ผลของเคอร์คิวมินและเตตระไฮโดรเคอร์คิวมินต่อการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กที่เหงือกในหนูเบาหวาน

นาย ดุสิต พรหมรักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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The effects of curcumin and tetrahydrocurcumin on gingival microvascular dysfunction in diabetic rats

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Physiology
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Thesis Title	THE EFFECTS OF CURCUMIN AND TETRAHYDROCURCUMIN ON GINGIVAL MICROVASCULAR DYSFUNCTION IN DIABETIC RATS
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Field of Study	Physiology
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ดุสิต พรหมรักษ์ : ผลของเคอร์คิวมินและเตตระไฮโดรเคอร์คิวมินต่อการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กที่เหงือกในหนูเบาหวาน. (The effects of curcumin and tetrahydrocurcumin on gingival microvascular dysfunction in diabetic rats) **อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. สุภัทรา อมาตยกุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร. สุทธิลักษณ์ ปทุมราช, รศ.ทพญ. ศิริพร โชติไพบูลย์พันธุ์**, 95 หน้า.

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาผลของสารเคอร์คิวมินและเตตระไฮโดรเคอร์คิวมินต่อการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กที่เหงือกในหนูเบาหวาน โดยวิธีฉีดสารสเตรปโตโซโตซินเข้าทางหลอดเลือดดำเพียงครั้งเดียวในขนาด 55 มิลลิกรัมต่อน้ำหนักตัวหนู 1 กิโลกรัม หนูวิสตาร์เพศผู้ น้ำหนัก 200-250 กรัม ได้ถูกแบ่งแบบสุ่มเป็น 6 กลุ่ม กลุ่มละ 6 ตัว คือ 1) กลุ่มควบคุม (CON) ที่ป้อนด้วยน้ำเกลือ (Normal saline solution) 2) กลุ่มควบคุมที่ได้รับการป้อนด้วยสารเคอร์คิวมิน (CON+CUR) ปริมาณ 100 มิลลิกรัมต่อน้ำหนักตัวหนู 1 กิโลกรัม 3) กลุ่มควบคุมที่ได้รับการป้อนด้วยสารเตตระไฮโดรเคอร์คิวมิน (CON+THC) ปริมาณ 100 มิลลิกรัมต่อน้ำหนักตัวหนู 1 กิโลกรัม 4) กลุ่มเบาหวาน (STZ) 5) กลุ่มเบาหวานที่ได้รับการป้อนด้วยสารเคอร์คิวมิน (STZ+CUR) ปริมาณ 100 มิลลิกรัมต่อน้ำหนักตัวหนู 1 กิโลกรัม 6) กลุ่มเบาหวานที่ได้รับการป้อนด้วยสารเตตระไฮโดรเคอร์คิวมิน (STZ+THC) ปริมาณ 100 มิลลิกรัมต่อน้ำหนักตัวหนู 1 กิโลกรัม

ทำการทดลองหลังจากสัตว์ทดลองได้รับการฉีดสเตรปโตโซโตซินไปแล้ว 8 สัปดาห์ วันที่ทำการทดลองหนูถูกชั่งน้ำหนักและทำให้สลบจากนั้นทำการวัดการไหลเวียนเลือดที่ไปเลี้ยงบริเวณเหงือกโดยใช้เลเซอร์ ดอปเปลอร์ โฟลเมตรี นับการเกาะติดของเม็ดเลือดขาวที่หลอดเลือดโพสแคปิลารี่ เวนูล โดยวิธีอินทราไวทัล ฟลูออเรสเซ็นท์ ไมโครสโคปี้ เมื่อสิ้นสุดการทดลองทำการเก็บเลือดจากหลอดเลือดที่ท้องเพื่อนำไปวัดระดับน้ำตาลในเลือด ไกลโคซิเลทเท็ดฮีโมโกบิล และระดับ TNF-α ในซีรัม จากนั้นทำการตัดเก็บเนื้อเยื่อของเหงือกทันทีเพื่อนำมาใช้วิเคราะห์หาระดับมาลอนไดอัลดีไฮด์

จากผลการทดลองพบว่าหนูกลุ่มเบาหวานมีการลดลงของการไหลเวียนเลือดที่ไปเลี้ยงบริเวณเหงือก ในขณะที่การเกาะติดของเม็ดเลือดขาวที่หลอดเลือด ระดับ TNF-α ในซีรัมและระดับมาลอนไดอัลดีไฮด์สูงขึ้น อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับหนูกลุ่มควบคุม ในขณะที่หนูกลุ่มเบาหวานที่ได้รับการป้อนเคอร์คิวมินและหนูกลุ่มเบาหวานที่ได้รับการป้อนเตตระไฮโดรเคอร์คิวมินพบว่ามีแนวโน้มในการเพิ่มการไหลเวียนเลือดที่ไปเลี้ยงบริเวณเหงือกแต่ยังไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามหนูทั้งสองกลุ่มมีการลดลงของการเกาะติดของเม็ดเลือดขาวที่หลอดเลือด ระดับ TNF-α ในซีรัมและระดับมาลอนไดอัลดีไฮด์ เมื่อเปรียบเทียบกับหนูกลุ่มเบาหวาน นอกจากนี้พบว่าหนูกลุ่มเบาหวานที่ได้รับการป้อนเคอร์คิวมินและหนูกลุ่มเบาหวานที่ได้รับการป้อนเตตระไฮโดรเคอร์คิวมิน มีน้ำหนักตัวลดลงและมีระดับน้ำตาลในเลือดกับไกลโคซิเลทเท็ดฮีโมโกบิลเพิ่มขึ้นเมื่อเปรียบเทียบกับหนูกลุ่มควบคุม

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นว่า สารเคอร์คิวมินและเตตระไฮโดรเคอร์คิวมินสามารถป้องกันการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กที่เหงือกในหนูเบาหวานได้ โดยผลการป้องกันเกิดจากการยับยั้งภาวะออกซิเดทีฟสเตรส อย่างไรก็ตามเมื่อเปรียบเทียบระหว่างเคอร์คิวมินและเตตระไฮโดรเคอร์คิวมินพบว่า ผลของการป้องกันความผิดปกติไม่มีความแตกต่างกัน

สาขาวิชา.....สรีรวิทยา.....ลายมือชื่อนิสิต .....

ปีการศึกษา.....2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5087137120 : MAJOR PHYSIOLOGY

KEYWORDS : DIABETES / CURCUMIN / TETRAHYDROCURCUMIN / GINGIVAL MICROVASCULAR / OXIDATIVE STRESS

DUSIT PROMRUG : THE EFFECTS OF CURCUMIN AND TETRAHYDROCURCUMIN ON GINGIVAL MICROVASCULAR DYSFUNCTION IN DIABETIC RATS. ADVISOR : ASSOC.PROF. SUPATHRA AMATYAKUL, Ph.D., CO-ADVISOR : ASSOC.PROF.SUTHILUK PATUMRAJ, Ph.D., ASSOC.PROF.SIRIPORN CHOTIPAIBULPAN, D.D.S. 95 pp.

To study the effect of curcumin and tetrahydrocurcumin (THC) on gingival microvascular dysfunction in diabetic rats, the animal model of streptozotocin (STZ)-induced diabetic rats (a single intravenous injection of STZ; 55 mg/kg BW) was used. Male Wistar rats were randomly divided into six group (n=6/group). Group 1 (CON) rats were fed with normal saline (NSS) daily via an intragastric tube. Group 2 and 3 (CON+CUR; CON-THC) rats were fed with curcumin and THC dissolved in NSS (100 mg/kg BW), respectively. Group 4 (STZ) diabetic rats were induced by STZ and were fed with NSS. Group 5 and 6 (STZ+CUR; STZ+THC), diabetic rats were fed with curcumin and THC (100 mg/kg BW), respectively.

The experiment was performed at 8th week after injection of STZ. On the day of experiment the rats were weighed and anesthetized. Gingival blood-flow (GBF) was measured by Laser Doppler Flowmetry and leukocyte adhesion was examined by intravital fluorescence microscopy. At the end of each experiment, blood samples were collected from abdominal aorta to measure blood glucose, glycosylated hemoglobin, TNF-α level. After that, the gingival tissue was isolated to determine malondialdehyde (MDA) level.

GBF was significantly decreased in STZ rats while the number of adherent leukocytes, TNF-α level and MDA level were increased compared with CON rats. In the STZ+CUR, the GBF seem to be increased but not significantly, whereas the leukocyte adhesion, TNF-α level and MDA level were significantly less than those in the STZ. The same result was observed for STZ+THC rats. Body weight were lower, while blood glucose level, glycosylated hemoglobin were increased in STZ, STZ+CUR and STZ+THC rats compared with CON rats.

In conclusion, the present study has demonstrated that curcumin and THC could prevent gingival microvascular dysfunction in diabetic rats by reducing oxidative stress. In addition, the results explored the protective effect of curcumin is as potent as THC.

