ผลกระทบของสารสกัดจากบุหรี่ต่อการตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์เมื่อกระตุ้นด้วยลิโพพอลิแซ็กคาไรด์ของพอร์ไฟโรโมแนสจิงจิวาลิส

นางสาวปารณีย์ ไพรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MODULATING EFFECT OF CIGARETTE SMOKE EXTRACT ON THE RESPONSE OF HUMAN CORONARY ARTERY ENDOTHELIUM TO *PORPHYROMONAS GINGIVALIS* LPS

Miss Bharani Phairat

A Thesis Submitted in Partial Fulfillment of the Requirements

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ปารณีย์ ไพรัตน์ ผลกระทบของสารสกัดจากบุหรี่ต่อการตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์เมื่อกระตุ้นด้วยลิโพพอลิแซ็กคาไรด์ของพอร์ไฟโรโมแนสจิงจิวาลิส. (MODULATING EFFECT OF CIGARETTE SMOKE EXTRACT ON THE RESPONSE OF HUMAN CORONARY ARTERY ENDOTHELIUM TO *PORPHYROMONAS GINGIVALIS* LPS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. รังสินี มหานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.สาธิต พิชญางกูร, 60 หน้า.

บุหรี่เป็นปัจจัยเสี่ยงที่สำคัญของโรคปริทันต์และโรคหลอดเลือดแดงแข็ง อย่างไรก็ดียังไม่มีการศึกษาถึงบทบาทของบุหรี่และเชื้อก่อโรคปริทันต์ต่อโรคหลอดเลือดแดงแข็ง ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลกระทบของสารสกัดจากบุหรี่ต่อการตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์เมื่อกระตุ้นด้วยโทลล์ไลค์รีเซพเตอร์ไลแกน 2-ลิโพพอลิแซ็กคาไรด์ของพอร์ไฟโรโมแนสจิงจิวาลิส *(P.gingivalis* LPS) โดยประเมินจากระดับของอินเตอร์ลิวคิน-8 (IL-8) และ โมโนไซต์คีโมแอทแทรกแทนท์โปรตีน-1 (MCP-1) ในห้องปฏิบัติการ การแสดงออกของโทลล์ไลค์รีเซพเตอร์ไลแกนวัดโดยวิธีรีเวอร์สทรานส์คริปเตส-พอลิเมอเรส (reverse transcriptase-polymerase chain reaction) การตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์เมื่อถูกกระตุ้นด้วยโทลล์ไลค์รีเซพเตอร์ไลแกน และ/หรือ ทูเมอร์เนโครซิสแฟคเตอร์ อัลฟ่า (TNF-α) รวมถึงสารสกัดจากบุหรี่ วัดจากระดับ IL-8 และ MCP-1 โดยวิธีอีไลซา (ELISA) ซึ่งพบว่าเซลล์หลอดเลือดหัวใจมนุษย์มีการแสดงออกของเมสเซนเจอร์อาร์เอนเอ (mRNA) ของโทลล์ไลค์รีเซพเตอร์ 1, 2, 3, 4, 5, 6, 9 และ 10 แต่ไม่พบการแสดงออกของเมสเซนเจอร์อาร์เอนเอของโทลล์ไลค์รีเซพเตอร์ 7 และ 8 เมื่อกระตุ้นเซลล์หลอดเลือดหัวใจมนุษย์นี้ด้วยไลแกนของโทลล์ไลค์รีเซพเตอร์ 2, 3, 4, 5 พบว่าสามารถทำให้ระดับ IL-8 และ MCP-1 เพิ่มขึ้น นอกจากนี้เมื่อกระตุ้นเซลล์ร่วมกันระหว่าง *P. gingivalis* LPS และ TNF-α พบว่า สามารถกระตุ้นให้เซลล์หลอดเลือดหัวใจมนุษย์ผลิต IL-8 มากขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับการกระตุ้นด้วย *P. gingivalis* LPS หรือ TNF-α อย่างเดียว ($p<0.05$) ในขณะที่ผลต่อการผลิต MCP-1 ค่อนข้างน้อย และสารสกัดจากบุหรี่เพียงอย่างเดียวกระตุ้นให้เซลล์หลอดเลือดหัวใจมนุษย์มีการผลิต IL-8 เพิ่มขึ้น ในขณะที่ผลิต MCP-1 ลดลง อย่างไรก็ตามสารสกัดจากบุหรี่จะยับยั้งการตอบสนองเซลล์หลอดเลือดหัวใจมนุษย์เมื่อถูกกระตุ้นด้วย *P. gingivalis* LPS โดยผลิต IL-8 และ MCP-1 ลดลง ($p<0.05$) ดังนั้นการศึกษานี้แสดงให้เห็นถึง การแสดงออกของโทลล์ไลค์รีเซพเตอร์ของเซลล์หลอดเลือดหัวใจมนุษย์ที่สามารถตอบสนองต่อการกระตุ้นด้วยโทลล์ไลค์รีเซพเตอร์ไลแกน และพบว่าการกระตุ้นด้วยสารสกัดจากบุหรี่ ร่วมกับ *P. gingivalis* LPS แทนที่จะเพิ่มการตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์โดยการผลิตไซโตไคน์เพิ่มขึ้น แต่ผลการศึกษาพบว่าการผลิตของ IL-8 และ MCP-1 ลดลงเมื่อกระตุ้นร่วมกัน อย่างไรก็ตามเป็นที่น่าสนใจว่าสารสกัดจากบุหรี่เพียงอย่างเดียวจะเพิ่มการตอบสนองของเซลล์หลอดเลือดหัวใจมนุษย์โดยการผลิต IL-8 เพิ่มขึ้น ดังนั้นความสัมพันธ์ระหว่าง *P. gingivalis* LPS สารสกัดจากบุหรี่ และเซลล์หลอดเลือดหัวใจมนุษย์ ที่แสดงถึงความเชื่อมโยงระหว่างโรคปริทันต์ การสูบบุหรี่และโรคหลอดเลือดแดงแข็ง มีลักษณะซับซ้อนซึ่งต้องการการศึกษาต่อในอนาคต

ภาควิชา .....ปริทันตวิทยา..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ปริทันตศาสตร์..... ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEY WORD: ATHEROSCLEROSIS / TOLL-LIKE RCEPTORS / CIGARETTE SMOKE EXTRACT / ENDOTHELIAL CELLS / PERIODONTITIS

BHARANI PHAIRAT: MODULATING EFFECT OF CIGARETTE SMOKE EXTRACT ON THE RESPONSE OF HUMAN CORONARY ARTERY ENDOTHELIUM TO *PORPHYROMONAS GINGIVALIS* LPS. THESIS PRINCIPAL ADVISOR: ASSO. PROF. RANGSINI MAHANONDA, Ph.D, THESIS CO-ADVISOR : SATHIT PICHYANGKUL, Ph.D, 60 pp.

Cigarette smoking is well known as a strong common risk factor of periodontitis and atherosclerosis. To the best of our knowledge, no previous studies have assessed the role of smoking and periodontal infection in atherosclerosis. *Objectives:* In this study, we used an *in vitro* model of human coronary artery endothelial cell (HCAEC) culture to investigate the immune modulating effects of cigarette smoke extract (CSE) on the response of HCAECs to a Toll-like receptor(TLR) 2 ligand-*Porphyromonas gingivalis* lipopolysaccharide(LPS) as assessed by measurement of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) production. TLR expression of HCAECs was analyzed by RT-PCR. The responses of HCAECs to specific-purified TLR ligand and/or tumor necrosis factor-alpha (TNF-α) were measured by IL-8 and MCP-1 production using ELISA. The effect of CSE on stimulated HCAECs was also evaluated. The results showed that HCAECs expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, 9 and 10, but not of TLRs 7 or 8. Stimulation of HCAECs with highly purified TLR2, 3, 4, 5 ligands led to IL-8 and MCP-1 production. Enhancement of IL-8 was observed in HCAECs after combined stimulation with *P.gingivalis* LPS and TNF-α, as compared with single stimulation ($p<0.05$), but the effect on MCP-1 production was minimal. CSE alone stimulated IL-8 response, whereas suppressed MCP-1 production in HCAECs. However, CSE markedly inhibited *P.gingivalis*-induced IL-8 and MCP-1 production. *Conclusion:* HCAECs expressed functional TLRs. Our results indicated that instead of enhancing cytokine response, the combination of CSE and *P.gingivalis* LPS suppressed IL-8 and MCP-1 production from HCAECs ($p<0.05$). However, it is interesting that CSE alone could promote HCAEC response by increasing IL-8 production. Such complex interaction between *P.gingivalis* LPS, CSE and the HCAEC response which links between periodontitis, smoking and atherosclerosis, needs further investigation.

Department ……..Periodontology…….. Student's signature.....……..……

Field of study……Periodontics…… Principal Advisor's signature……..

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
CpG ODN	cytidine-phosphate-guanosine oligonucleotide
C. pneumoniae	*Chlamydia pneumoniae*
CSE	cigarette smoke extract
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3–phosphate dehydrogenase
HBD-2	human β-defensin-2
HCAECs	human coronary artery endothelial cells
HDL	high-density lipoprotein
hEGF	human recombinant epidermal growth factor
hFGF-B	human fibroblast growth factor–basic with heparin
HGECs	human gingival epithelial cells
H. pylori	*Helicobacter pylori*
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
LDL	low-density lipoprotein
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MCP-1	monocyte chemoattractant protein-1
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MyD88	myeloid differentiation primary-response protein 88

NHANES	national health and nutrition examination survey
P. gingivalis	*Porphyromonas gingivalis*
PAMPs	pathogen–associated molecular patterns
PBMC	peripheral blood mononuclear cells
PGE_2	prostaglandin E_2
PMN	polymorphonuclear leucocytes
Poly I:C	polyinosine-polycytidylic acid
R3-IGF-1	human recombinant insulin-like growth factor
RNA	ribonucleic acid
RT-PCR	reverse transcriptase–polymerase chain reaction
SEM	standard error of mean
ssPolyU	single strand poly-uridine
S. typhimurium	*Salmonella typhimurium*
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
TLRs	Toll–like receptors
TNF	tumor necrosis factor
VCAM1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

CHAPTER I

INTRODUCTION

1.1 Background of present study

Periodontal disease is recognized as one of the global oral health problems and generally perceived as a non-life threatening disease. It is caused by bacterial infection which affects the tissue surrounding the teeth. Periodontitis, an advanced form, is very common in adults. It destroys deeper periodontal tissues and bone, and in severe cases lead to tooth loss (Williams, 1990). Atherosclerosis is a major component of cardiovascular disease, a well-known leading cause of death in the western society and predicted to be the major killer among the global population in 2020 (Murray and Lopez, 1997). Atherosclerosis involves thickening or hardening of the walls of large and medium-sized arteries which is caused by formation of atheromatous plaque. Its deadly end-stage complications or events include coronary thrombosis, acute myocardial infarction and stroke. **Interestingly, what periodontitis and atherosclerosis have in common is that both being a chronic inflammatory disease and it is likely to be contributed by the exaggerated activation of innate immune response** followed by adaptive immune response (Gibson et al., 2006). In addition, periodontitis patients share many of the same risk factors as atherosclerosis patients including age, gender, lower socioeconomic status, stress, and smoking (Paquette et al., 2007).

