradation." J Periodontol 82(7): 1071-1079.

Zhang, W., F. Song, et al. (2009). "Cigarette smoke condensate affects the collagen-degrading ability of human gingival fibroblasts." J Periodontal Res 44(6): 704-713.

Zhang, W., F. Song, et al. (2010). "Effects of tobacco and P. gingivalis on gingival fibroblasts." J Dent Res 89(5): 527-531.

Zhou, J., B. L. Olson, et al. (2007). "Nicotine increases the collagen-degrading ability of human gingival fibroblasts." Journal of Periodontal Research 42(3): 228-235.

Zhou, J. and L. J. Windsor (2006). "Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases." Journal of Periodontal Research 41(1): 47-54.

Zhou, J. and L. J. Windsor (2007). "Heterogeneity in the collagen-degrading ability of Porphyromonas gingivalis-stimulated human gingival fibroblasts." Journal of Periodontal Research 42(1): 77-84.

Zhou, J., J. Zhang, et al. (2012). "Porphyromonas gingivalis promotes monocyte migration by activating MMP-9." J Periodontal Res 47(2): 236-242.

CURRICULUM VITAE

Nouf Khider Al-Shibani

EDUCATION

Ph.D. in Dental Science (2012)

Indiana University, Indianapolis, IN, U.S.A.

M.S.D./Residency in Periodontics (2007)

Indiana University, Indianapolis, IN, U.S.A.

B.D.S. in Dental Sciences (2001)

College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

RESEARCH EXPERIENCE

Ph.D. research

August 2007- September 2012

Indiana University School of Dentistry, Indianapolis, Indiana

Project: Responses of Human Neutrophils to Tobacco Smoke Components.

Advisor: Dr. L. Jack Windsor (Ph.D.)

Masters research

August 2004 - May 2007

Indiana University School of Dentistry, Indianapolis, IN, U.S.A.

Project: Effects of *Porphyromonas gingivalis* on human gingival fibroblasts from healthy and inflamed tissues.

Advisor: Dr. L. Jack Windsor (Ph.D.)

Bachelor reseach

July 1996 - June 2001

College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

Project: A survey of dental anomalies in a group of Saudi population.

Advisor: Dr. Maysara Al-Shawwaf.

ACADEMIC MEMBERSHIPS

- Indiana Section of the American Association for Dental Research
- American Associationfor Dental Research (AADR)
- International Association for Dental Research (IADR)
- Mid-west Society of Periodontology
- Saudi Dental Club

SELECTED PEER REVIEWED PUBLICATIONS

1. Effects of *Calendula officinalis* on human gingival fibroblasts. Saini P, Al-Shibani N, Sun J et al. Homeopathy 2012;101:92-8.

2. Effects of cigarette smoke condensate on oral squamous cell carcinoma cells. Allam E, Zhang W, Al-Shibani N et al. Arch Oral Biol 2011;56:1154-61.

3. Responses of human neutrophils to nicotine and/or Porphyromonas gingivalis. Al-Shibani N, Labban N, Kowolik M et al. J Periodontol 2011;82:1504-1508.

4. Effects of provisional acrylic resins on gingival fibroblast cytokine/growth factor expression. Labban N, Song F, Al-Shibani N et al. J Prosthet Dent 2008;100:390-7.

5. Effects of Porphyromonas gingivalis on human gingival fibroblasts from healthy and inflamed tissues. Al-Shibani N and Windsor LJ. J Periodontal Res;2008:465-70.

CHAPTERS

1. Al-Shibani N, Labban N, Allam E, and Windsor LJ. Tobacco: A Risk Factor for Periodontal Disease. Periodontal Disease: Symptoms, Treatment and Prevention. Sho L Yamamoto. Nova Science Publishers, Inc 2011. Pp 121-135.

2. Allam E, Bottino M, Al-Shibani N, and Windsor LJ. Collagen Scaffolds: Tissue Engineering and Repair. Type I Collagen: Biological Functions, Synthesis and Medicinal Applications. Maria Eduarda and Marcio Pinto. Nova Science Publishers, Inc 2012.

CONFERENCES AND ABSTRACTS

1. Effects of Tobacco Smoke Components on Human Neutrophils. Al-Shibani N, Kowolik M, and Windsor LJ. American Association of Dental Reseach (AADR) 41st Annual Meeting. Tampa FL.

2. Effects of Calendula officinalis on Human Gingival Fibroblasts. Saini P, AL-Shibani N, Jun S et al. American Association of Dental Reseach (AADR) 41st Annual Meeting. Tampa FL.

3. Responses of Tobacco Smoke Components to Human Neutrophils: Part II. Al-Shibani N, Kowolik M, and Windsor LJ. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

4. Resolvin-Dl Blocks the Effects of *Porphyromona*s *Gingivalis* on Human Gingival Fibroblasts. Khaled M, Al-Shibani N, Labban N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

5. Metronidazole-Containing Devices: Effect on the Oxidative Burst of Human Neutrophils. Austin Starr, Al-Shibani N, Kowolik M et al. Indiana Section American Association of Dental Research Indiana University School of Dentistry Research Day April 16th, 2012.

6. Oxidative Burst of Human Neutrophils Induced by Doxycycline-Containing Devices. Hani Ahdab, Al-Shibani N, Kowolik M et al. IUSD research day on April 16, 2012, School of Dentistry, Indiana University.

7. Interactions of Human Endothelial Cells with Streptococcus mutans. Rodenbeck J, Gregory RL, Al-Shibani N et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

8. The Effects of Tobacco Treated *Porphyromonas gingivalis* on human epithelial cells. Tursunova R, Al-Shibani N, Windsor LJ et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

9. Interactions of Human Gingival Fibroblasts with Tobacco Treated *Porphyromonas gingivalis.* Lanier B, Al-Shibani N, Windsor LJ et al. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day April 16th, 2012.

10. Responses of Tobacco Smoke Components to Human Neutrophils: Part I. Al-Shibani N, Kowolik M, and Windsor LJ. Indiana Section American Association of Dental Research / Indiana University School of Dentistry Research Day, 2011.

11. Effects of Porphyromonas gingivalis on human gingival fibroblasts from healthy and inflamed gingival. Al-Shibani N and Windsor LJ. Hinman Research Symposium. The University of Tennessee, Memphis TN. 2008.