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ACh = Acetylcholine

ADP = Adenosine diphosphate

AGEs = Advanced glycation end products

ANG-II = Angiotensin II

ATP = Adenosine triphosphate

cAMP = cyclic Adenosine monophosphate

CAMs = Cell adhesion molecules

cGMP = cyclic Guanosine monophosphate

COX-2 = Cyclooxygenase-2

EC = Endothelial cell

EDRFs = Endothelial-derived relaxing factors

eNOS = Endothelial nitric oxide synthase

E-selectin = Endothelial leukocyte adhesion molecule

ET-1 = Endothelin-1

$FADH_2$ = Reduced form of flavin adenine dinucleotide

GSH = Glutathione

HLA = Human leukocyte antigen

HMECs = Human microvascular endothelial cells

HO-1 = Heme oxygenase-1

ICAM-1 = Intercellular adhesion molecule 1

ICAMs = Intercellular adhesion molecules

IL-1 = Interleukin-1

IL-6 = Interleukin-6

iNOS = Inducible nitric oxide synthase

IκB = NF-κB inhibitors

LDL = Low density lipoprotein

LPS = Lipopolysaccharide

L-selectin = Leukocyte adhesion molecule

NF-κB = Nuclear factor κ B

NHANES III = The Third National Health and Nutrition Examination Survey

NRF2 = Hormetic nuclear factor (erythroid-derived 2)-like 2

MCD = Lipogenic methionine and choline deficient

MCP-1 = Monocyte chemotactic protein-1

MDA = Malondialdehyde

NADH = Reduced form of nicotinamide adenine dinucleotide

nNOS = Neuronal nitric oxide synthase

NO = Nitric oxide

NOS = Nitric oxide synthase

NSS = 0.9% normal saline

O_2^- = Superoxide

OH = Hydroxyl

$ONOO^-$ = Peroxynitrite

ox-LDL = oxidised-LDL

PAI-1 = Plasminogen activator inhibitor-1

PECAM-1 = Platelet endothelial cell adhesion molecule-1

PGH_2 = Prostaglandin H2

PGI_2 = Prostacyclin

PMN = Polymorphonuclear leukocyte

PPARgamma = Peroxisome proliferator activated receptor gamma

R6G = Rhodamine 6G

RAGE = AGEs receptor

RBC = Red blood cell

RNS = Reactive nitrogen species

ROS = Reactive oxygen species

SDS = Sodium dodecyl sulphate

SEM = Standard errors of mean

SOD = Superoxide dismutase

STZ = Streptozotocin

TCA = Tricarboxylic acid

THC = Tetrahydrocurcumin

TNF-α = Tumour necrosis factor-α

t-PA = tissue Plasminogen activator

TPA = Tetradecanoylphorbol acetate

UCP = Uncoupling proteins

VCAM-1 = Vascular cell adhesion molecule-1

VCAMs = Vascular cell adhesion molecules

VSMC = Vascular smooth muscle cel

CHAPTER I

INTRODUCTION

Diabetes mellitus is worldwide health problem affecting people in all stages of life. It is a dramatic impact on health, causing a high degree of morbidity and mortality in affected individuals as well as placing an economic burden on the health-care system. Diabetes mellitus is a syndrome of abnormal carbohydrate, fat, and protein metabolisms that results in acute and chronic complications due to the absolute or relative lack of insulin (WHO Expert Committee on Diabetes Mellitus: second report, 1980: 1-80; Zimmet, Alberti, and Shaw, 2001: 782-787). In the metabolic dysregulation of diabetes, persisting hyperglycemia associated with both type 1 and type 2 diabetes mellitus in the development of multiple macrovascular and microvascular complications such as retinopathy, nephropathy, neuropathy and periodontitis (Shlossman et al., 1990: 532-536; Jakus and Rietbrock, 2004: 131-142; Kirkman et al., 2006: 75-80).

Gingival microvascular dysfunction causes the pathology in oral organ such as periodontal diseases, which are a group of inflammatory diseases that affect the supporting tissues of the dentition including, alveolar bone, periodontal ligament, cementum, and gingiva. The relation of diabetes and periodontal disease has been explored by many researchers over the years. Periodontal diseases are one of the serious oral health problems noticed in patients with diabetes. This is often considered the sixth complication of diabetes (Loe, 1993: 329-334). Several studies have demonstrated a relation between diabetes and gingival microvascular dysfunction (Cianciola et al., 1982: 653-660; Shlossman et al., 1990: 532-536; Pontes Andersen et al., 2007: 1264-1275). There is substantial evidence to support considering diabetes as a risk factor for periodontal disease. The prevalence and severity of periodontal disease in diabetic patient were higher than people without diabetes (Soskolne, 1998: 3-12; Dental visits among dentate adults with diabetes--United States, 1999 and 2004, 2005: 1181-1183). In a large epidemiological study in the U.S. (The Third National Health and Nutrition Examination Survey or NHANES III), the degree of glycemic control was associated with prevalence of severe periodontitis. Patients with poorly controlled diabetes had significantly higher prevalence of severe periodontitis. Conversely,

patients with well-controlled diabetes had no significant increase in the risk for periodontitis (Tsai, Hayes, and Taylor, 2002: 182-192). Moreover, severe periodontal disease involve the severity of diabetes and the degree of metabolic control, this refer to a two-way relationship between periodontal disease and diabetes mellitus (Grossi and Genco, 1998: 51-61).

In the current study regarding the biologic link between diabetes and gingival microvascular dysfunction supports persisting hyperglycemia leading to an exaggerated inflammatory response to the pathogenic gingival microvascular (Southerland et al., 2006: 130-143; Lamster et al., 2008: 19S-24S). Hyperglycemia in diabetes leads to the generation of reactive oxygen species (ROS) from the metabolic pathway. In high blood glucose levels condition, glucose transports into endothelial cells and produces electron carrier molecules, which enter in to mitochondria and release of electrons that react with oxygen molecules to form superoxides by electron-transport chain (Brownlee, 2001: 813-820; Akalin et al., 2008: 44-52). Therefore, non-enzymatic glycosylation of proteins that result in greater formation of advanced glycation end products (AGEs) can produce ROS by binding to cell-surface receptor for these AGEs, termed RAGE (receptor for AGEs) (Vlassara and Palace, 2002: 87-101). The elevation of ROS causes an oxidative stress that may contribute to endothelial dysfunction, which one of important reason for the development of gingival tissue inflammation and destruction.

Oxidative stress activates the secretion of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Chapple and Matthews, 2007: 160-232). These mediators trigger the inflammation process and impaired capillary function in gingival tissue. Oxidative stress also enhances the expression of a range of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and Inter cellular adhesion molecule 1 (ICAM-1) on endothelial cell surface. These refer to elevate leukocyte adhesion and migration through endothelial cell, which implicate the inflammation of gingival tissue in periodontal disease (Zitzmann et al., 2002: 490-495; Southerland et al., 2006: 130-143). Moreover, oxidative stress causes diabetic complication through altering the hemodynamic in vessel. This shows the reduction of blood flow to various organs in the diabetes patients (Hile and Veves, 2003: 446-451;

Burgansky-Eliash et al., 2010: 765-773). The reduction of Nitric oxide (NO) production and availability are explained to the important factor for the hemodynamic alteration (Honing et al., 1998: 241-249). The blood flow reduction is observed in periodontal disease. That is representing the one of abnormality in vascular gingival tissue.

Curcumin (diferuloylmethane) is a yellow phenolic compound which extract from the rhizome of the *Curcuma longa* Linn. Which plant is a spice originating from Southeast Asia. Widely used in foods, for cosmetic, and medicinal purposes. Curcumin has a number of biological applications with an antioxidant activity both in vitro and in vivo (Ruby et al., 1995: 79-83; Suryanarayana et al., 2007: BR286-BR292). Moreover, curcumin is also shown to have anti carcinogenic (Xia et al., 2007: 2161-2169), anti-inflammatory including an inhibitory effect on the production of TNF-α, IL-1b, and IL-8 by lipopolysaccharide (LPS)-stimulated monocytes and alveolar macrophages (Kawamori et al., 1999: 597-601; Literat et al., 2001: 253-267), and antidiabetic/hypoglycemic effect (Arun and Nalini, 2002: 41-52; Mahesh, Sri Balasubashini, and Menon, 2004: 639-644). Previous study performed by Patumraj et al. (2006: 481-489), they demonstrated that supplementation of curcumin could protect endothelial dysfunction in the iris tissue of STZ-induced diabetic rats by the parameter of blood glucose levels, number of leukocytes adhesion, and iris blood perfusion. However, no study that demonstrates the effect of curcumin on gingival microvascular dysfunction in diabetic rats.

Tetrahydrocurcumin (THC), one of the major metabolites of curcumin, is produced by the rapidly metabolized curcumin during absorption from the intestine. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin. Interestingly, it has been reported to be a more potent antioxidant than curcumin (Sugiyama, Kawakishi, and Osawa, 1996: 519-525; Atsumi, Fujisawa, and Tonosaki, 2005: 236-242). Numerous studies in experimental animals indicate that tetrahydrocurcumin also has anti-cancer effect (Yoysungnoen et al., 2008: 2003-2009), hypoglycemic effect (Pari and Murugan, 2007: 323-329), anti-inflammatory effect (Hong et al., 2004: 1671-1679), anti atherosclerotic effect (Naito et al., 2002: 243-250), and anti hepatotoxicity (Pari and Murugan, 2004: 481-486). Moreover, THC supplementation has been reported to prevent cerebral endothelial dysfunction in

streptozotocin (STZ)-induced diabetic rats (Jariyapongskul, Patumraj, and Suksumrarn, 2008: 151-155).

To our knowledge, so far no other investigations have been carried out on the effect of curcumin on gingival microcirculation of experimental diabetic model. Since THC also contribute to the physiological and pharmacological activities of curcumin, the present investigation aims to assess the effect of long-term feeding of curcumin and THC on gingival microvascular dysfunction in streptozotocin-induced diabetic rats. The study of curcumin in comparison with THC is performed in order to find out which one is more effective in protecting the abnormality of gingival microcirculation.

Research questions

1. Could curcumin and tetrahydrocurcumin prevent gingival microvascular dysfunction in diabetes-induced rat model?

2. Do oxidative stress and/or hypoglycemic action involve in the protective effect?

3. Is tetrahydrocurcumin more potent than curcumin in the protective effect?

Research objectives

1. To study the protective effects of curcumin and tetrahydrocurcumin on gingival microvascular dysfunction in diabetes-induced rat model.

2. To study the mechanism underlines the beneficial effect of curcumin and tetrahydrocurcumin in diabetes-induced rat model.

3. To compare the protective effects of curcumin and tetrahydrocurcumin on gingival microvascular dysfunction in diabetes-induced rat model.

Hypothesis

Curcumin and tetrahydrocurcumin can prevent gingival microvascular dysfunction in diabetic rats by decreasing oxidative stress and/or blood glucose level. The protective effect of tetrahydrocurcumin is more potent than curcumin.

CHAPTER II

REVIEW OF THE LITERATURE

Diabetes mellitus

Definition

Diabetes mellitus is a state of chronic hyperglycemia (Hyperglycemia; the state of having an overmuch concentration of glucose in blood) (WHO Expert Committee on Diabetes Mellitus: second report, 1980: 1-80). The cause of diabetes mellitus usually due to hereditary and/or environment. Insulin is a hormone synthesized and secreted by the β-cells of the islets of Langerhans in the pancreas; a hormone is a major regulator glucose concentration in the blood. Hyperglycemia may be the result of insufficient production of insulin or resistance of cell to insulin effect. This condition leads to abnormalities of carbohydrate, protein, and lipid metabolism.