Innate immune system plays a critical role as the first line of defense. It recognizes invading pathogens via pattern recognition receptors such as Toll-like receptors (TLRs). On the other hand, an exaggerated activation of innate immune responses can lead to inflammatory disease (Meneghin and Hogaboam, 2007). Cells of periodontal tissues are known to express a variety of TLRs (Mahanonda and Pichyangkul, 2007). TLR triggering by bacterial plaque organisms in periodontal cells

including epithelium and fibroblasts as well as PMNs play an integral role in periodontal homeostasis and disease (Mahanonda et al., 2007). Once the epithelial barrier is breached and invaded by gram negative periodontopathic bacteria, persistent and excessive activation of innate immune response in the connective tissue coupled with the responses from adaptive immunity would lead to destruction of periodontal tissue and bone. Lesion of periodontitis consists of dense inflammatory infiltrates of activated macrophages, mast cells, T and B cells and high levels of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-8, IL-17, prostaglandin E_2 (PGE_2), and matrix metalloproteinases(MMPs) (Page et al., 1997). Like periodontitis, activation of the innate immune system via TLR triggering has been suggested to contribute to the development and progression of atherosclerosis (Frantz et al., 2007; Oude Nijhuis et al., 2007). In humans, atherosclerotic plaques contain a large number of foam cells (lipid-laden macrophages) and activated T cells, as well as vascular endothelial cells, smooth muscle cells, extracellular matrix, lipids and acellular lipid-rich debris. In addition, increased cytokine and chemokine expression such as TNF-α and monocyte chemoattractant proteint-1 (MCP-1) have also been detected (Hansson and Libby, 2006; Nelken et al., 1991; Takeya et al., 1993). Recent observations demonstrate TLR expression in vascular as well as myocardial cells (Frantz et al., 2007). Up-regulation of TLR2, and TLR4 expression has been observed in atherosclerotic lesion as compared to normal vascular tissue (Edfeldt et al., 2002). Decreased atherosclerosis development was demonstrated in *in vivo* TLR4 knockout mice (Michelsen et al., 2004). Epidemiologic studies indicate that bacterial infection and atherosclerosis are connected, and have suggested there could be a link between atherosclerosis and the activation of the primary innate immune receptors -TLRs.

Recent clinical and epidemiologic studies suggest modest association between periodontitis and cardiovascular diseases (Paquette et al., 2007). Low-grade chronic systemic inflammation from distant site such as periodontitis, may be linked to pathogenesis of atherosclerosis. ***Porphyromonas gingivalis***, ***Aggregatibacter actinomycetemcomitans***, ***Tannerella forsythia,*** **the key periodontopathic bacteria, were found in patients' atheroma** (Haraszthy et al., 2000). Data from *in vitro* and *in vivo*

experimental animal models have shown that these pathogens, which act as TLR ligands, promote up-regulation of endothelial adhesion molecules (vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM-1), proinflammatory cytokines and chemokines (IL-8, MCP-1) production, platelet aggregation, foam-cell formation, and the development and aggravation of atheroma (Cybulsky and Gimbrone,1991, Gibson et al., 2006). Recently, intensive periodontal intervention trials by removing all the bacterial loads in severe periodontitis patients resulted in periodontal health and at the same time improvement in endothelial function (reducing the risk of atherosclerosis) (Tonetti et al., 2007). Hence, periodontal infection may link with vascular lesion of atherosclerosis by affecting the innate immune response of the vessels, to include endothelial cells.

Cigarette smoking is well recognized as a common as well as important risk factor for both periodontitis and atherosclerosis (Hujoel et al., 2002). It can alter cell function and promote the development and severity of both diseases (Laan et al., 2004; Masubuchi et al., 1998; Oltmanns et al., 2005; Su et al., 1998; Vassallo et al., 2005). Recent observation in our laboratory demonstrated the immune modulating effect of cigarette smoke extract (CSE) on human gingival epithelial cells, the initial barrier of periodontal tissues. The CSE suppressed protective function of HBD-2 production from human gingival epithelial cells (HGECs) in response to LPS derived from periodontopathic bacteria-*P. gingivalis* (TLR2 ligand) and TNF-α. Whereas the CSE enhanced inflammatory responses by increasing pro-inflammatory IL-8, therefore promoting susceptibility to periodontal disease. To the best of our knowledge, no previous studies have assessed the role of smoking and periodontal infection in atherosclerosis. In this study, we will use an *in vitro* model of human coronary artery endothelial cell (HCAEC) culture to investigate the immune modulating effect of CSE on the response of HCAECs to a TLR2 ligand-*P.gingivalis* LPS as assessed by measurement of IL-8 and MCP-1 production.

1.2 Objectives

We investigated the modulating effect of cigarette smoke extract on the response of HCAECs to *P. gingivalis* LPS. The specific aims of the present study were

1.2.1 To investigate a full panel of TLR expression (TLR: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) on HCAECs.

1.2.2 To determine functional TLRs of HCAECs after stimulation with specific ligands and measure IL-8 and MCP-1.

1.2.3 To determine the effect of CSE on IL-8 and MCP-1 production from *P. gingivalis* LPS-stimulated HCAECs.

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1.3 Hypothesis

1.3.1 HCAECs express a variety of TLRs and produce IL-8 and MCP-1 upon TLR ligation, TNF-α, or the combination of *P. gingivalis* LPS and TNF-α stimulation.

1.3.2 Cigarette smoke extract modulates function of stimulated HCAECs by increasing IL-8 and MCP-1 production.

1.4 Field of research

Investigation the TLR expression of HCAECs and IL-8 and MCP-1 production in response to TLR ligands, cytokine and CSE.

1.5 Criteria inclusions

1.5.1 Primary human coronary artery endothelial cells were purchased from Clonetics.

1.5.2 Peripheral blood mononuclear cells (PBMC), a positive control for TLR expression, were obtained from previous DNA collection of our laboratory.

1.5.3 Analysis of TLR expression (TLRs 1-10) was determined by reverse transcriptase polymerase chain reaction (RT-PCR).

1.5.3 The responses of HCAECs to different TLR ligands, TNF-α, the combination of *P. gingivalis* LPS and TNF-α, and CSE (IL-8 and MCP-1 production) were measured by ELISA.

1.6 Limitation of research

This study cannot investigate the innate response of other types of cardiovascular blood vessels such as aorta due to high expenses.

1.7 Application and expectation of research

1.7.1 New scientific information: how CSE and periodontal pathogen affect the innate immune response of human endothelial cells. A better understanding about the link between cigarette smoking and oral diseases such as periodontitis and cardiovascular disease.

1.7.2 Publication in the international peered-reviewed journal.

1.7.3 The experimental data would be applicable for the anti-cigarette smoking campaign.

1.8 Keywords

Atherosclerosis, Periodontal disease, Cigarette smoke extract, Endothelial cells, Toll-like receptor

CHAPTER II

LITERATURE REVIEW

2.1 Periodontitis

Periodontitis is a chronic bacterial infection of tooth supporting structures. It causes destruction of periodontal connective tissue and bone, in severe cases, tooth loss. Gram negative oral bacteria including *P. gingivalis, A. actinomycetemcomitans*, and *T. forsythia* are recognized as etiologic agents in periodontitis. The disease initiation and progression results from host response to dental plaque bacteria.

Innate immune system plays a critical role as the first line of defense. It recognizes invading pathogens via pattern recognition receptors such as TLRs. Cells of periodontal tissues are known to express a variety of TLRs (Mahanonda and Pichyangkul, 2007). TLR triggering by bacterial plaque organisms in periodontal cells including epithelium and fibroblasts as well as PMNs play an integral role in periodontal homeostasis and disease (Mahanonda et al., 2007). Once the epithelial barrier is breached and invaded by gram negative periodontopathic bacteria, persistent and excessive activation of innate immune response in the connective tissue coupled with the responses from adaptive immunity would lead to destruction of periodontal tissue and bone. Lesion of periodontitis consists of dense inflammatory infiltrates of activated macrophages, mast cells, T and B cells. In addition high levels of inflammatory mediators such as TNF-α, IL-1, IL-8, IL-17, PGE_2, and MMPs can be detected in inflamed gingival tissues and gingival crevicular fluid (Seymour, 1991; Page et al., 1997). Like other infectious diseases, periodontitis is multifactorial in nature. Genetics and environmental factors such as stress and smoking can influence disease initiation and severity (Page et al., 1997).

Cigarette smoking is a well-known risk factor for periodontal disease that has been investigated extensively in both cross-sectional and longitudinal studies (Ah et al., 1994; Bergstrom, 1989; Haber et al., 1992, 1993, 1994; Preber et al., 1992; Sopori, 2002; Tonetti, 1998). A meta-analysis of six studies concluded that smokers are almost three times as likely to have severe periodontitis compared to non-smokers (Papapanou, 1996). The most recent and largest epidemiological study, the National Health and Nutrition Examination Survey (NHANES) III, showed that after adjusting for age, race or ethnicity, income and educational level, current smokers were four times as likely to have periodontitis as compared to non-smokers (Tomar et al., 2000). In 12 month-longitudinal study, smokers were shown to be at a significantly greater risk for further attachment loss when compared to non-smokers, the odd ratio was 5.4 (Machtei et al., 1997). When a group of light smokers were compared to that of heavy smokers in relation to periodontal attachment loss, the odds for severe attachment loss were ranged from 2.05 to 4.75 for light and heavy smokers respectively and those for severe bone loss from 3.25 to 7.28 (Grossi et al., 1994, 1995).

2.2 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease affecting large and medium-sized arterial blood vessels. It is a major disease of cardiovascular. The atherosclerotic lesions (also known as atherosclerotic plaque) typically present as asymmetrical thickenings of the intima (the innermost layer of the artery) which contain vascular endothelial cells, immune cells (mainly macrophages and T cells), smooth muscle cells, extracellular matrix, lipids and acellular lipid-rich debris. Fatty streaks are early stage of plaque formation with accumulation of lipid droplets and immune cells, particularly a large number of lipid-laden macrophages, known as foam cells. Fatty streaks are prevalent in young individuals, may never have symptoms and disappear with time, or they may progress into mature atherosclerotic plaques with more complex structure. Mature plaque can cause clinical complications such as flow-limiting stenoses, but the most severe life-threatening clinical events, including myocardial infarction, ischemic

stroke, and aneurysms, follow the rupture of a plaque and thrombosis (Hansson and Libby, 2006; Shah, 2003).

Traditional risk factors for atherosclerosis include cigarette smoking, hypertension (>140/90 mmHg), high low-density lipoprotein cholesterol (100 mg/dl), low high-density lipoprotein cholesterol (<40 mg/dl), diabetes mellitus, family history of premature coronary heart disease, age (men>45 years, women >55 years), obesity (body mass index >30 kg/m2), physical inactivity and an atherogenic diet (Greenland et al., 2003; Smith, 2006). However, about 50% of the atherosclerosis patients lack these identified factors and despite continued preventive efforts addressing modifiable risk factors, mortality rates from cardiovascular disease remain virtually unchanged over the past decade in develop countries (Vita and Losclazo, 2002). What triggers and accelerates this life threatening disease is not as yet fully understood (Ross, 1999). Current understanding of this chronic inflammatory disease reveals intense immunological activity which participates in every phase of the disease. Like periodontitis, activation of the innate immune system via TLR triggering has been suggested to contribute to the development and progression of atherosclerosis (Frantz et al., 2007; Oude Nijhuis et al., 2007). Recent observations demonstrate TLR expression in vascular as well as myocardial cells (Frantz et al., 2007). Up-regulation of TLR2 and TLR4 expression has been observed in atherosclerotic lesion as compared to normal vascular tissue (Edfeldt et al., 2002). Decreased atherosclerosis development was demonstrated in *in vivo* TLR4 knockout mice. (Michelsen et al., 2004).