Classification

Type 1 diabetes mellitus

Type 1 diabetes mellitus is characterized by loss of the insulin-producing β-cells in the pancreas leading to insulin deficiency. Type 1 diabetes is called “insulin dependent” or “juvenile” diabetes because it shows majority of the diabetes cases in children. Autoantibodies against β-cells and insulin are major causes of type 1 diabetes mellitus. These autoantibodies include islet cell antibodies, insulin autoantibodies, and glutamic acid decarboxylase antibodies. Type 1 diabetes associates with human leukocyte antigen (HLA) phenotypes on chromosome 6 responsible for class-II histocompatability complexes that is DR3, DR4, and DR3/DR4. Environmental factors are related in the pathogenesis of type 1 diabetes such as viruses (mumps, Coxsackie’s B, and rubella), dietary substances (bovine milk albumin and gluten), severe or prolonged stress and drug (phenytoin, diuretics, oral contraceptive steroids, and

β-adrenergic agonists). Pathogenesis of type 1 diabetes model shows that environmental factors trigger an autoimmune response against the pancreas, leading to chronically over years to progressive loss of β-cell mass and impairment of insulin secretion. In the event that the β-cell mass is approximately at 20% of its original content, clinical presentation of type 1 diabetes patients often occur with diabetic ketoacidosis. Over the next one or two years β-cell function may improve and within this period; patients may use of little or no exogenous insulin. This is known as "Honeymoon period" of type 1 diabetes. Nevertheless, months or years later, autoimmune β-cell destruction will be complete. There is no endogenous insulin secretion extant and the patient will be declared "insulin-dependent" (Figure 2.1).

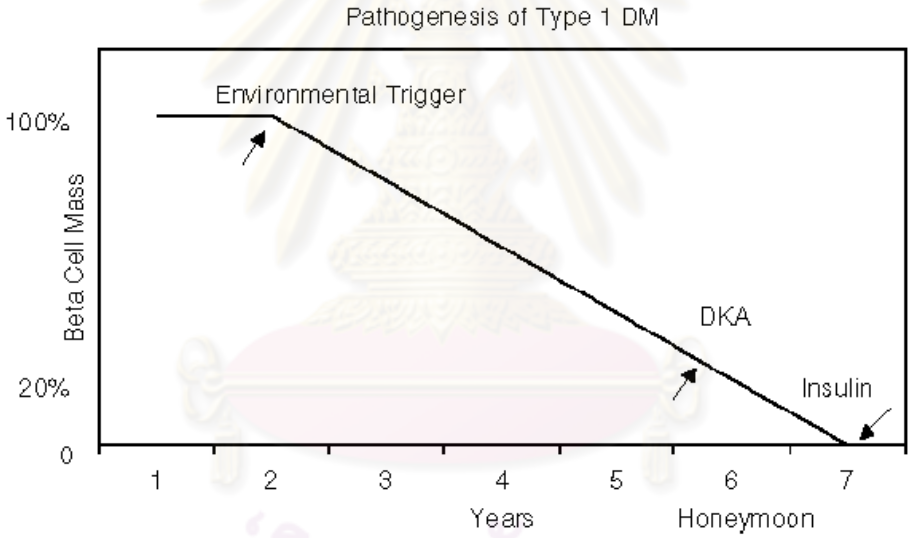


Figure 2.1 Schema of the development of type 1 diabetes in a model individual. After activation by environmental trigger, the autoimmune attack against pancreatic β-cell results in progressive loss of β-cell mass over several years. Clinical presentation often occurs with DKA when the β-cell mass is approx 20% of its original content. Over the next 1 or 2 years, the autoimmune attack persists resulting in complete loss of β-cell mass and true insulin-dependence. The honeymoon period is the term for this temporary time when use of little or no insulin is required (Veves, Giurini, and LoGerfo, 2006)

Type 2 diabetes mellitus

Type 2 diabetes mellitus is characterized by reduced response of target tissues to insulin that is called “insulin resistance”. Insulin levels are often quite high and resistance to the hormone is counteracted by increased stimulation of the hormone receptor. The pathogenesis of type 2 diabetes is both genetic and environmental cause. Several observations showed that a usual family history of individual with diabetes, typically involving in a first-degree relative and the prevalence of diabetes is highly correlated with a contemporaneous increase in the prevalence of obesity. There are many theories describing the cause and mechanism of type 2 diabetes. Central obesity (fat concentrated around the waist in relation to abdominal organs, but not subcutaneous fat) is known to predispose individuals to insulin resistance. Abdominal fat stimulates activities and secreting a group of hormones called adipokines that may possibly impair glucose tolerance (Veves et al., 2006).

Vascular complication in diabetes mellitus

The development of diabetic complications is a major cause of morbidity and mortality in diabetic patients. Clinical studies have shown a strong relationship between hyperglycemia and diabetic microvascular and macrovascular complication such as atherosclerosis, retinopathy, nephropathy, periodontitis (Jakus and Rietbrock, 2004: 131-142). Moreover, these complications are seen in both type 1 and type 2 diabetes. The incidence and progression of these complications are recognized to be due, in a large part, to persisting poor glycemic control (Tsai, Hayes, and Taylor, 2002: 182-192).

Microvascular function

The microcirculation is a complex system that regulates the distribution of red blood cell (RBC) and plasma throughout individual organs. Blood flow into an organ is controlled by arteriolar network, which vessels are surrounded by smooth muscle that either constricts or relaxes in response to the balance between pressure and dilatory

stimulation. In the event of downstream of the arterioles, RBC flow is distributed throughout the capillary networks. O_2 and nutrients are diffused from the RBC and exchanges CO_2 and other waste products from neighboring tissues (Bateman, Sharpe, and Ellis, 2003: 359-373). The endothelial cell (EC) is a critical component of the integrated microvascular system, which lines the interior surface of blood vessels wall and separates the lumen from the vascular smooth muscle cell (VSMC). Structure and function of EC are important in the maintenance of the microcirculatory function. As a physical barrier, the EC is semi-permeable and controls small and large molecules transport between blood and tissue (Sumpio, Riley, and Dardik, 2002: 1508-1512).

In addition to maintain vascular homeostasis, the EC produces a variety of regulatory chemical mediators (Table 2.1) and membrane exposure molecules (Table 2.2) that are responsible for one or more of the following.

Substances released by endothelial cells	Effects on vascular homeostasis
Plasminogen activator inhibitor	Antifibrinolytic
Tissue plasminogen activator	Fibrinolytic
Protein S	Anticoagulant, profibrinolytic
Tissue factor pathway inhibitor	Anticoagulant
Endothelin-1	Vasoconstrictor
von Willebrand factor	Coagulant (protects factor VIII), platelet adhesion
Nitric oxide	Vasodilator, inhibits platelet aggregation and adhesion
Prostacyclin	Vasodilator, inhibits platelet aggregation and adhesion
Cytokines (including interleukin-1, -6, -8, monoyte chemoattractant protein, and colony-stimulating factor)	Leukocyte function

Table 2.1 Summary of the main substances released by endothelial cells and their most important effects on vascular homeostasis (Ribeiro et al., 2009: 1121-1151)

Exposed on the luminal surface of endothelial cells	Effects on vascular homeostasis
Antithrombin III P2y and ET_B receptors	Anticoagulant Vasodilation, platelet inhibition
Angiotensin-converting enzyme	Vasoconstriction
Plasmin receptors	None in the absence of fibrinolysis
Ectonucleotidases	Vasodilation, platelet inhibition
Coagulation factor receptors	None in the absence of fibrinolysis
Thrombin receptors	Anticoagulant, vasodilation, platelet inhibition
Annexin V	Anticoagulant
Heparan sulfate	Anticoagulant
Thrombomodulin	Anticoagulant
Platelet activating factor	Platelet and leukocyte activation
Tissue factor (thromboplastin)	Coagulant
Cell adhesion molecules	Leukocyte adhesion and migration, platelet activation

Table 2.2 Substances expressed on the surface on endothelial cells and their most important effects on vascular homeostasis (Ribeiro et al., 2009: 1121-1151)

1. Regulation of vascular tone through vasodilatation or vasoconstriction for regulation of organ blood flow and blood pressure

Under physiological circumstances, the regulation of vascular tone is a balanced by the synthesis and secretion of endothelial-derived relaxing factors (EDRFs) such as nitric oxide (NO) and prostacyclin (PGI_2), and contracting factors such as endothelin-1 (ET-1), and angiotensin II (ANG-II).

1.1 Nitric oxide (NO)

NO is diffusible molecular messenger in the vascular and immune system. It is generated through oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS) which classified as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Table 2.3)

Isoform	Protein weight (KDa)	Ca^{2+} dependency	Expression	Tissue distribution
nNOS	160	Ca^{2+} dependent	constitutive	Central and peripheral nervous system
iNOS	130	Ca^{2+} independent	inducible	Macrophage
eNOS	135	Ca^{2+} dependent	constitutive	Endothelial cells

Table 2.3 Comparison of three nitric oxide synthase isoforms (Nahrevanian, 2009: 440-448)

nNOS (NOS type I, NOS-I and NOS-1) is found in neuronal tissue (central nervous system, parasympathetic ganglia and nonadrenergic noncholinergic peripheral autonomic nerve fibers). nNOS is important in regulation of central nervous system blood flow, peripheral and central transmission of pain signals, neurotransmitter release from cholinergic nerve fibers and neurotransmitter that is involved in memory formation (Melikian et al., 2009: 256-262).

iNOS (NOS type II, NOS-II and NOS-2) is Ca^{2+} calmodolin independent and contributes to pathology in the vessel. It is rapidly and durably expressed by inflammatory cell (such as macrophages) in response to endotoxins and inflammatory cytokines. An excess iNOS-induced NO during inflammation can mediate cell and tissue injury (Skaleric et al., 2006: 7010-7013; Nahrevanian, 2009: 440-448).

eNOS (NOS type III, NOS-III and NOS-3) is necessary for the regulation of vascular tone and regional blood flow in physiologic microvascular function. NO produced by eNOS in the endothelium diffuses to the VSMC where it activates the enzyme guanylate cyclase through binding between NO and heme moiety of guanylate cyclase. The effect of interaction increases in cyclic guanosine monophosphate (cGMP) production which mediates relaxation of VSMC. Thus the net effect of an increase in NO is vasodilatation. Several stimuli can activate eNOS to produces NO, including mechanical shear stress, estrogens, insulin, acetylcholine, and other receptor-dependent agonists. Moreover, eNOS contributes to inhibition of platelets aggregation, modulation of leukocyte–endothelial interactions, and modulation of VSMC proliferation (Figure 2.2) (Atochin and Huang, 2010: 965-974).

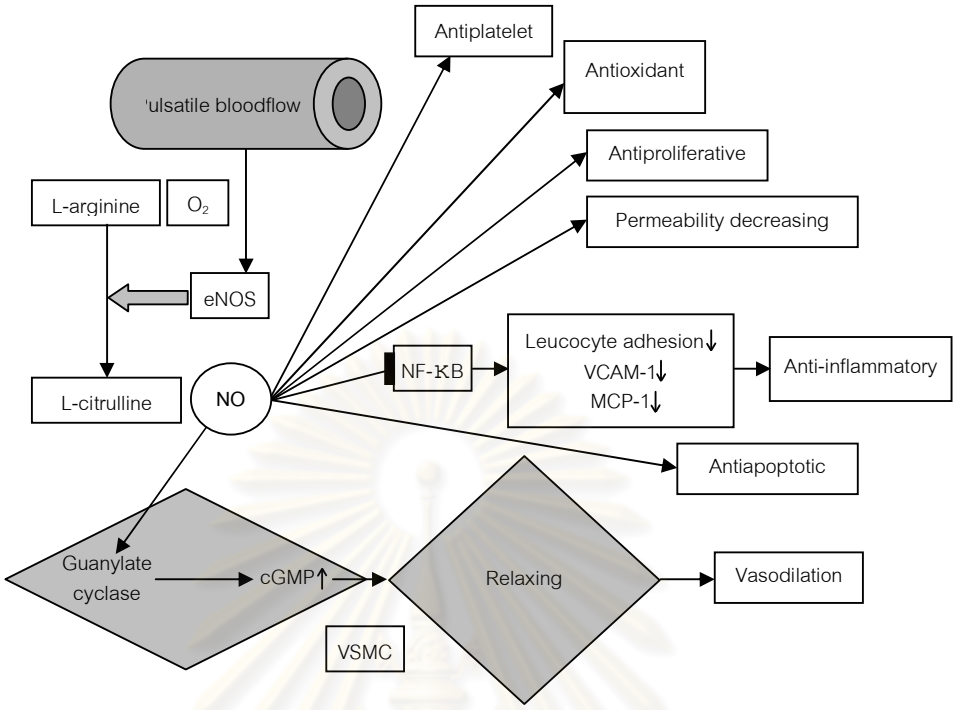


Figure 2.2 Properties and production process of NO (nitric oxide) as important factor in endothelial function (van den Oever et al., 2010: 1-15)

1.2 Prostacyclin (PGI_2)

PGI_2 is one of a family of lipids derived from arachidonic acid, prostaglandins. PGI_2 synthase synthesizes it from prostaglandin H2 (PGH_2), which is produced by hydrolysis of arachidonic acid by cyclooxygenase-2 (COX-2). It is synthesized from EC in response to various substances, including shear stress bradykinin, thrombin, adenosine triphosphate (ATP), and adenosine diphosphate (ADP). PGI_2 is described as a substance inhibiting platelet aggregation and a potent vasodilator; these properties are mediated by stimulation of adenyl cyclase to produce cyclic adenosine monophosphate (cAMP) in platelets and VSMC (Figure 2.3) (Ribeiro et al., 2009: 1121-1151).

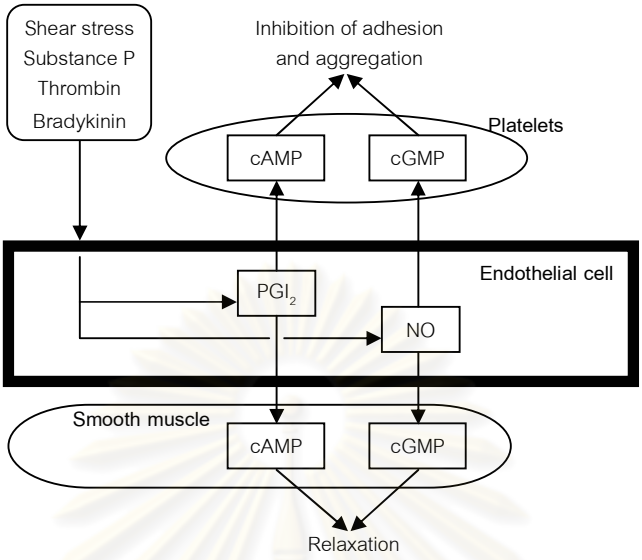


Figure 2.3 Stimuli for the release of PGI_2 and NO by EC. High levels of cAMP activate protein kinase A, which then activates myosin light chain kinase, promoting smooth muscle relaxation and vasodilation. cAMP inhibits undue platelet activation such as may occur with binding to thromboxane A_2. cGMP acts as a second messenger in a similar fashion to cAMP, by activating intracellular protein kinases (Ribeiro et al., 2009: 1121-1151)

1.3 Endothelin-1 (ET-1)

ET-1, a potent vasoconstrictor peptide is synthesized and stored inside secretory vesicles in endothelial cells. Several physical and chemical stimuli that include hypoxia, angiotensin II (ANG-II), growth factors and other cytokines can activate EC to release large quantities of ET-1. In physiologic endothelial function there is a constant balance between the production of NO and ET-1, with NO's ability to limit ET-1 production counterbalanced by ET-1's stimulation of endothelial production of NO and other vasodilator peptides. In addition, ET-1 also includes stimulating growth and proliferation of VSMC in the vessel wall (Ribeiro et al., 2009: 1121-1151).

Angiotensin II (ANG-II)

ANG-II, one of mediator that induces vasoconstriction is synthesized by EC. It has important regulation of vascular tone and fluid volume in cardiovascular circulation. ANG-II binds to and activates specific angiotensin receptors for regulates several VSMC functional activities including contraction, growth, proliferation and differentiation. On the whole, the effects of ANG-II oppose those of NO (de Gasparo, 2002: 347-358).

2. Regulation of platelet function, coagulation and fibrinolysis for maintenance of fluidity of blood and avoidance of bleeding

Normal endothelium function is anticoagulant and antithrombotic. It regulates hemostasis and thrombosis depending on the expression and release of several molecules. PGI_2 and NO play important role in inhibiting platelet aggregation which is essential for protecting against various prothrombotic conditions. Endothelium limits activation of the coagulation cascade by thrombomodulin/protein C and thromboplastin/tissue factor inhibitor interactions. Moreover, it regulates fibrinolysis through tissue plasminogen activator (t-PA) and its physiological inhibitor plasminogen activator inhibitor-1 (PAI-1) (Ribeiro et al., 2009: 1121-1151; van den Oever et al., 2010: 1-15).

3. Regulation of leukocyte traffic through pro-inflammatory cytokines and adhesion molecules those involved in the inflammatory reaction.

Under basal conditions, EC minimally expresses adhesion molecules that encourage interaction of circulating leukocytes such as intramolecular cell adhesion molecules-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1). However, thrombin, endotoxins or inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) can activate EC to induce surface expression of adhesion molecules that are necessary for the adhesion, rolling and migration of leukocytes in the bloodstream to damaged tissue (Ribeiro et al., 2009: 1121-1151). NO generated from EC inhibits interaction of

leukocytes as well as cytokine induced expression of adhesion molecules, possibly through the inhibition of the transcription factor nuclear factor κ B (NF-κB) (van den Oever et al., 2010: 1-15).

Microvascular dysfunction in diabetes mellitus

Several studies have demonstrated that type 1 and type 2 diabetes are presence of microvascular dysfunction, which is effected of endothelium abnormality. This shows imbalance in the production of mediators that regulate vascular tone, platelet aggregation, coagulation, fibrinolysis and increase leukocyte adhesion in experimental models of both type diabetes (Goldberg, 2009: 3171-3182; Ribeiro et al., 2009: 1121-1151).

1) Impairment of endothelium-dependent vasodilation is a feature of resistance arteries in experimental diabetic animal models that arise from insufficient availability of EDRFs especially NO, which can be caused by diminished eNOS gene expression, L-arginine deficiency and uncoupling of eNOS and tetrahydrobiopterin deficiency (Mayhan, 1989: H621-H625; Atochin and Huang, 2010: 965-974). In streptozotocin (STZ)-induced diabetic rats, reduced endothelium-dependent vasodilation to acetylcholine (ACh) of isolated thoracic aorta have been exhibited after 4 weeks of diabetes (Baluchnejadmojarad and Roghani, 2008: 1-5). Moreover, enhanced vascular response to vasoconstrictors is associated with increased vascular tone in diabetes. Yousif et al. (2009: 1-12) demonstrated that administration of norepinephrine, ET-1 and ANG-II in the isolated mesenteric vascular bed and renal artery increased vasoconstrictor responses in STZ-induced diabetic rats.

2) Coagulation abnormalities occurred in diabetes. Coagulation factors such as PAI-1, von Willebrand's factor, and fibrinogen are elevated in diabetics with poor glycemic control conditions, which inhibit fibrinolysis and conduce to plaque progression. In addition, platelet aggregation and adhesion to endothelial cells increases in association with diabetes (Kassab, McFarlane, and Sower, 2001: 249-255). The importance of NO in platelet function was explained by *in vitro* experiments such as inhibition of platelet aggregation by NO donors (de Belder et al., 1994: 691-694). In

diabetes, the reduction of NO bioactivity is associated with the platelet activation, which suggests as a potential mechanism contributing to the accelerated atherosclerosis seen in diabetic patients by detrimental effects such as capillary microembolization, local progression of vascular lesions, and triggering of acute arterial thrombosis (Schafer et al., 2004: 1720-1726).

The impairment of endothelium-dependent vasodilation and coagulation abnormalities contribute to blood flow reduction in diabetes. The reduction of blood flow was found in various organs including, cerebral, ocular, coronary, renal, forearm, hindlimb and other skeletal muscle. The alterations of blood flow were found in type 1 and type 2 diabetes in experimental animals and humans (Toda, Imamura, and Okamura, 2010: 189-209).

Hyperglycemia induced the upregulation of proinflammatory mediators and adhesion molecules. Glucose is known to increase generation of ROS that enhance binding of NF-ĸB to transcriptional targets and subsequently, to increase systemic levels of immune parameters like TNF-α, IL-6, and ICAM-1 (Kempf et al., 2007: 389-396). Inflammation and leukocyte-endothelial interactions are critical in the development of diabetic retinopathy and nephropathy pathogenesis (Adamiec and Oficjalska-Mlynczak, 2005: 330-333; Galkina and Ley, 2006: 368-377). These cause the elevation of capillary permeability, capillary occlusion, and destruction.

Inflammatory cytokines such as TNF-α activates endothelial cells to induce expression of a series of molecules that are essential for the adhesion, rolling, and migration of leukocytes in the bloodstream to damaged tissue, on endothelia cells surface. The process of leukocyte traffic is mediated by cell adhesion molecules (CAMs), which are glycoprotein that expressed on the surface of activated cells that are involved in cell-cell and cell-matrix binding. There are three main groups of regulation leukocyte traffic, 1) the immunoglobulin superfamily includes intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), 2) the selectins includes the endothelial leukocyte adhesion molecule (E-selectin), P-selectin and the leukocyte adhesion molecule (L-selectin), and 3) the integrin family includes integrin β1, β2 and β7.