Cigarette smoking is reported to be associated with average 70% increase in the risk of death from coronary artery disease (United States Surgeon General, 1983). Smoking also nearly doubles the risk of ischemic stroke (Shinton and Beevers, 1989). It has been hypothesized that smoking may accelerate the pathogenesis of atherosclerosis through a number of different mechanisms, which collectively contribute to increased risk. For example, smoking was shown to reduce antioxidant vitamins, creating a proatherogenic lipoprotein profile (elevated levels of LDL and VLDL, and a decrease in HDL), and augmenting monocyte adherence to endothelial cells (Craig et

al., 1989; Dovgan et al., 1994; Taylor et al., 1998). The constituents in cigarette smoke were able to induce oxidative stress, thus increasing endothelium dysfunction (Taylor et al., 1998) and resulting in the enhancement of blood monocyte/macrophage migration into sites susceptible to atherosclerosis. Exposure to oxidized LDL in the intima could promote macrophage to transform into foam cells (Taylor et al., 1998). A recent study evaluated from 130 autopsies demonstrated that smoking associated with a formation of preatherosclerotic intima hyperplasia, the early phase of lesion development (Cizek et al., 2007). Moreover, smoking has been reported to increase platelet activation, fibrinogen levels and blood viscosity, resulting in a pro-coagulant condition (Taylor et al., 1998).

2.3 Association between periodontitis and atherosclerosis

Compiling evidence demonstrate association between periodontitis and atherosclerosis (Beck et al., 1996, 1998, 2001; Hung et al., 2004; Khader et al., 2004; Meurman et al., 2004; Vettore et al., 2004). A large proportion of patients with periodontal disease exhibit cardiovascular disease (Umino and Nagao, 1993). Both types of patients share many of the same risk factors including age, gender (predominantly male), low socioeconomic status, stress, and smoking (Beck et al., 1998). And both diseases also have in common a characteristic of chronic inflammation, a disease hallmark.

Among worldwide population studies, consistent observations and meta-analyses indicate modest but statistically significant increase in the risk of cardiovascular disease with periodontitis (Abnet et al., 2005; Beck et al., 1998, 2001; Hung et al., 2004; Khader et al., 2004; Meurman et al. 2004; Vettore et al., 2004). For example, in the Atherosclerosis Risk in Communities study and the Health Professional Follow-up Study, clinical signs of periodontitis such as clinical attachment loss and number of tooth loss are associated with clinical cardiovascular disease and subclinical atherosclerosis (carotid artery intima-media wall thickness using B-mode ultrasound) (Beck et al., 2001; Elter et al., 2004; Hung et al., 2004). The odd ratios for periodontitis

with a high attachment loss (>=10% of sites with attachment loss > 3mm) and coronary artery heart disease was 1.5 (95% CI 1.1-2.0) and 1.8 (95% CI 1.4-2.4) for periodontitis with high tooth loss and coronary artery heart disease (Elter et al., 2004). Regarding stroke, high risk ratio varying from 2.85 (95% CI 1.78-4.56) to 1.74 (95% CI 1.08-2.81) was reported among periodontitis patients in a meta-analysis study (Meurman et al., 2004). In addition, periodontitis was found to be a principal independent predictor of carotid atherosclerosis with an odds ratio of 4.64 (95% CI 1.64-13.10) (Soder et al., 2005). Exposure to periodontal pathogens significantly increase risk for atherosclerosis. Patients with seropositive to *A. actinomycetemcomitans* and *P. gingivalis* showed an odds ratio of 2.6 (95% CI 1.0-7) for a secondary stroke (Pussinen et al., 2004).

How periodontitis influences initiation/progression of atherosclerosis remains to be explained. Like other infections such as *Chlamydia pneumoniae*, cytomegalovirus, herpes simplex virus*, Helicobacter pylori*, periodontal pathogens are candidates for triggering and perpetuating the inflammatory responses of the arterial wall (O'Connor et al. 2001). The nature of periodontal infections result in low-grade bacteremias and endotoxemias in affected patients (Sconyers et al., 1973; Silver et al., 1977). Dental treatment could also contribute to bacteremia (Messini et al.,1999). In atherosclerosis patients, periodontal pathogens such as *P. gingivalis, A. actinomycetemcomitans, T. forsythia, Treponema denticola* were identified in atherosclerotic lesions using DNA techniques (PCR and fluorescence *in situ* hybridization) (Cavrini et al., 2005; Fiehn et al., 2005; Haraszthy et al., 2000; Okuda et al., 2001; Padilla et al., 2006; Pucar et al., 2007). Live *A. actinomycetemcomitans* could be cultured from both atherosclerotic plaque and subgingival plaque of the same patient (Padilla et al., 2006). This detection of live periodontal pathogen therefore suggests the effect on vascular pathology via the bacterial exposures appear biologically plausible.

Among several periodontal pathogens, *P. gingivalis* has been the key periodontal pathogen that mostly studied regarding their virulence factors and association with atherosclerosis (Gibson et al., 2006; Paquette et al., 2007). This organism can induce platelet aggregation by secreting collagen-like platelet

aggregation associated proteins as demonstrated *in vitro* and *in vivo* studies (Herzberg and Meyer, 1996, 1998). This ability may involve the development of thrombosis. Besides being molecularly identified in atherosclerotic plaque, it was able to invade and replicate in aortic and heart endothelial cells, thus suggesting a direct mechanism by which this microorganism may contribute to the development and/or progression of disease (Deshpande et al., 1998). When macrophages were incubated with *P. gingivalis* and low-density lipoprotein, the bacteria potentially induced the uptake by the macrophages and led to foam cell transformation (Giacona et al., 2004). Further support for the role of *P. gingivalis* in atherosclerosis comes from animal models using $Apoe^{-/-}$ mice lack of apolipoprotein E (genetically hyperlipidemic mice). Oral challenge with invasive strains of *P. gingivalis* in apolipoprotein-E-deficient mice resulted in *P. gingivalis* bacteremia and localization in aortic tissue. These mice subsequently exhibited increased aortic atherosclerosis (Chi et al., 2004; Gibson et al., 2004; Lalla et al., 2003; Li et al., 2002). In pigs with normal cholesterol after *P. gingivalis* challenges, the animals developed both coronary and aortic lesions (Brodala et al.,2005). This implies that *P. gingivalis* bacteremias may exert an atherogenic stimulus independent of the high cholesterol levels.

2.4 Endothelial cell response

The endothelium is located at the interface between circulating blood and the surrounding tissues and serves a multitude of functions that help to maintain organ homeostasis, including vasoregulation, vascular permeability, provision of an anticoagulant surface (Grandel and Grimminger, 2003; Hippenstiel and Suttorp, 2003; Jaffe, 1987). In physiological condition, endothelium does not generally support the binding of leukocytes. In early atherosclerotic plaque formation (animal model), endothelial cells play a critical role in the innate immune response, expressing adhesion molecules in responses to cholesterol accumulation in the intima (the innermost layer of vessel). These molecules are VCAM-1 and ICAM-1 which bind to monocytes and lymphocytes (Davies et al., 1993; Kerr, 1999; O'Brien et al., 1993). They are not expressed on un-stimulated endothelial cells (Bevilacqua et al., 1989) and their

expressions are transient after stimulation (Bevilacqua et al., 1994). Cytokines such as TNF-α, IL-1, oxidized LDL, bacterial infection such as *Chlamydia, P. gingivalis* were able to activate endothelial cells to express adhesion molecules and cytokines as well as chemokines including IL-8, MCP-1 and macrophage colony-stimulating factor (M-CSF) (Khlgatian et al., 2002; Nassar et al., 2002; Price and Loscalzo, 1999; Tedgui and Mallat, 2006). Interleukin-8, a C-X-C chemokine released by many cell types, exerts diverse biological properties such as chemotaxis of neutrophils and lymphocytes (Baggiolini et al., 1989), regulation of cell adhesion (Oppenheim et al., 1991) and activation of neutrophils (Huber et al., 1991). MCP-1, a member of the CC chemokine family, attracts and activates blood monocytes (Baggiolini et al., 1997; Mantovani et al., 1997). It recruits monocytes into the subendothelial layers of the vessel wall (Nelken et al., 1991; Takeya et al., 1993). Abundant amounts of IL-8 as well as MCP-1 have been detected in atherosclerotic lesions (Djeu et al., 1990; Simonini et al. 2000; Wang et al. 1996). The increased expression of MCP-1 mRNA has been noted in endothelial cells, macrophages, and vascular smooth muscle cells of human atherosclerotic arteries (Seino et al., 1995; Yla-Herttula et al., 1991). In addition, IL-8 mRNA was expressed in foam cells of human atherosclerotic lesion (Wang et al. 1996). Recent studies in MCP-1-deficient (Gu et al., 1998) and MCP-1 receptor-deficient (Boring et al., 1998) mice have demonstrated decreased numbers of macrophages and reduced development of atherosclerotic lesions.

Shortly after adhesion molecule and chemokine induction, immune cells are recruited and populated in subendothelial layer containing smooth muscle cells, collagen fibers and lipids. Most of these cells are activated cells and produce cytokines, proteases, prothrombic molecules and vasoactive substances, all of which can affect plaque inflammation and vascular function. Without complications, an intact endothelium covers the plaque. If the complications occur, atherosclerotic lesions may result in episodes of plaque rupture and superficial endothelial erosion leading to thrombus formation, which is the proximate event responsible for acute ischemic syndromes.

2.5 TLR and endothelial function

Inflammation plays a continuous role from endothelial cell expression of adhesion molecules to the development of the fatty streak, established plaque, and finally plaque rupture. TLRs, a key receptor of the innate immune response, recognize and distinguish highly conserved structures present in large groups of microorganisms known as pathogen-associated molecular patterns (PAMPs) (Takeda and Akira, 2005). To date 10 TLRs in humans and 12 TLRs in mice have been described (Beutler et al., 2004). Each of the 10 known human TLRs is believed to respond to a distinctive aspect of microbial infection. As specificity for broad categories of PAMPs is provided by a relatively limited diversity of TLRs, in some cases combinations of TLRs are required for recognition of certain PAMPs (Hajjar et al., 2001; Ozinsky et al., 2000). For example, TLR2 forms heterophilic dimers with TLR1 and TLR6 to recognize diacyl and triacyl lipopeptides, respectively (Takeda and Akira, 2005). Many other human TLRs and their ligands are known as shown in table 1. For example TLR2 recognizes peptidoglycan, TLR3 recognizes viral double-stranded RNA, TLR4 recognizes LPS, TLR5 recognizes flagellin, TLR7 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial DNA. It should be noted that cell surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) seem to recognize microbial products whereas intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize nucleic acids. (Table 1)

Table 1: Human Toll-like receptors and their ligands

Receptor	Ligand	References
TLR1	Triacyl lipopeptides	Takeuchi et al.,2002
TLR2	Lipoprotein/lipopeptides	Aliprantis et al., 1999
	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Porphyromonas gingivalis lipopolysaccharide	Hirschfeld et al., 2001
	Porphyromonas gingivalis fimbriae	Asai et al., 2001
	Mycobacterial lipoarabinomannan	Means et al., 1999
	Zymosan	Underhill et al., 1999
TLR3	Double-stranded RNA	Alexopoulou et al., 2001
	Polyinosine-polycytidylic acid	Alexopoulou et al., 2001
TLR4	*Escherichia coli* Lipopolysaccharide	Tapping et al., 2000
	Porphyromonas gingivalis LPS	Darveau et al., 2004
TLR5	Flagellin	Gewirtz et al., 2004
TLR6	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Diacyl lipopeptides	Takeuchi et al.,2002
	Zymosan	Ozinsky et al., 2000
TLR7	Imidazoquinoline	Hemmi et al., 2002
TLR8	Single-stranded RNA	Heil et al., 2004
	Imidazoquinoline	Jurk et al., 2002
TLR9	Bacterial DNA	Bauer et al., 2001
	CpG Oligonucleotides	Hemmi et al.,2000
TLR10	N.D.	