The conventional multistep paradigms of the leukocyte-endothelial interactions are shown in Figure 2.4 (Galkina and Ley, 2006: 368-377; Ribeiro et al., 2009: 1121-1151).

1). Selectin-dependent leukocyte rolling on the endothelial layer: selectins are expressed on the surface of endothelial cells, leukocytes and platelets. It stabilizes leukocyte-endothelium interactions by promoting cell-cell adhesion. Selectins bind reversibly to leukocytes, capturing them from the bloodstream, slowing their movement and initiating their rolling over the endothelial surface.

2). Chemokine-dependent integrin activation with subsequent leukocyte adhesion: firm binding of leukocytes to the endothelium by the immunoglobulins including ICAM-1 and VCAM-1, which interact with integrins on the surface of rolling leukocytes and bring about a stable bond to the endothelium. After expression of VCAM-1, cells with VCAM-1 receptors adhere preferentially to the sites of expression, and are subsequently stimulated to migrate through endothelial junctions into the subendothelial space.

3). Diapedesis: the final stage of leukocyte migration between endothelial cells involves the plateletendothelial cell adhesion molecule-1 (PECAM-1), which is concentrated at endothelial junctions and facilitates leukocyte diapedesis.

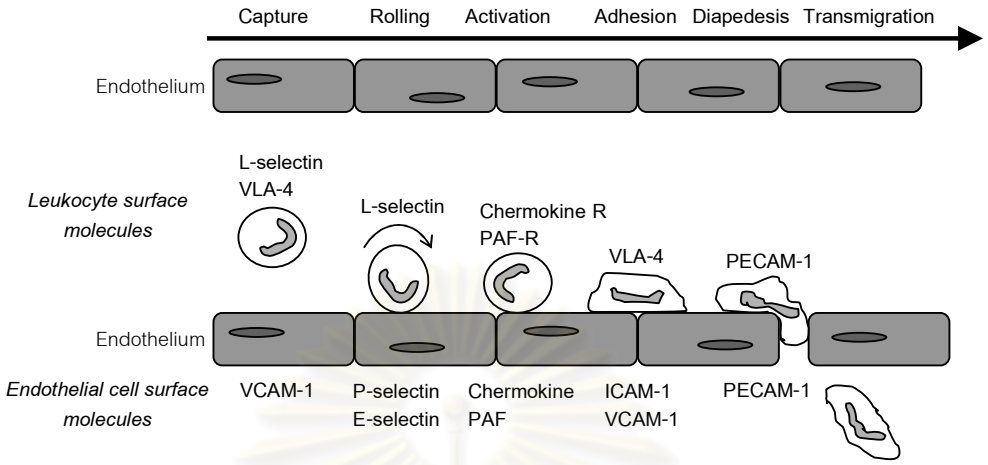


Figure 2.4 Sequential multistep model of leukocyte-endothelial adhesion. Extravasculation of leukocyte from blood into tissues mediated by cascade of adhesive interactions between leukocytes and EC (Ribeiro et al., 2009: 1121-1151).

Gingival microvascular dysfunction in diabetes mellitus

Gingiva is the mucous membrane extending from the cervical portion of the tooth to the zone of the alveolar mucosa, holding the jaw and teeth roots in place and protecting them from infections (Goldman and Cohen, 1969). Gingivitis and periodontitis are the 2 major forms of inflammatory diseases affecting the gingiva. Chronic gingivitis is the very common inflammatory reaction occurring in the gingival tissues, which exhibit signs of inflammation, redness and swelling, but without involvement of the periodontium. The periodontium comprises the connective tissue, cementum, periodontal ligaments, and the alveolar bone. Periodontitis occurs when inflammation spreads to the periodontium that is characterized by loss of connective tissue attachment and alveolar bone (Treatment of plaque-induced gingivitis, chronic periodontitis, and other clinical conditions, 2001: 1790-1800).

Gingival microvascular dysfunction causes the periodontitis, which is diabetic microvascular pathology (Nassar, Kantarci, and van Dyke, 2007: 233-244). Several studies have demonstrated a relation between diabetes and gingival microvascular dysfunction. Comprehensive evaluation indicates that diabetes has two to three times higher risk for incidence of periodontitis (Taylor, 2001: 99-112). Moreover, poorly controlled diabetes has a significantly higher prevalence of severe periodontitis (Seppala, Seppala, and Ainamo, 1993: 161-165; Tsai et al., 2002: 182-192). It has been reported that the expression of inflammatory cells is increased in gingival tissue twelve weeks after the diabetic induction in rats (Claudino et al., 2007: e1320). The biologic link between diabetes and gingival microvascular dysfunction was caused by hyperglycemia lead to the generation of ROS and AGEs (Vlassara and Palace, 2002: 87-101; Akalin et al., 2008: 44-52). The AGE-RAGE interaction and ROS can induce oxidative stress that may contribute to gingival inflammation, periodontal tissue destruction, and alveolar bone loss.

Oxidative stress as a pathogenesis of diabetic

Hyperglycemia is a condition to which an excessive amount of glucose circulates in the blood plasma. This exists and relates causally to pathogenesis of microvascular and macrovascular complications in diabetes. Possible mechanism of hyperglycemia detrimental effect was suggested that hyperglycemia induces a variety of biochemical changes in vascular endothelial cells by numerous pathways (Brownlee, 2005: 1615-1625). A major mechanism is produced by glucose metabolites in glycolysis, electron transport in mitochondria and the other mechanism involves in AGEs (Aronson and Rayfield, 2002: 1-10). These pathways may contribute to increased oxidative stress, which is a common pathway related to pathogenesis of complication in diabetes (Baynes, 1991: 405-412; Giugliano, Ceriello, and Paolisso, 1996: 257-267; Kashiwagi et al., 1996: S84-S86).

Oxidative stress

Oxidative stress has been defined as a disturbance in the balance between antioxidants and pro-oxidants, with increased levels of pro-oxidants leading to tissue damage. Pro-oxidants are reactive species that can be divided into reactive nitrogen species (RNS) and reactive oxygen species (ROS). The well-characterized reactive species are free radicals such as superoxide (O_2^-), nitric oxide (NO), hydroxyl (OH), and peroxynitrite ($ONOO^-$). They have been defined as any species capable of independent existence that contain one or more unpaired electrons. Naturally, highly reactive and diverse species, capable of extracting electrons and thereby oxidizing a variety of biomolecules vital to cell and tissue function (Annuk, Zilmer, and Fellstrom, 2003: S50-S53) (Figure 2.5).

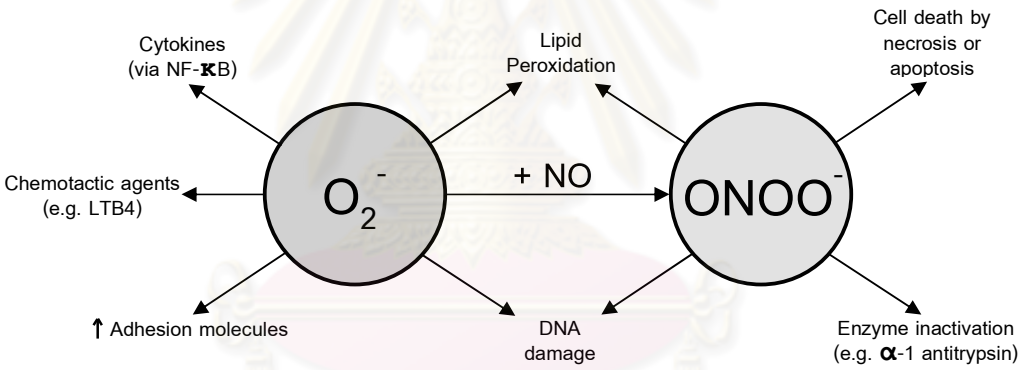


Figure 2.5 The interactions between superoxide and nitric oxide to form the peroxynitrite anion and the molecular and cellular effects of these reactive species (Chapple and Matthews, 2007: 160-232)

ROS are intermediary metabolites that are normally produced in the course of oxygen metabolism. Under physiological conditions, ROS play a critical role as signal molecules, and ROS are produced by activated leukocytes and macrophages are essential for the defense against invading microorganisms (Zalba et al., 2007: 24-29). In addition to a mitochondrial origin, ROS can be generated by enzymes including oxidases, cyclooxygenases and lipoxygenases.

The hyperglycemia-induced intracellular ROS are increased through several pathways. A major mechanism is produced by the proton electromechanical gradient generated by the mitochondrial electron transport chain, thus resulting in increased production of superoxide (Aronson and Rayfield, 2002: 1-10). The other mechanism involves the AGEs.

1. Oxidative stress produced by glucose metabolic pathway

In normal cell metabolism, glucose oxidation starts with glycolysis in the cytoplasm and produces the reduced form of nicotinamide adenine dinucleotide (NADH) and pyruvate. Pyruvate can be transported into the mitochondria for produce CO_2, H_2O, one molecule of the reduced form of flavin adenine dinucleotide ($FADH_2$) and four molecules of NADH by the oxidation of tricarboxylic acid (TCA) cycle. $FADH_2$ and NADH provide energy for ATP production through oxidative phosphorylation by the electron-transport chain.

The mitochondrial electron-transport chain consisted with four inner membrane-associated enzyme complexes called complex I, II, III and IV, plus cytochrome c and the mobile electron carrier ubiquinone. NADH obtained from both glycolysis and TCA cycle activity donates electron to complex I (NADH:ubiquinone oxidoreductase) and $FADH_2$ obtained from TCA cycle activity donates electron to complex II (succinate:ubiquinone oxidoreductase). Both complex I and II eventually transfer electrons to ubiquinone, then the ubisemiquinone radical-generating Q cycle transfer electrons from reduced ubiquinone to complex III (ubiquinol:cytochrome c oxidoredutase). Electron transport then progresses through cytochrome c, complex IV (cytochrome c oxidase) and finally to O_2 molecule, which they reduce to water.

During transportation of electrons through complex I, II and IV, some energies of those electrons are used to pump proton across the mitochondrial inner membrane in these complexes. This generates proton gradient that drives the synthesis of ATP by ATP synthase. Otherwise, uncoupling proteins (UCP) can bleed down the proton gradient to generate heat as the way of maintenance the rate of ATP generation constant.