N.D. = not determined

Human vascular endothelial cells express a variety of TLRs, TLR1, 2, 3, 4, 5, and 9 (Bulut et al., 2002; Dunzendorfer et al., 2004; Faure et al., 2000; Faure et al., 2001; Frantz et al., 1999; Li et al., 2004; Loos et al., 2006;. Maaser et al., 2004; Opitz et al., 2006, Tissari et al., 2005). Due to their strategic location, endothelial cells continually

expose to blood-born pathogens and sense pathogen invasion via TLRs, a critical process of pathogen elimination. Binding between PAMPs and TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate inflammatory responses including release of cytokines and chemokines and upregulation of cell adhesion molecules. Activation of innate immunity further instructs the development of antigen-specific acquired immunity. Current information suggests that TLR signaling pathways are divergent, although Myeloid differentiation primary-response protein 88 (MyD88), a key adaptor molecule, is used by most TLRs and this signaling pathway is called MyD88-dependent. Stimulation of TLR3 and some TLR4 utilize different adaptor molecules and the signaling pathway is called MyD88-independent pathways (Takeda and Akira, 2005).

It may be postulated that a link between atherosclerosis and periodontitis involve activation of these primary innate immune receptors-TLRs of the vascular endothelial cells by bacterial PAMPs of periodontal infection, leading to/promoting inflammatory events in atherosclerosis. Gibson et al. (2004) reported that TLR2 and TLR4 were up-regulated on the surfaces of HCAECs challenged with invasive strains of live *P. gingivalis*. *In vitro* HCAEC cultured with *P. gingivalis* produced high levels of adhesion molecules (ICAM-1, VCAM-1, and selectin) (Gibson et al., 2006; Khlgatian et al., 2002) as well as IL-8 and MCP-1 (Gibson et al., 2006; Nassar et al., 2002). All of these molecules/mediators are possible mechanisms by which *P. gingivalis* activates vascular inflammatory response and pathology.

Different factors may interact with each other and increase the risk of atherosclerosis in patients. A recent study investigated the dual roles of tobacco smoke-exposure and infection e.g. *C. pneumoniae* as atherogenic risk co-factors. Smokers more commonly demonstrated antibodies to *C. pneumoniae* than nonsmokers (Hahn and Golubjatnikov, 1992; Karvonen et al., 1994) and this organism was detected more often in smokers' atherosclerotic plaque (Berger et al., 2000). They found that tobacco smoke induce *C. pneumoniae* persistence in HCAECs (Wiedeman et al., 2005). However, the mechanism of interaction between the two atherogenic risk factors has not

yet been elucidated. Similar to *C. pneumoniae*, a majority of periodontitis patients smoke (Tomar et al., 2000). In our study, we will investigate the link between periodontal infection and atherosclerosis by using an *in vitro* HCAEC culture stimulated with highly purified *P. gingivalis* LPS (TLR2 ligand). To the best of our knowledge, no previous studies have assessed the combined effect of smoking and periodontal infection on endothelial responses. Therefore, we will also investigate the immune modulating effect of cigarette smoke extract (CSE) on the response of HCAECs to *P. gingivalis* LPS as assessed by measurement of IL-8 and MCP-1 production.

CHAPTER III

MATERIALS AND METHODS

3.1 Medium

HCAEC culture medium (EGM-2-MV, Clonetics, San Diego, USA) supplemented with hEGF, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, gentamicin, amphotericin-B, and 5%FBS was used throughout the study

3.2 Reagents

3.2.1 TLR ligands

We used specific ultrapure TLR ligands for TLRs 2, 3, 4, 5, 7, 8 and 9 obtained from InvivoGen (San Diego, CA, USA) as shown in table 2.

Table 2: Toll-like receptor-specific ligands used in stimulating HCAECs

TLR ligand	Ligand	Dose
TLR2	Ultrapure LPS from *P. gingivalis*	50 μg/ml
TLR3	Polyinosine-polycytidylic acid (poly I:C)(Synthetic analog of double stranded RNA)	100 μg/ml
TLR4	Purified LPS from *Escherichia coli* K12 strain	10 μg/ml
TLR5	Purified flagellin from *Salmonella typhimurium*	5 μg/ml
TLR7	Loxoribine (Guanosine analog)	100 μM
TLR8	Single-stranded polyU oligonucleotide complexed with LyoVec™	5 μg/ml
TLR9	CpG oligonucleotide (CpG ODN) 2006	10 μg/ml

3.2.2 TNF-α

TNF-α was purchased from R&D Systems, Inc. (Minneapolis, MN,USA).

3.2.3 Preparation of CSE

CSE was prepared as previously described (Hoshino *et al*., 2005), with modification. Briefly, the mainstream smoke from five cigarettes ("Krongtip 90," Thailand Tobacco Monopoly, filtered type) was bubbled through 30 ml of pre-warmed (37^0C) medium at a constant negative pressure of 290 mmHg. Medium containing CSE was filtered through 0.2-μm filters. The filtrate was considered to be undiluted CSE, and was stored at -20^0C until use.

3.3 Human coronary artery endothelial cell culture

Primary human coronary artery endothelial cells were purchased from Clonetic. HCAECs were grown in an endothelial cell growth medium (EGM-2-MV, Clonetics, San Diego, USA), which was supplemented with trace elements, growth factors and antibiotics. The cells were grown at 37^0C in a humidified 95% air 5% CO_2 atmosphere. At confluence, the cells were detached from the culture flasks using trypsin-EDTA, washed twice, and resuspended in complete endothelial cell growth medium. The HCAECs culture at passage 3-5 was used for all the experimental assays.

3.4 mRNA expression of TLRs in HCAECs

HCAECs (2 $X10^5$ cells) were washed twice. Total RNA was isolated by using RNeasy Mini kit from Qiagen (Chatsworth, CA, USA), according to the manufacturer's instructions. The RNA samples were further purified by successive treatment with DNase I (Qiagen, Chatsworth, CA, USA). 1 μg of total RNA will be reverse transcripted using ImProm-II Reverse Transcription System for RT-PCR, according to the manufacturer's

instructions (Promega, Madison, WI, USA). TLR primer sequences shown in Table 3 were used. PCR amplification was performed using Taq DNA polymerase (Qiagen, Chatsworth, CA, USA), by Mastercycler gradient (Eppendorf, Germany) for 35 cycles of 94°C for 30 seconds, 55°C (TLRs 1-2, 4-8, 10), or 50°C (TLR3) or 65°C (TLR9) for 30 seconds, 72°C for 1 min, and then a final extension of 72°C for 2 min. Forward and reverse primer pairs selected for human TLRs 1-10 were purchased from Proligo (Singapore). The PCR products were separated on a 1.2% agarose gel containing ethidium bromide and visualized by UV illimination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. As a negative control, a PCR reaction was performed without an RT sample. Human PBMC was used as a positive control. The methods of RT-PCR for measuring mRNA expression of TLR on PBMC were followed the same procedure as those of HCAECs.

Table 3: Primer sequence of TLRs and GAPDH

Product	Forward primer	Reverse primer	Amplicon size (bp)
TLR1*	CGTAAAACTGGAAGCTTTGCAAGA	CCTTGGGCCATTCCAAATAAGTCC	885
TLR2*	GGCCAGCAAATTACCTGTGTG	CCAGGTAGGTCTTGGTGTTCA	614
TLR3*	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	319
TLR4*	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGTT	622
TLR5##	CCTCATGACCATCCTCACAGTCAC	GGCTTCAAGGCACCAGCCATCTC	355
TLR6$	ACTGACCTTCCTGGATGTGG	TGGCACACCATCCTGAGATA	404
TLR7$	ACAAGATGCCTTCCAGTTGC	ACATCTGTGGCCAGGTAAGG	207
TLR8*	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	636
TLR9##	GCGAGATGAGGATGCCCTGCCCTACG	TTCGGCCGTGGGTCCCTGGCAGAAG	510
TLR10$	GGCCAGAAACTGTGGTCAAT	AACTTCCTGGCAGCTCTGAA	287
GAPDH#	TGATCTCTGCCCCCTCTGCTG	GCCTGCTTCACCACCTTCTTG	435

(* primer sequence by Saikh et al., 2003, ## primer sequence by Schaefer et al., 2004, $ nucleotide sequences were determined from PubMed (National Center for Biomedical Information), # primer sequence by Nukaga et al., 2004 and the primers were custom designed using primer 3 software.)

3.5 TLR ligation on HCAECs after treatment with different TLR ligands and/or TNF-α

HCAECs (1x 10^4 cells/ml) in 96-well plates (Corning Glass) were treated with either 1) various single TLR ligands: *P. gingivalis* LPS (50 μg/ml), poly(I:C) (100 μg/ml), *E. coli* LPS (10 μg/ml), *Salmonella typhimurium* flagellin (5 μg/ml); loxoribine (100 μM), ssPolyU (5 μg/ml), and CpG ODN 2006 (10 μg/ml); 2) TNF-α (2 ng/ml), 3) TLR ligand and cytokine combination: *P. gingivalis* LPS (50 μg/ml) plus TNF-α (2 ng/ml). After stimulation with TLR ligand(s) and/or TNF-α for 24 h, the culture supernatants were collected for measurement of IL-8 and MCP-1 expression.

3.6 CSE treatment of stimulated HCAECs

3.6.1 Cell viability test by MTT

Preliminary experiments were conducted to determine non-toxic concentrations of CSE, in order to ensure that any of their observed effects on HCAEC function did not result from cellular toxicity or cell death. Viability and appearance of treated and untreated cells after 24 h incubation were assessed and compared by MTT assay and light microscopy. The cell did not differ with CSE at 1:16, 1:32 and 1:64 dilution so these levels were selected for use in the study.

3.6.2 CSE treatment of stimulated HCAECs and determination of IL-8 and MCP-1

HCAECs (1 x10^4 cells) were added to each well of a 96 –well flat-bottom microtitier plate and allowed to adhere for 24 h. Following adherence, *P. gingivalis* LPS and/or TNF-α and/or CSE was added to the HCAECs and the final volume of the culture medium was adjusted to 0.2 ml with complete medium. All incubations were carried out at 37°C in 5% humidified CO_2 for 24 h. After incubation, culture supernatants were

collected and assayed for IL-8 and MCP-1 using specific ELISA kits, according to the manufacturer's instructions (R& D Systems).