In hyperglycemia, glucose enters the endothelial cell via the facilitative transporter. This is not insulin-sensitive and continuously transports glucose. Intracellular hyperglycemia increases glucose oxidation in the TCA cycle, this effect pushes more NADH and $FADH_2$ into the electron-transport chain. High activity of electron-transport chain that causes increased proton gradient across the inner mitochondrial membrane until a critical threshold is arrived. At this point, electron transfer to complex III is blocked, causing ubiquinone donates the electron to molecule O_2, in that way generating O_2^- (Brownlee, 2005: 1615-1625) (Figure 2.6).

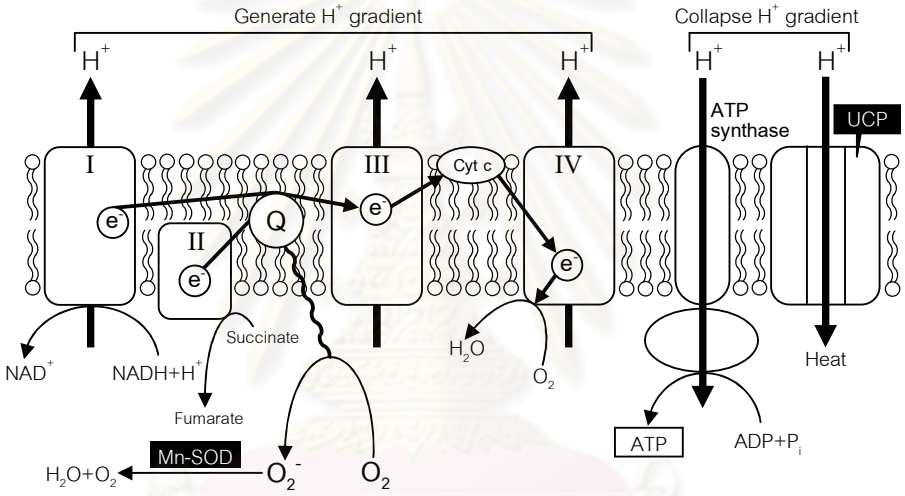


Figure 2.6 Hyperglycemia-induced production of O_2^- by the mitochondrial electron transport chain (Brownlee, 2005: 1615-1625)

2. Oxidative stress produced by increasing advanced glycation end products (AGEs)

Hyperglycemia can increase AGEs from non-enzymatic reactions between extracellular proteins and glucose. Glucose forms chemically reversible early glycosylation products with reactive amino groups of circulating or vessel wall proteins (Schiff bases), which subsequently rearrange to form the more stable Amadori-type

early glycosylation products. Some of the early glycosylation products on long-lived proteins (e.g. vessel wall collagen) continue to undergo complex series of chemical rearrangement to form AGEs (Figure 2.7). The cellular interactions of AGEs are mediated through a specific receptor for AGEs (RAGE) determinants on cell surfaces. AGEs interaction with RAGE on endothelial cells, resulting in the induction of oxidative stress by upregulate oxidative stress response genes and release oxygen radicals that caused microvascular complications (Aronson and Rayfield, 2002: 1-10) (Figure 2.8).

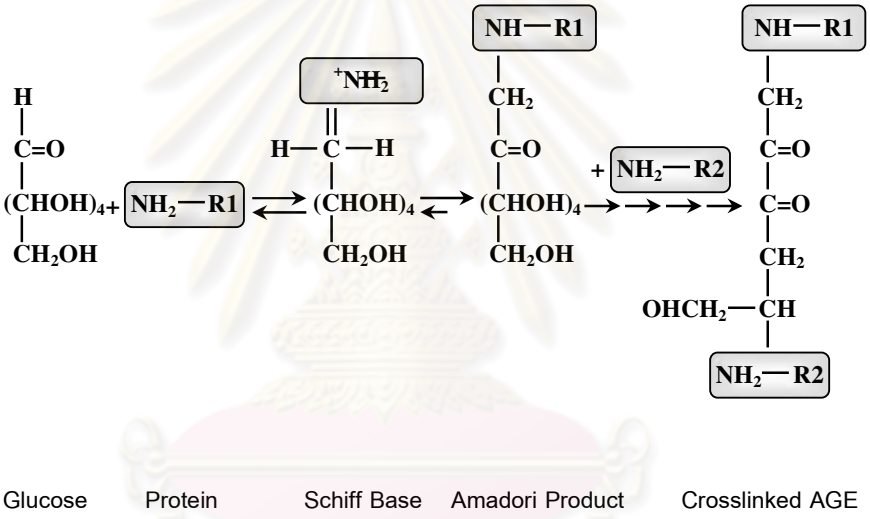


Figure 2.7 The formation of advanced glycosylation end products (Aronson and Rayfield, 2002: 1-10)

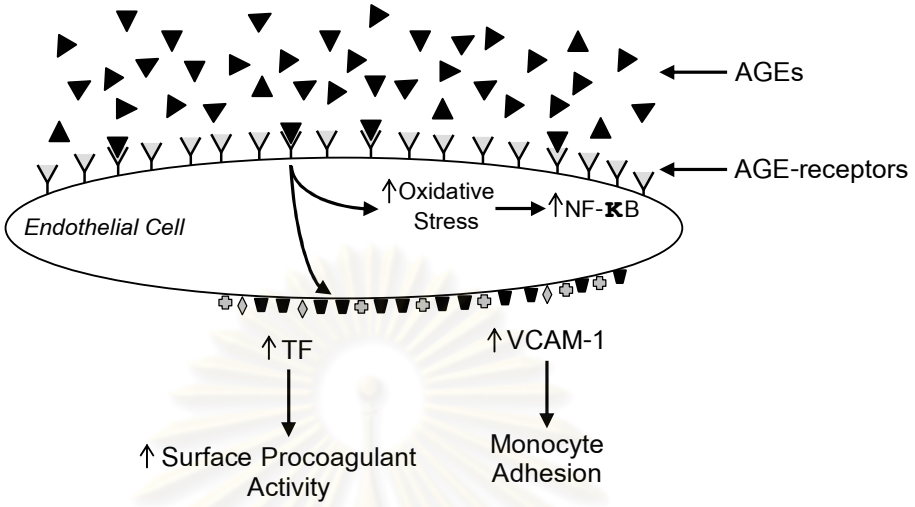


Figure 2.8 Consequence of AGEs interaction with the AGE receptors on endothelial cell (Aronson and Rayfield, 2002: 1-10)

Curcumin

Curcumin occurs naturally in the root of turmeric (*Curcuma longa* Linn) (Figure 2.10). Turmeric contains curcumin along with other chemical constituents known as the "curcuminoids". The major curcuminoids present in turmeric are curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%). The curcuminoids are polyphenols and are responsible for the yellow colour of turmeric (Figure 2.9).

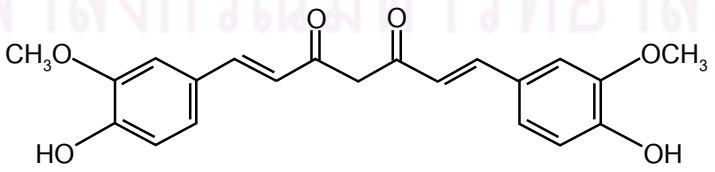


Figure 2.9 Chemical structure of curcumin

Figure 2.10 *Curcuma longa* Linn

(http://learners.in.th/blog/egg20/325478)

Curcumin is poorly absorbed from the gut because curcumin is transformed during absorption from the intestines, and the transformed products, which is more polar and colorless than curcumin, enters the serosal side. Curcumin was first biotransformed to dihydrocurcumin and tetrahydrocurcumin and that these compounds subsequently were converted to monoglucuronide conjugates. Thus, curcumin–glucuronide, dihydrocurcumin–glucuronide, tetrahydrocurcumin–glucuronide, and tetrahydrocurcumin are major metabolites of curcumin in mice plasma (Lin, Pan, and Lin-Shiau, 2000: 153-158). Most of the curcumin administered was reduced by an endogenous reductase system and subsequently glucuronidated by UDP-glucuronosyl transferases. The main biliary metabolites of curcumin are glucuronide conjugates of tetrahydrocurcumin and hexahydrocurcumin (Holder, Plummer, and Ryan, 1978: 761-768) (Figure 2.11).

Curcumin is unstable at neutral and basic pH values and is degraded to ferulic acid and feruloylmethane. More than 90% of curcumin decomposes rapidly in buffer systems at neutralbasic pH conditions. Curcumin is unstable in phosphate buffer at pH 7.4 therefore curcumin should be stable in the stomach and small intestines because the pH is between 1 and 6, and degradation of curcumin is extremely slow in these conditions (Oetari et al., 1996: 39-45; Wang et al., 1997: 1867-1876).

Curcumin has a potent antioxidant activity, the antioxidant mechanisms of curcumin have been the focus of interest of free radicals chemists and biologists. Curcumin is free-radical scavenging activity, which contains a phenolic group (Figure 2.9). Curcumin is superb electron donor by donating the H-atom from the phenolic group to free radical molecule for neutralizing free radicals forming stable products (Menon and Sudheer, 2007: 105-125). In addition, several studies have shown that curcumin possesses anti-oxidant (Ruby et al., 1995: 79-83; Suryanarayana et al., 2007: BR286-BR292), anti-inflammatory (Kawamori et al., 1999: 597-601), and anti-carcinogenic activity (Rao et al., 1995: 259-266).

Several studies have reported that curcumin can reduce blood glucose levels but the mechanism is unclear (Arun and Nalini, 2002: 41-52; Mahesh, Sri Balasubashini, and Menon, 2004: 639-644; Murugan and Pari, 2006: 122-127). However, some experiments show that curcumin has no effect on blood glucose level (Majithiya and

Balaraman, 2005: 697-705; Suryanarayana et al., 2005: 2092-2099; Seo et al., 2008: 995-1004).

Toxicological studies have shown that curcumin is characterized by low toxicity. When administered orally doses up to 5 g/kg of body weight. No apparent toxic effects were seen in rats (Wahlstrom and Blennow, 1978: 86-92).