3.7 Statistic analysis

The data were analyzed using the computer program SPSS V11.5 software. Independent-Samples *t*-test was used to determine the effect TLR ligands and/or combination with TNF-α on IL-8 and MCP-1 production for normally distributed data. One way analysis of variance was used to analyze the modulating effect of CSE. If statistical difference was detected, a pairwise multiple comparison test (Bonferroni *t*-test) was used to identify the difference among the groups. A critical level of 0.05 was employed. Thus, p-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

4.1 mRNA expression of TLRs on HCAECs

TLRs are predominantly expressed on many cells and known to play a central role in pattern recognition in the innate immune system. To evaluate the expression of TLRs in HCAECs, total RNA from commercial available primary coronary artery endothelial cells of normal human tissues was analyzed by RT-PCR using a panel of specific primers of TLRs 1-10. We found the mRNA expression of TLRs 1, 2, 3, 4, 5, 6, 9, and 10 on HCAECs but not TLRs 7 and 8 (Figures 1A and 1B). However, TLR10 mRNA expression was very minimal. The results were reproducible in two separate experiments. Human PBMC, obtained from previous DNA collection (healthy donor) of our laboratory, were used as a positive control and shown to express all mRNA of TLRs 1-10 (Figure 1C). As a negative control, a PCR reaction was performed without an RT sample. GAPDH expression was used to confirm the quality of all RNA preparation for RT-PCR.

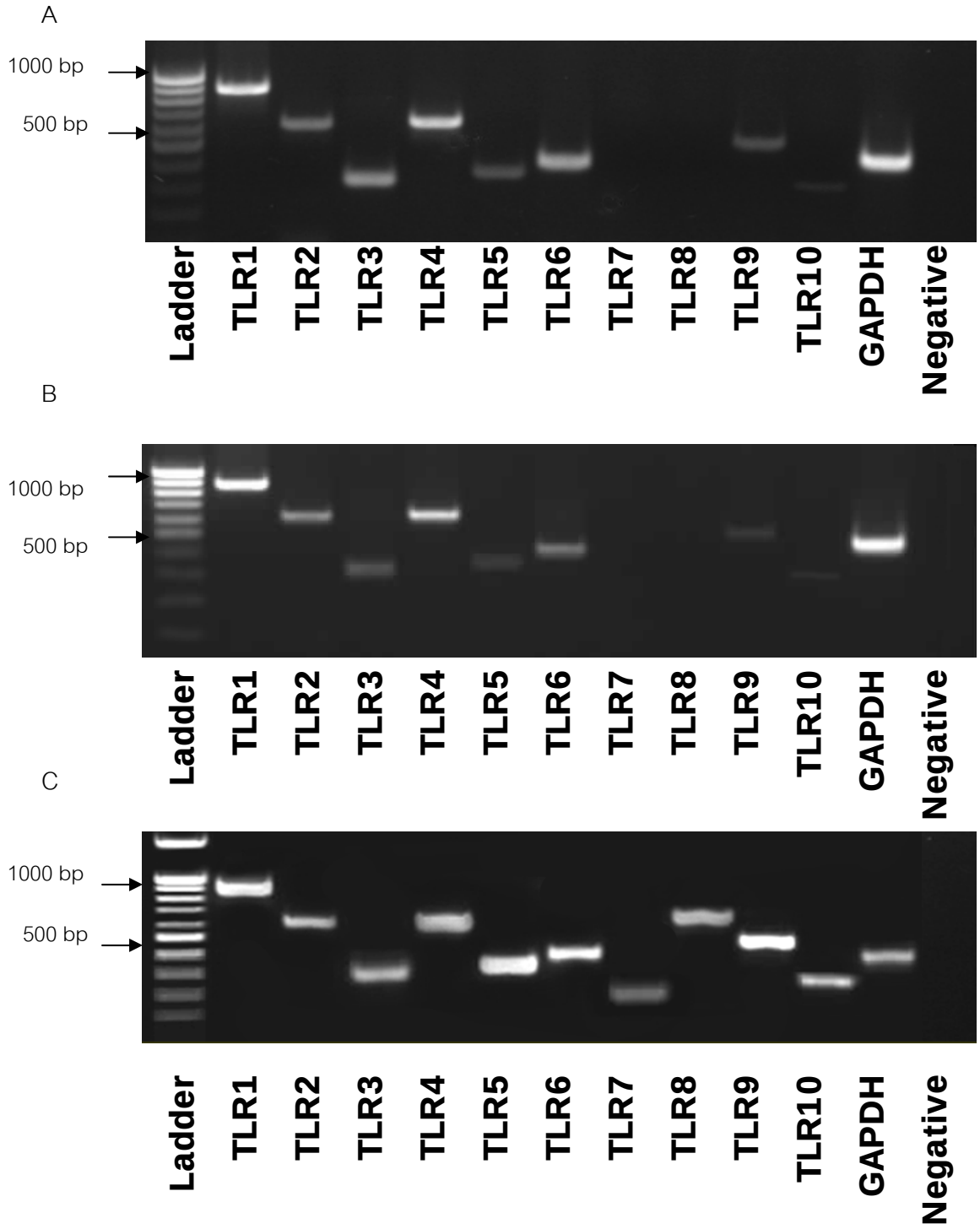
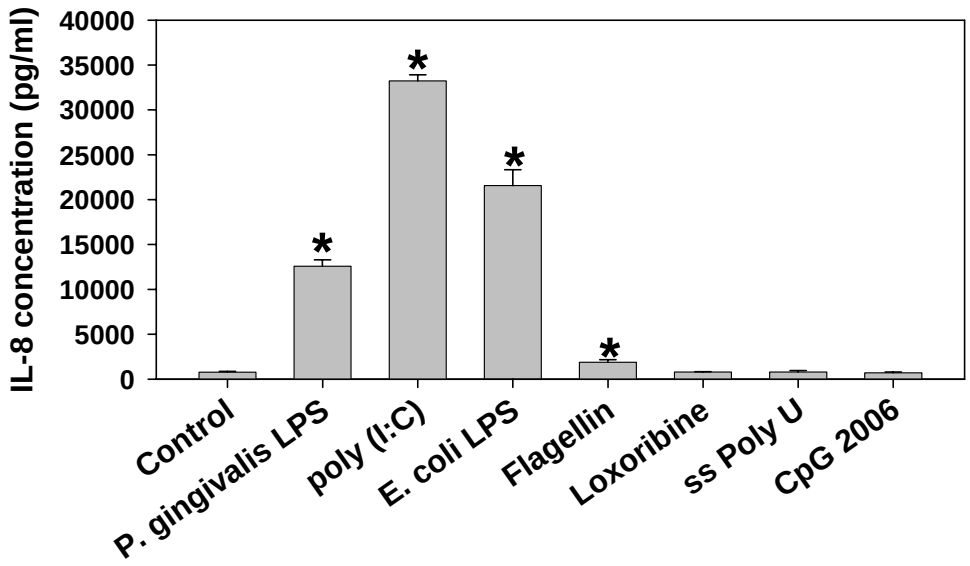


Figure 1. TLR expression in HCAECs. TLRs1-10 mRNA was determined in cultured HCAECs by RT-PCR (A and B). PBMC mRNA was used as positive control (C). GAPDH mRNA was used as an internal control. A non-RT sample was used as a negative control.

4.2 TLR ligands stimulate IL-8 and MCP-1 production

To characterize the functional relevance of TLRs in HCAECs, IL-8 and MCP-1 production was determined after stimulation with a variety of highly purified TLR ligands. As depicted in Figures 2A and 2B, un-stimulated HCAECs constitutively expressed low levels of IL-8 and MCP-1. HCAEC production of IL-8 and MCP-1 was induced by stimulation with *P. gingivalis* LPS, poly I:C, *E. coli* LPS and *S. typhimurium* flagellin , respective ligands for TLRs 2, 3, 4 and 5 , which coincided with their mRNA expression. Eventhough the cells expressed TLR9 mRNA, IL-8 and MCP-1 were not up-regulated in HCAECs stimulated with CpG ODN 2006, (Figures 2A and 2B).

A



B

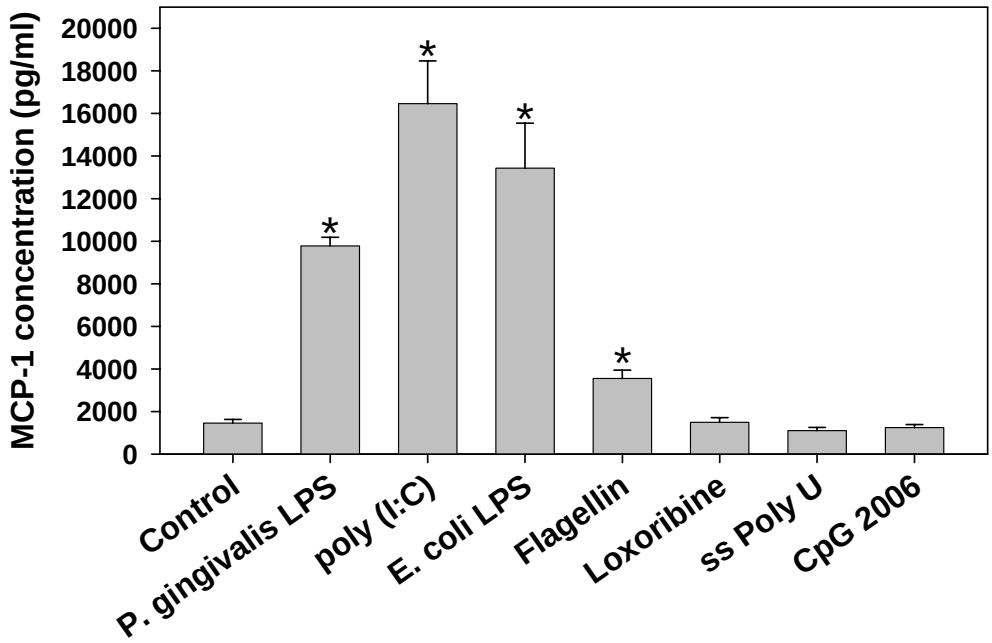
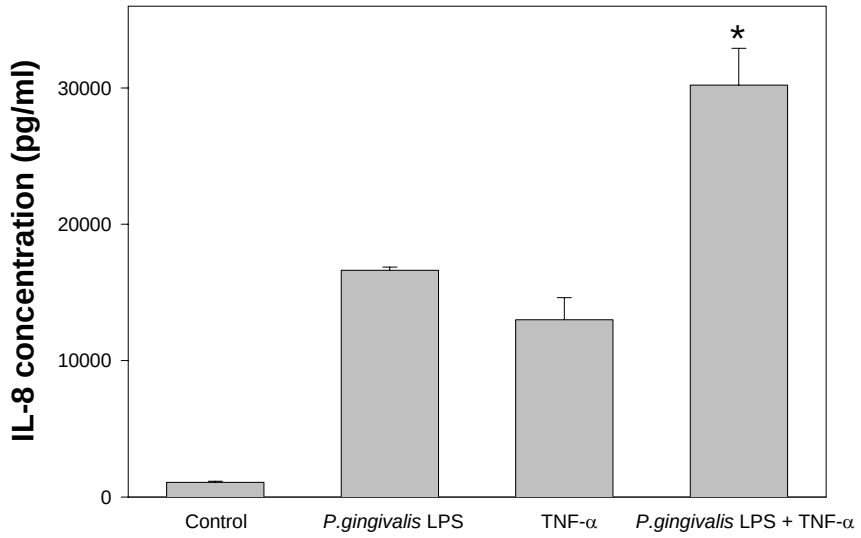


Figure 2. IL-8 and MCP-1 production from HCAECs after stimulation with various TLR ligands. HCAECs were cultured in 96-well plates, and stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly I:C (TLR3 ligand); *E. coli* LPS (TLR4 ligand); *S. typhimurium* (TLR5 ligand); Loxoribine (TLR7 ligand); polyU (TLR8 ligand); CpG ODN 2006 (TLR9 ligand). Culture medium was used as a control. After 24 h incubation, the culture supernatants were harvested for measurement of IL-8 (A) and MCP-1 (B) production by ELISA. Data are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with un-stimulated control).