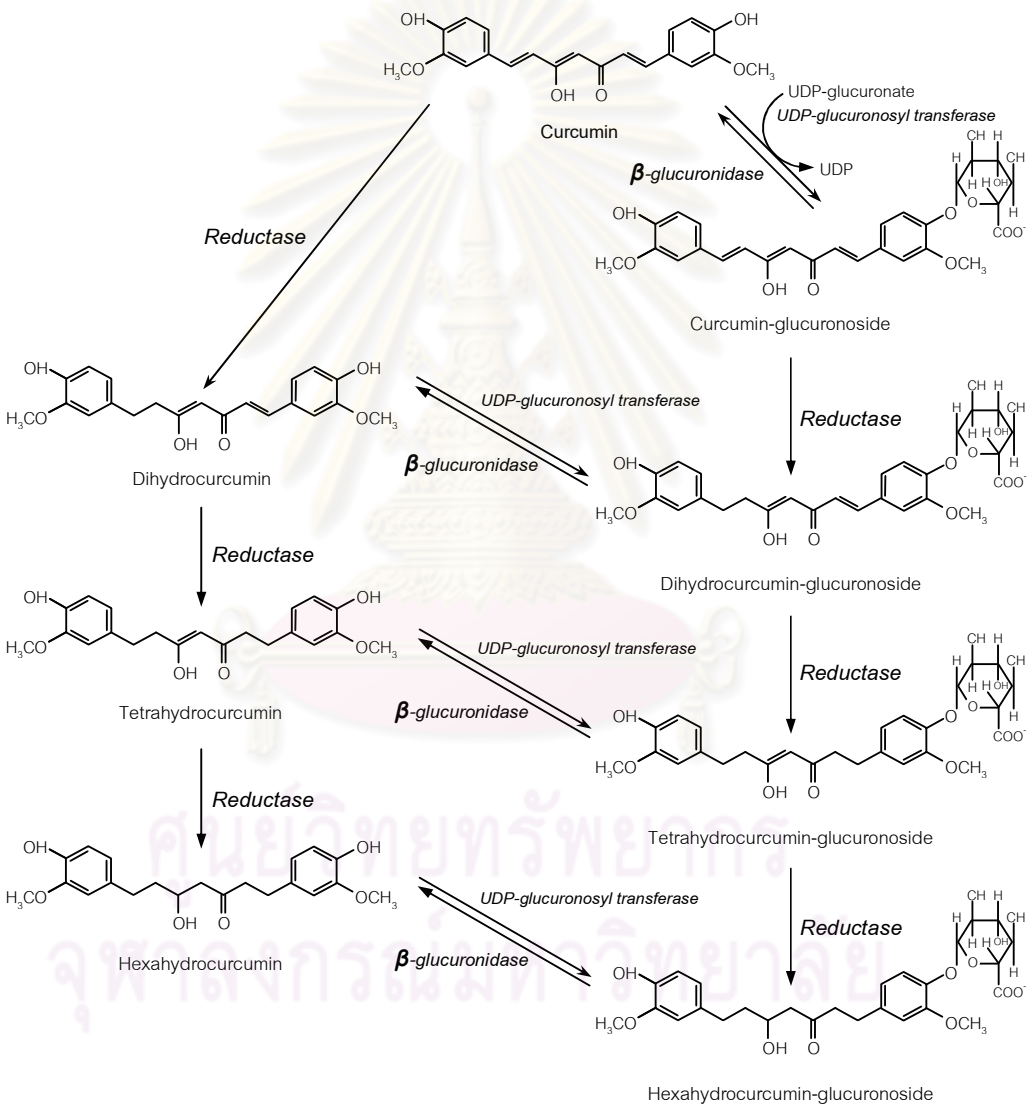


Figure 2.11 Proposed biotransformation and metabolites of curcumin (Pan, Huang, and Lin, 1999: 486-494)

Tetrahydrocurcumin

Tetrahydrocurcumin (THC) is one of the major metabolites of curcumin (Figure 2.12). THC is the rapidly metabolized product of curcumin during absorption from the intestine. It is a colorless product and shows appreciable hydrophillicity, unlike curcumin. THC possesses hydroxyl groups that make it a typical substrate for glucuronide conjugation in intestinal and hepatic. The final reduction of THC to hexahydrocurcuminol may occur in microsomes, possibly by cytochrome P450 reductase (Sugiyama, Kawakishi, and Osawa, 1996: 519-525). THC more stable than curcumin in buffer solutions of physiologic (pH 7.2) and basic pH. THC was also stable in rat plasma. These suggest that THC serve as the available forms of curcumin in vivo (Pan et al., 1999: 486-494). Structurally, THC and curcumin have identical beta-diketone structures and phenolic groups, but differ in that THC lacks the double bonds. THC exhibits physiological and pharmacological properties of protective effects on oxidative stress as similar to those of curcumin (Murugan and Pari, 2006: 122-127).

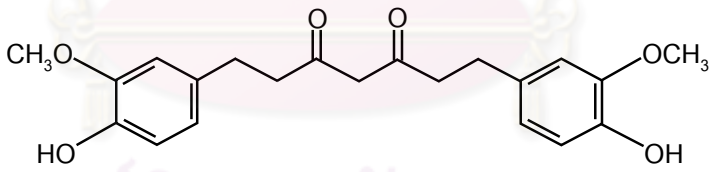


Figure 2.12 Chemical structure of tetrahydocurcumin

In *in vitro* studies, THC showed the strongest antioxidant activity of other curcuminoids (Osawa et al., 1995: 1609-1612). THC can reduce lipid peroxidation in tert-butylhydroperoxide - induced erythrocyte membrane model due to the structure of THC (Sugiyama et al., 1996: 519-525).

In *in vivo* study, Okada et al. (2001: 2090-2095) demonstrated the effect of curcumin and THC on oxidative stress-induced renal injury mice after feeding with a rat chow containing 0.5 g/100 g curcumin or THC for 4 weeks. THC showed stronger inhibitory effects of oxidative stress than curcumin and the concentrations of THC and its conjugates in the liver and serum were higher. They suggested that the antioxidant effects of THC were greater than those of curcumin because THC may be more easily absorbed from the gastrointestinal tract.

In addition, several studies in experimental animals indicate that THC also prevent cancer (Lin et al., 2000: 153-158), as well as a protective agent against inflammation (Nakamura et al., 1998: 361-370; Hong et al., 2004: 1671-1679), atherosclerotic lesions (Naito et al., 2002: 243-250) and hepatotoxicity (Pari and Murugan, 2004: 481-486).

CHAPTER III

MATERIALS AND METHODS

Animal preparation

Male Wistar rats (National Laboratory Animal Center of Salaya Campus, Mahidol University) weighing 200-250 g were divided randomly into diabetic and non-diabetic group. The animals were housed in a group of three per 1 cage. The animals were kept in well-ventilated room which the temperature 22±4°C with a 12 hours light and dark cycle which provide darkness from 7.00 PM to 7.00 AM. All animals were fed with regular dry rat chow and tap water *ad libitum* through the experimental period.

All experiments were conducted in accordance with the guidelines of The National Research Council of Thailand (1999) for experimental animals.

Diabetic induction

Diabetes was induced by a single intravenous injection of streptozotocin (STZ; Sigma chemical Co. USA, 55 mg/kg BW). Streptozotocin was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co., USA) and immediately injected into the tail vein. The same volume of citrate buffer pH 4.5 was injected by the same route to non-diabetic control animal. Diabetic condition is defined as a plasma glucose concentration equal to or greater than 200 mg/dL at 48 hours after streptozotocin injection. A glucometer (One Touch Ultra, a Johnson & Johnson, USA) was used for evaluation of plasma glucose from tail vein blood sample. Sample was analyzed by applying a drop of blood to a control strip inserted into the monitor. Rats treated with streptozotocin that did not exhibit an elevation of blood glucose level at 48 hours ($\geq$200 mg/dL) were excluded from the study (Sridulyakul, Chakraphan, and Patumraj, 2006: 315-321).

Curcumin and Tetrahydrocurcumin supplementation

Supplementation of the rats with curcumin (Cayman Chemical Co., USA) and tetrahydrocurcumin (THC) (Department of Chemical, Faculty of Science, Ramkamhang University) started 10 days after the administration of streptozotocin. Curcumin and tetrahydrocurcumin were prepared daily by dissolving in 0.9% normal saline (NSS) (Pari and Murugan, 2004: 481-486; Xia et al., 2006: 938-944). The rats were fed daily by gavage at a concentration of 100 mg/kg BW (Banerjee et al., 2003: 213-224; Jariyapongskul, Patumraj, and Suksumrarn, 2008: 151-155).

Experimental design

Each diabetic (STZ) and control (CON) group was subdivided into three groups (n=6): rats fed NSS, rats fed curcumin (Cayman Chemical Co., USA) 100 mg/kg BW dissolved in NSS, and rats fed THC (Department of Chemical, Faculty of Science, Ramkhamhaeng University) 100 mg/kg BW dissolved in NSS. Ten days after STZ injection or citrate buffer, the animals were fed with curcumin or THC to each group by gavage.

The experiment was carried out on the 8th week after the injection of streptozotocin or citrate buffer. At the end of experiment, the rats were weighed and anesthetized with sodium pentobarbital (60 mg/kg BW, i.p.) and a tracheotomy was performed. They were ventilated mechanically with room air and supplemental oxygen.

The rats were randomly divided into six groups of six animals each.

Group 1 CON : non-diabetic rats treated with NSS

Group 2 CON+CUR : non-diabetic rats treated with curcumin (100 mg/kg BW)

Group 3 CON+THC : non-diabetic rats treated with THC (100 mg/kg BW)

Group 4 STZ : diabetic rats treated with NSS

Group 5 STZ+CUR : diabetic rats treated with curcumin (100 mg/kg BW)

Group 6 STZ+THC : diabetic rats treated with THC (100 mg/kg BW)