4.3 Combination of *P.gingivalis* LPS and TNF-α stimulates expression of IL-8 and MCP-1

We next evaluated whether the TLR-triggering HCAEC response could be enhanced by cytokine. Here we used TNF-α, a pro-inflammatory cytokine which has been consistently detected in periodontitis and atherosclerosis lesions (Seymour, 1991; Page et al., 1997; Hanson and Libby et al., 2006; Tedgui and Mallat, 2006). We investigated IL-8 and MCP-1 production from HCAECs when stimulated with the combination of this cytokine and LPS from *P. gingivalis*, a product of a key periodontal pathogen. Cells were treated with *P. gingivalis* LPS (50 μg/ml), TNF-α (2 ng/ml), or a combination of *P. gingivalis* LPS (50 μg/ml) and TNF-α (2 ng/ml) for 24 h, and the IL-8 and MCP-1 production was analyzed by ELISA. The combination of TLR-2 ligand-*P. gingivalis* LPS and TNF-α significantly increased IL-8 production as compared to those of individual stimulations ($p < 0.05$) (81.66% increased as compare to *P. gingivalis* LPS alone stimulation and 132.47% increased as compared to TNF-α single stimulation) (Figure 3A). However, MCP-1 production remained relatively unchanged in combined stimulation as compared to individual stimulation (Figure 3B)

A



B

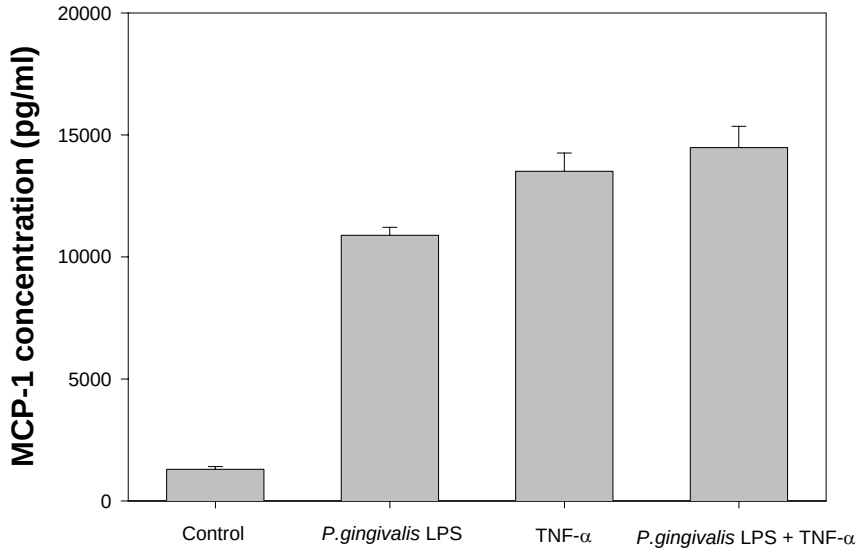


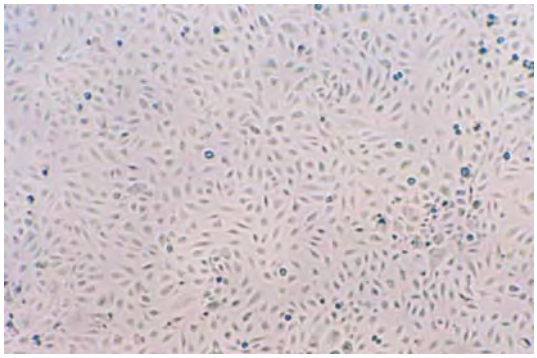
Figure 3. Expression of IL-8 and MCP-1 from HCAECs after stimulation with *P. gingivalis* LPS and TNF-α combination. HCAECs were cultured in 96-well plates and stimulated with *P. gingivalis* LPS, TNF-α, or *P. gingivalis* LPS and TNF-α. Culture medium was used as a control. After 24 h incubation, the culture supernatants were harvested for measurement of IL-8 (A) and MCP-1 (B) production by ELISA. Data are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with single stimulation).

4.4 Non-toxic doses of CSE

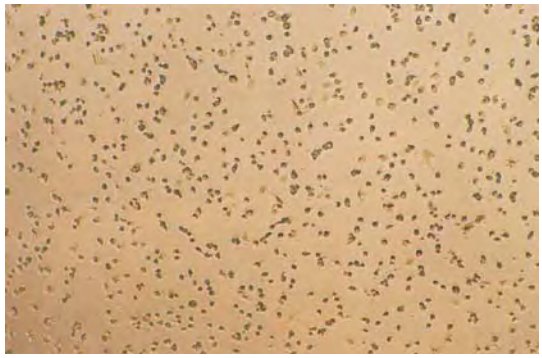
Our preliminary experiments were conducted to determine non-toxic concentrations of CSE, in order to ensure that any of their observed effects on HCAEC function did not result from cellular toxicity or cell death. Viability and appearance of treated and untreated cells were assessed in 2 separated experiments and compared by MTT assay and Trypan blue exclusion. HCAECs were untreated or treated with different dilutions of CSE (undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 dilution) for 24 h. The results of mean ± SEM of % cell viability is presented in table 4. At undiluted CSE, 1:2, 1:4, and 1:8 dilution, more than 30% of the HCAECs died. In contrast, at 1:16, 1:32, and 1:64 dilution of CSE, the cultured HCAECs were more than 100% viable as compared with the untreated control cells. The observation of the treated cells under microscope confirmed the MTT results (Figure 4). Therefore, these non-toxic concentrations of CSE at 1:16, 1:32, 1:64 dilution were used throughout the study.

Table 4: Cell viability of CSE-treated HCAECs by MTT

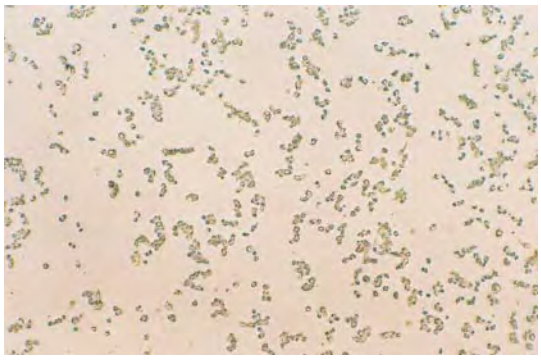
Dilutions of CSE	% cell viability (mean ± SEM)
Control (untreated HCAECs)	100.00
undiluted CSE	31.06 ± 8.27
CSE 1:2	27.45 ± 9.75
CSE 1:4	27.36 ± 9.22
CSE 1:8	67.66 ± 11.91
CSE 1:16	106.97 ± 2.10
CSE 1:32	132.11 ± 19.00
CSE 1:64	128.73 ± 16.84



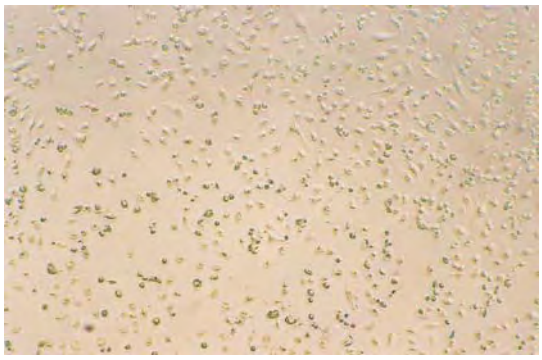
Control (Untreated HCAECs)



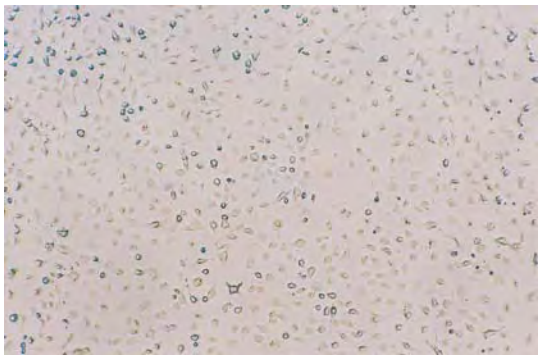
undiluted CSE



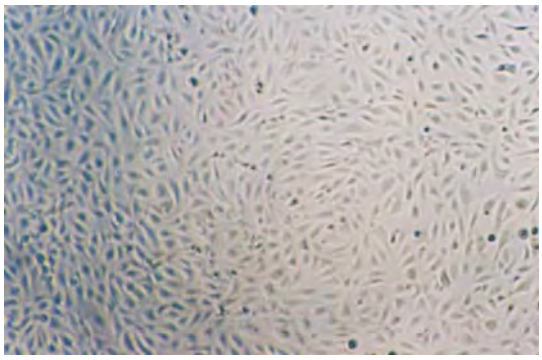
CSE 1:2 dilution



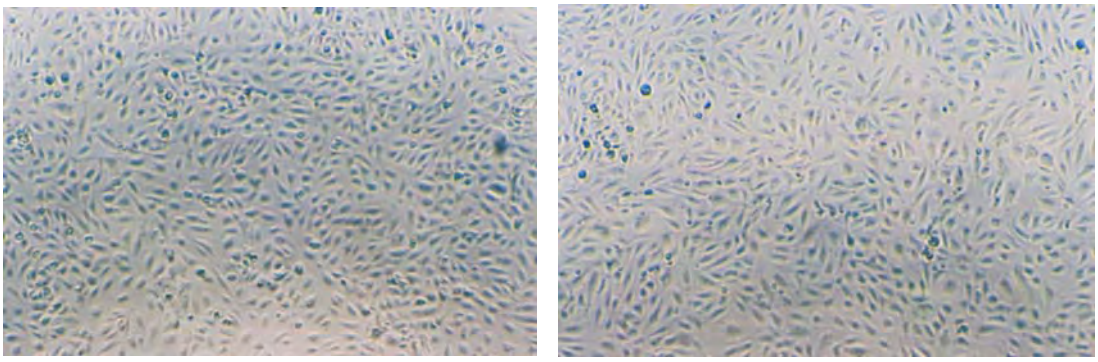
CSE 1:4 dilution



CSE 1:8 dilution



CSE 1:16 dilution



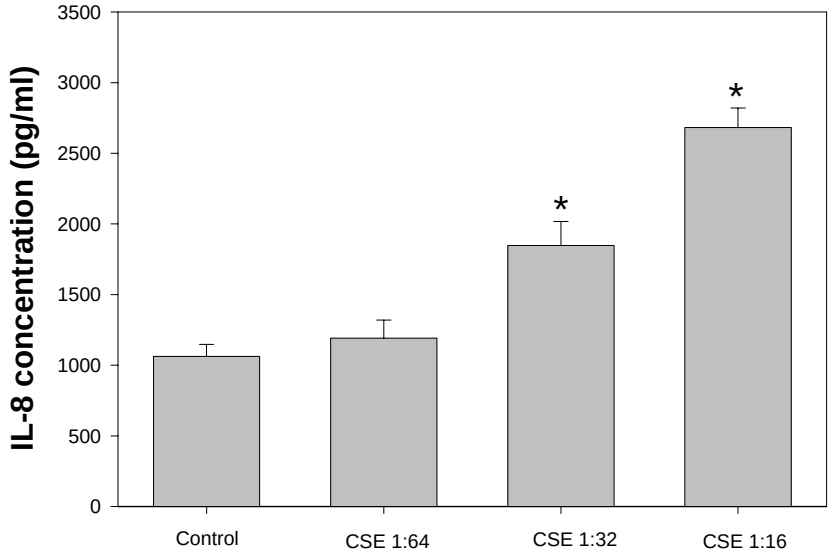
CSE dilution 1:32 CSE dilution 1:64

Figure 4. Toxicity test of CSE on HCAEC culture. CSE at 0, undiluted, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilution was added to HCAEC culture. The cell condition was observed under microscope (4x)

4.5 The effect of CSE on IL-8 and MCP-1 production in unstimulated HCAECs

We evaluated the effect of CSE on IL-8 and MCP-1 production in unstimulated HCAECs. HCAECs were treated with 1:16, 1:32, and 1:64 dilution of CSE for 24 h and IL-8 and MCP-1 concentrations were measured in the culture supernatants by ELISA. CSE alone at these non-toxic concentrations led to an increase in IL-8 production (Figure 5A). CSE at higher concentrations (1:32 and 1:16 dilution) significantly increased IL-8 production (73.9% and 152.4% respectively) as compared to those with controls (no CSE treatment) ($p < 0.05$) (Figure 5A). Interestingly, the results of MCP-1 production were quite different. MCP-1 production at low concentration (1:64 dilution) remained relatively unchanged from controls, while those at higher concentrations of 1:32 and 1:16 dilutions demonstrated a reduced MCP-1 production (26.47% and 65.85% respectively) (Figure 5B). However, a significant reduction of MCP-1 production could be observed at 1:16 dilution ($p < 0.05$) (Figure 5B).

A



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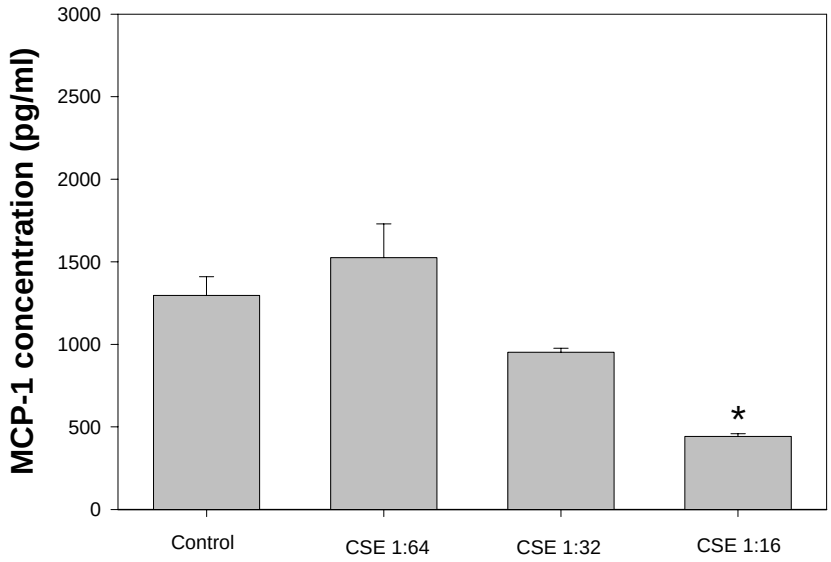
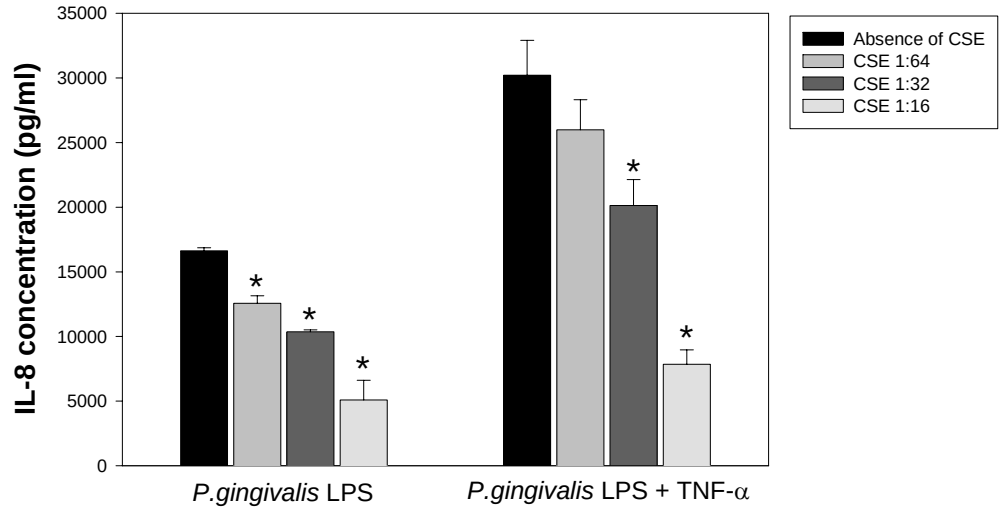


Figure 5. The effect of CSE on IL-8 and MCP-1 production in unstimulated HCAECs. HCAECs were treated with different dilution of CSE (1:64, 1:32 and 1:16). Culture medium was used as a control. After 24 h incubation, the culture supernatants were harvested for measurement of IL-8 (A) and MCP-1 (B) production by ELISA. Data are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with controls in the absence of CSE).

4.6 The effect of CSE on IL-8 and MCP-1 production in stimulated HCAECs

Next the immune modulating effect of CSE was evaluated in HCAECs which had been stimulated with *P.gingivalis* LPS alone or the combination of *P.gingivalis* LPS and TNF-α. Unlike in unstimulated HCAECs, CSE treatment at non-toxic concentrations in stimulated culture led to a decrease in IL-8 production (Figure 6A). The significant reduction in IL-8 production was observed in all concentrations of CSE in *P. gingivalis* LPS stimulated culture (24.41% at 1:64 dilution, 37.69% at 1:32 dilution, and 69.4% at 1:16 dilution) and in higher concentrations in combination stimulated culture (33.36% at 1:32 dilution and 74.03% at 1:16 dilution) as compared to those with no CSE treatment ($p < 0.05$) (Figure 6A). Similar to IL-8, The significant reduction in MCP-1 production was observed in all concentrations of CSE in *P. gingivalis* LPS stimulated culture (17.61% at 1:64 dilution, 55.37% at 1:32 dilution, and 91.94% at 1:16 dilution) and in higher concentrations in combination stimulated culture (49.73% at 1:32 dilution and 91.99% at 1:16 dilution) as compared to those with no CSE treatment ($p < 0.05$) (Figure 6B).

A



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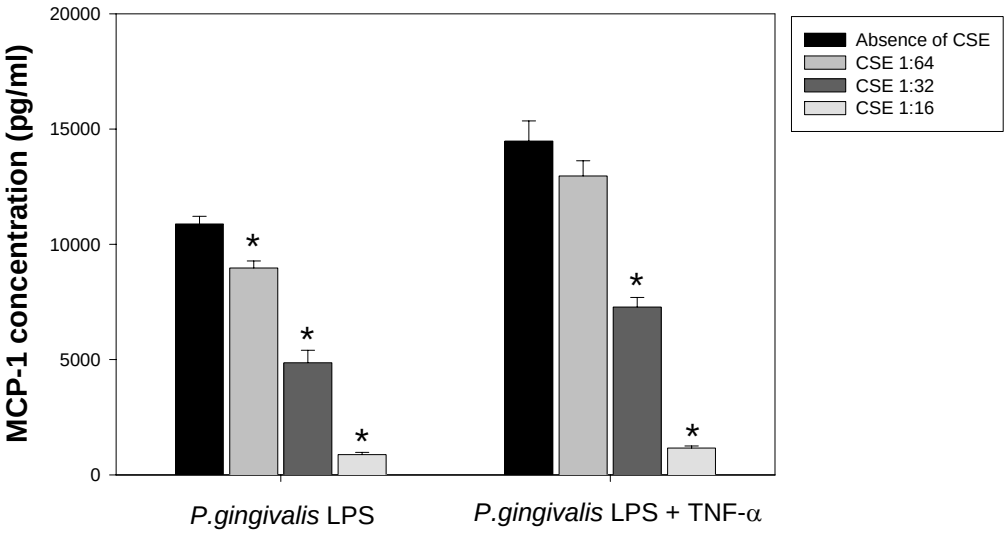

Figure 6. The effect of CSE on IL-8 and MCP-1 production in stimulated HCAECs. CSE (dilution 1:16, 1:32, 1:64) was added into HCAECs which were stimulated with *P.gingivalis* LPS or the combination of *P.gingivalis* LPS and TNF-α. Culture medium was used as a control. After 24 h incubation, the culture supernatants were harvested for measurement of IL-8 (A) and MCP-1 (B) production by ELISA. ($p < 0.05$). Data are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with cultures stimulated with *P.gingivalis* LPS or the combination of *P.gingivalis* LPS and TNF-α in the absence of CSE).

CHAPTER V

DISCUSSION AND CONCLUSION

Microbial infections are postulated to be cofactors involved in atherosclerosis, a major component of cardiovascular disease. Recent reports have suggested that bacteria, such as *C. pneumoniae* and *H. pylori*, and viruses, such as cytomegalovirus, herpes simplex virus, and hepatitis A virus, and periodontal pathogen may be associated with vascular insults that trigger atherogenesis (Shen et al., 2004; Volzke et al., 2004). Epidemiological studies suggest that chronic oral infection, and especially periodontitis, may be an important risk factor for cardiovascular disease (Madianos et al., 2002; Pussinen et al., 2007, Scannapieco et al., 2003;). Periodontal pathogens may enter the circulation during chewing, tooth brushing, flossing and gentle mastication (Forner et al., 2006; Sconyers et al., 1973; Silver et al., 1977). It is known that host immune response employs TLR and non-TLR pathways to recognize pathogens and commensal bacteria (Akira et al., 2004; Rakoff-Nahoum et al., 2004). This recognition leads to expression of mediators that limit microbial invasion. In the blood vessel, the endothelial cell is the first layer exposing to many PAMPs, so its innate immune response must be highly orchestrated and delicately balanced, in order to maintain homeostasis. Previous studies reported TLRs1, 2, 4 and 6 expression in primary HCAECs (Dunzendorfer et al., 2004, Erridge et al., 2007; Zeuke et al., 2002), therefore in this study we extended fully investigated a panel of TLR expression on this cell. We confirmed their observations and found that HCAECs expressed mRNA of TLRs 1, 2, 3, 4, 5, 9, and minimally expressed TLR10, but did not express TLRs 7 and 8. However, the expression of TLR10 mRNA was very minimal. These results were reproducible (n=2).

In line with the TLR mRNA expression, HCAECs produced IL-8 and MCP-1 in response to *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5. Similar finding of *P. gingivalis*-induced IL-8

and MCP-1 production was also observed in both HCAECs and human aortic endothelial cells (Erridge et al., 2007; Takahashi et al., 2006). It has been shown that highly purified *P. gingivalis* LPS possess lipid A heterogeneity, which may contribute to their ability to interact with either TLR2 or TLR4 (Darveau et al., 2004). *P. gingivalis* LPS at a concentration of 50 μg/ml used in our study predominantly stimulated HCAECs via TLR2 and to a lesser extent via TLR4 (InvivoGen product information). Poly(I:C) appeared to induce higher IL-8 and MCP-1 production than the other TLR ligands. Surprisingly, CpG ODN 2006, a potent ligand for human TLR9, did not enhance IL-8 or MCP-1 production. This is similar to previous observations in our laboratory showed that both human gingival fibroblasts and human gingival epithelial cells which also express TLR9 mRNA, but do not respond to CpG ODN 2006 (Mahanonda et al., 2007; Mahanonda et al., 2008 in press). The reason for this remains unclear and requires further investigation.

The finding that HCAECs expressed TLRs 2, 4, and 5 supports their role in the innate immune response against pathogens including bacteria and viruses which may lead to inflammatory events in atherosclerosis. Oral plaque bacteria are known to have PAMPs that are recognized by TLRs 2, 4 and 5. For example, *P. gingivalis* LPS and *P. gingivalis* fimbriae are recognized by TLR2 (Asai et al. 2001; Davey et al., 2008; Erridge et al., 2004, 2007; Hirschfeld et al. 2001; Kikkert et al., 2007; Zhou et al., 2005), LPS from *A. actinomycetemcomitans* and *Bacteroides fragilis* are recognized by TLR4 (Gutierrez-Venegas et al., 2006; Kikkert et al., 2007; Yoshimura et al., 2002). Flagellin of *T. denticola* is most likely recognized by TLR5. The expression of TLR3 in HCAECs is interesting since TLR3 recognizes double stranded RNA, a by-product of viral replication and transcription (Alexopoulou et al. 2001). A possible role of herpesviruses in etiology and severity of atherosclerosis has been reported (O'Connor et al. 2001). The presence of TLR3 thus indicates a role of HCAECs in antiviral response. The analysis of our data on TLR expression and TLR triggering of HCAECs suggest that this cell could play important role in the innate response to the microbial invasion in the blood vessel.

Cigarette smoking is well-known as a strong common risk factor of cardiovascular disease and periodontitis (Grossi et al., 1989; Hubert et al., 1982; Hujoel et al., 2002; Shinton and Beevers, 1994; Tomar et al., 2000). In this study we created an experimental model reproducing a situation of periodontitis subject who smokes using a HCAEC line to better understand whether the exposure of cigarette smoke may enhance the response of HCAECs to periodontal infection. To the best of our knowledge, no previous studies have assessed the effect of CSE on the response of HCAECs to key periodontal pathogen-*P. gingivalis* LPS. Our results showed that CSE had immune modulating effects on HCACEs both by itself and with other stimuli: *P. gingivalis* LPS or combined *P. gingivalis* LPS and TNF-α. The observed effects of CSE in this study were not due to cellular toxicity, since the concentrations used were in the non-toxic range. Treatment on HCAECs with CSE alone led to a dose dependent increase in IL-8 production but a decrease in MCP-1 production. However, an enhancement of IL-8 following CSE treatment was not due to a contamination of endotoxin since a level of bacterial endotoxin in undiluted CSE was less than 1 EU/ml which was lower than the endotoxin level in culture medium from limulus amebocyte lysate assay. Thus, the data suggest that the expression of these two mediators may be controlled by different pathways. Simultaneous enhancing and suppressing effect of smoking exposure on mediator production could also be observed in other cardiovascular endothelial cells. Treatment of unstimulated human aortic endothelial cells with smoke condensate enhanced IL-8 and IL-4 but inhibited MCP-1 production (Nordskog et al., 2005). In contrast to the effect of CSE alone on IL-8, CSE treatment of stimulated HCAECs with either *P. gingivalis* LPS or combined *P. gingivalis* LPS and TNF-α resulted in a reduced IL-8 production. This suppressive effect of CSE is unexpected since HCAECs with CSE alone treatment or stimulated HCAEC (*P. gingivalis* LPS or combined *P. gingivalis* LPS and TNF-α) without CSE resulted in increase IL-8 production. The reason for this finding required further investigation. Like IL-8, MCP-1 production was decreased in CSE treatment of stimulated HCAECs with either *P. gingivalis* LPS or combined *P. gingivalis* LPS and TNF-α. Our results agreed with the study of Patton et al. (2006) using

stimulated HCAECs. They reported a decrease in IL-8 and MCP-1 in *E. coli* LPS stimulated HCAECs after nicotine treatment (Patton et al., 2006).

In conclusion, our study demonstrates the role of HCAECs in the innate immune response of vascular system. They express an array of TLRs which can respond to specific microbial components, leading to the recruitment of inflammatory cells via production of IL-8 and MCP-1 for pathogen elimination. Our findings showed that CSE did not enhance *P. gingivalis* LPS-stimulated HCAEC response. The data does not seem to support our hypothesis that periodontal infection and smoking play role as risk factor in atherosclerosis by promoting more inflammation of the blood vessel. However, it is interesting to note that CSE alone could promote HCAEC response by increasing IL-8 production. Such complex interaction between *P. gingivalis* LPS, CSE and the HCAEC response which links between periodontitis, smoking and atherosclerosis, needs further investigation.

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APPENDICES

Table 5: The effect of TLR ligands on IL-8 production by HCAECs (n=4)

TLR ligands	IL-8 production (pg/ml)			
	1	2	3	4
Control	610.31	965.98	834.67	760.66
P. gingivalis LPS 50 µg/ml (TLR2)	13540.76	13792.24	12310.61	10,668.40
poly (I:C) 100 µg/ml (TLR3)	32812.87	34338.37	31536.73	34,247.74
E. coli LPS 10 µg/ml (TLR4)	20269.32	26811.70	19309.30	19,898.52
Flagellin 5 µg/ml (TLR5)	1462.52	1432.42	2101.69	2,567.81
Loxoribine 100 µM/ml (TLR7)	838.88	748.47	749.00	870.90
ss Poly U 5 µg/ml (TLR8)	1197.84	562.35	720.06	756.28
CpG 2006 10 µg/ml (TLR9)	544.37	881.17	597.51	860.28

Table 6: The effect of TLR ligands on MCP-1 production by HCAECs (n=4)

TLR ligands	MCP-1 production (pg/ml)			
	1	2	3	4
Control	1818.52	1680.54	1165.70	1162.964
P. gingivalis LPS 50 µg/ml (TLR2)	9619.13	10974.18	9250.53	9281.968
poly (I:C) 100 µg/ml (TLR3)	19160.43	20645.56	13120.62	12898.09
E. coli LPS 10 µg/ml (TLR4)	17998.20	16011.70	10271.41	9439.346
Flagellin 5 µg/ml (TLR5)	4398.47	4013.74	2763.98	3024.435
Loxoribine 100 µM/ml (TLR7)	1903.91	1839.83	1108.39	1146.555
ss Poly U 5 µg/ml (TLR8)	1428.18	1297.86	1321.86	874.7032
CpG 2006 10 µg/ml (TLR9)	1501.49	1470.04	1247.06	1021.75

Table 7: The effect of the combination of *P.gingivalis* LPS and TNF-α on IL-8 production by HCAECs (n=4)

Stimuli	IL-8 production (pg/ml)			
	1	2	3	4
Control	1130.45	1161.05	1148.81	808.71
P. gingivalis LPS 50 µg/ml (TLR2)	16156.00	16304.76	16994.17	17054.52
TNF-α 2 ng/ml	15623.74	15948.41	10292.80	10105.62
P. gingivalis LPS and TNF-α	34392.15	35337.34	25474.16	25614.50

Table 8: The effect of the combination of *P.gingivalis* LPS and TNF-α on MCP-1 production by HCAECs (n=4)

Stimuli	MCP-1 production (pg/ml)			
	1	2	3	4
Control	1039.11	1178.54	1438.64	1527.72
P. gingivalis LPS 50 µg/ml (TLR2)	10362.93	10274.04	11371.24	11529.48
TNF-α 2 ng/ml	14897.51	14706.97	12189.12	12257.75
P. gingivalis LPS and TNF-α	16712.59	15016.89	12948.00	13226.19

Table 9: The effect of CSE on IL-8 production in unstimulated HCAECs (n=4)

CSE	IL-8 production (pg/ml)			
	1	2	3	4
Control	1130.45	1161.05	1148.81	808.71
CSE 1:64	1290.13	1364.29	808.71	1302.47
CSE 1:32	1345.72	1989.05	1982.62	2072.96
CSE 1:16	2459.82	2479.76	2728.03	3056.65

Table 10: The effect of CSE on MCP-1 production in unstimulated HCAECs (n=4)

CSE	MCP-1 production (pg/ml)			
	1	2	3	4
Control	1039.11	1178.54	1438.64	1527.72
CSE 1:64	1168.19	1308.26	1527.72	2097.47
CSE 1:32	931.05	951.61	1018.50	910.50
CSE 1:16	459.26	479.82	412.94	418.09

Table 11: The effect of CSE on IL-8 production in stimulated HCAECs (n=4)

CSE	IL-8 production (pg/ml)			
	1	2	3	4
P. gingivalis LPS	16156.00	16304.76	16994.17	17054.52
CSE 1:64 + *P. gingivalis* LPS	14085.30	12811.38	11483.47	11895.12
CSE 1:32 + *P. gingivalis* LPS	10292.80	10078.93	10776.92	10292.80
CSE 1:16 + *P. gingivalis* LPS	7494.49	1889.95	7853.31	3114.30
P. gingivalis LPS + TNF-α	34392.15	35337.34	25474.16	25614.50
CSE 1:64 + *P. gingivalis* LPS + TNF-α	31215.67	27907.64	20382.04	24432.41
CSE 1:32 + *P. gingivalis* LPS + TNF-α	25334.16	19303.14	20254.03	15623.74
CSE 1:16 + *P. gingivalis* LPS + TNF-α	7622.40	5605.97	10939.20	7214.02

Table 12: The effect of CSE on MCP-1 production in stimulated HCAECs (n=4)

CSE	MCP-1 production (pg/ml)			
	1	2	3	4
P. gingivalis LPS	10362.93	10274.04	11371.24	11529.48
CSE 1:64 + *P. gingivalis* LPS	8260.28	8693.20	9699.01	9216.17
CSE 1:32 + *P. gingivalis* LPS	4465.98	3827.02	6384.36	4755.59
CSE 1:16 + *P. gingivalis* LPS	946.25	883.32	1071.69	608.35
P. gingivalis LPS + TNF-α	16712.59	15016.89	12948.00	13226.19
CSE 1:64 + *P. gingivalis* LPS + TNF-α	14091.65	14044.57	11484.23	12234.86
CSE 1:32 + *P. gingivalis* LPS + TNF-α	7958.75	7980.25	6870.36	6300.12
CSE 1:16 + *P. gingivalis* LPS + TNF-α	1029.93	1321.16	1300.43	988.12

BIOGRAPHY

Miss Bharani Phairat was born on 22nd of September 1981 in Bangkok. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Chulalongkorn University in 2004, and became a staff member of the Faculty of Dentistry, Srinakharinwirot University in 2004 - 2006. She studied in Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2006.