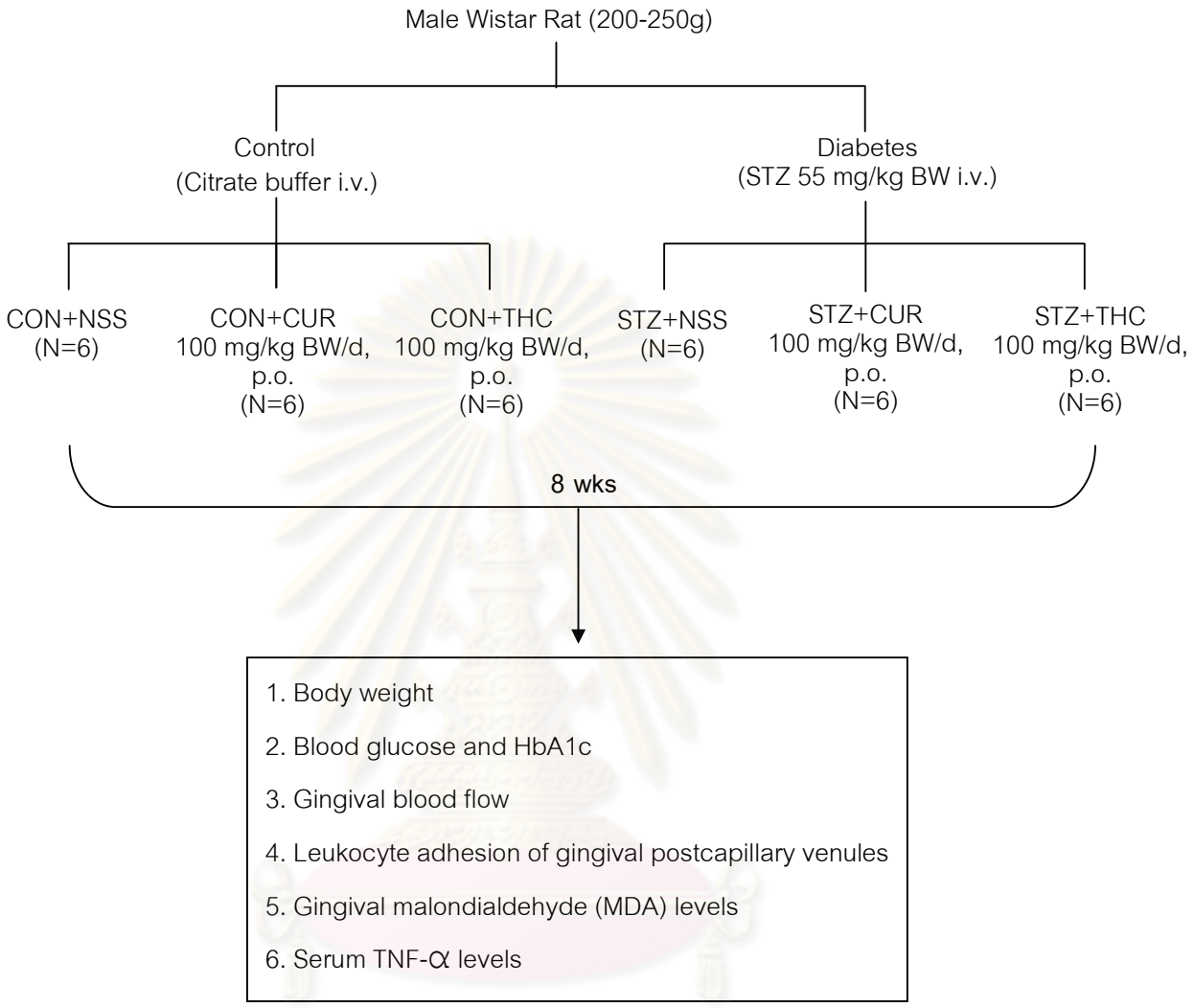


Figure 3.1 The diagram demonstrates the dividing of experimental animal groups

Principle of laser Doppler Flowmetry technique

Laser Doppler flowmetry is an established technique for the real-time measurement of microvascular red blood cell (or erythrocyte) perfusion in tissue. Perfusion is sometimes also referred to as microvascular blood flow or red blood cell flux.

A laser Doppler instrument output often gives flux, velocity and concentration of the moving blood cells. These parameters are extracted from the power spectrum of the photocurrent fluctuations produced by reflected light illuminating a photodetector.

Laser Doppler flowmetry works by illuminating the tissue under observation with low power laser light from a probe containing optical fiber light guides. Laser light from one fiber is scattered within the tissue and some is scattered back to the probe. Another optical fiber collects the backscattered light from the tissue and returns it to the monitor (Figure 3.2).

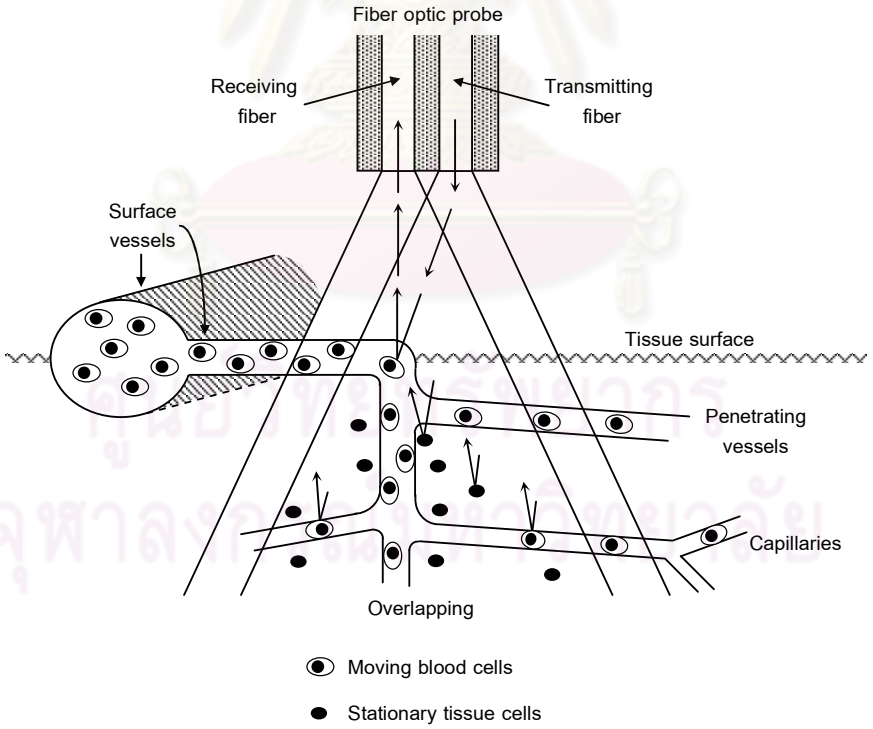


Figure 3.2 Schematic diagram of Laser Doppler probe, skin surface and skin microcirculation

Gingival blood flow measurement

Gingival blood flow was measured using a Laser Doppler Flowmeter with the fiber optic probe (wavelength 780 nm) (MoorLAB server/satellite, Moor Instruments, UK). The needle probe was fixed perpendicularly above the lower interdental papilla about 1 mm and measured for 1 minutes (Figure 3.3). The readings of the laser Doppler signals were recorded into a computer and analyzed with MoorSoft for Windows/moorLAB v1.31. The Highlighted black area of the GBF graph (duration of 1 minute) was used to calculate for the mean of GBF (Figure 3.4). The measurements were taken twice to ensure reproducibility and the mean was then calculated for each animal (Morozumi et al., 2004: 267-272).

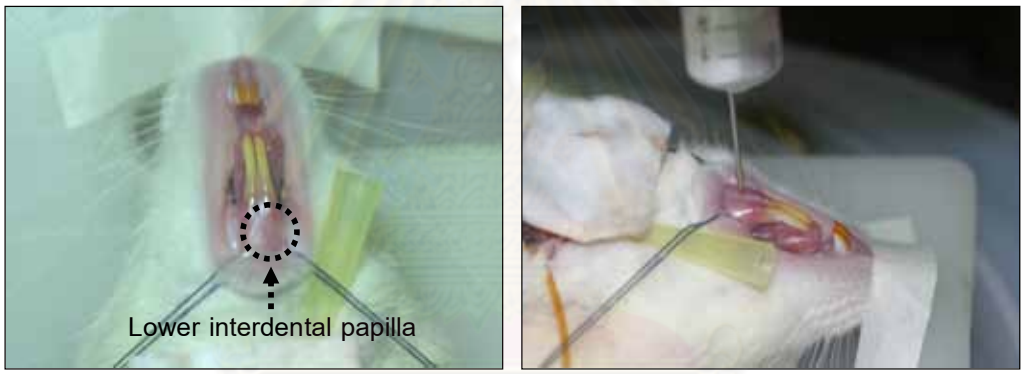


Figure 3.3 A photograph of the Laser Doppler Flowmeter measuring point in rat mandible

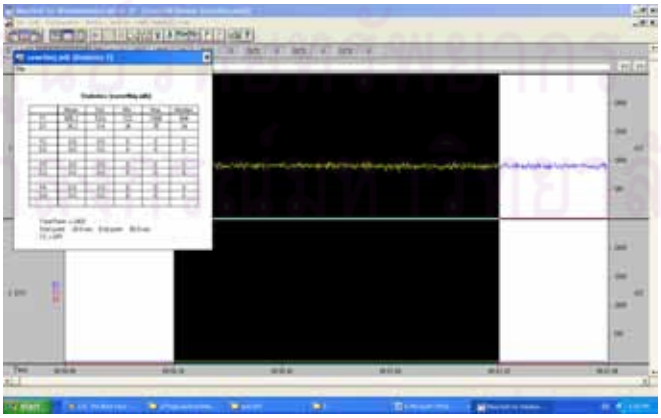


Figure 3.4 A photograph of GBF calculation by MoorSoft for Windows/moorLAB v1.31

Principle of intravital fluorescence microscopy technique

Intravital fluorescence microscopy has been used to observe the microvascular of parenchymatous organ, such as, brain, heart, lung, liver, pancreas, gut, and kidney.

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths. The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen (Figure 3.5).

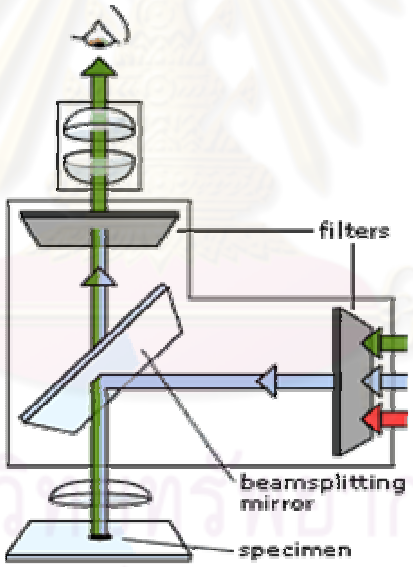


Figure 3.5 Schematic of a fluorescence microscope

Leukocyte adhesion measurement

The gingival microcirculation was observed by an intravital fluorescence microscope with a 20x objective lens (Nikon, Japan). A catheter was inserted into a femoral vein for injection of rhodamine 6G (R6G; Sigma chemical Co., USA) 0.15 mg/kg BW. The emission wavelength of R6G lies between 530 and 540 nm. The observation was done in three gingival areas for two minutes in each area immediately after the injection of R6G, adherent leukocytes were recorded in real time in the postcapillary venule (20 to 30 μm diameter), by a CCD video camera (Hamamatsu, Japan), a camera controller (Hamamatsu, Japan), and a video recorder (SONY, Japan). The data output of this observation is the numbers of adherent leukocytes in each gingival area (Figure 3.6).

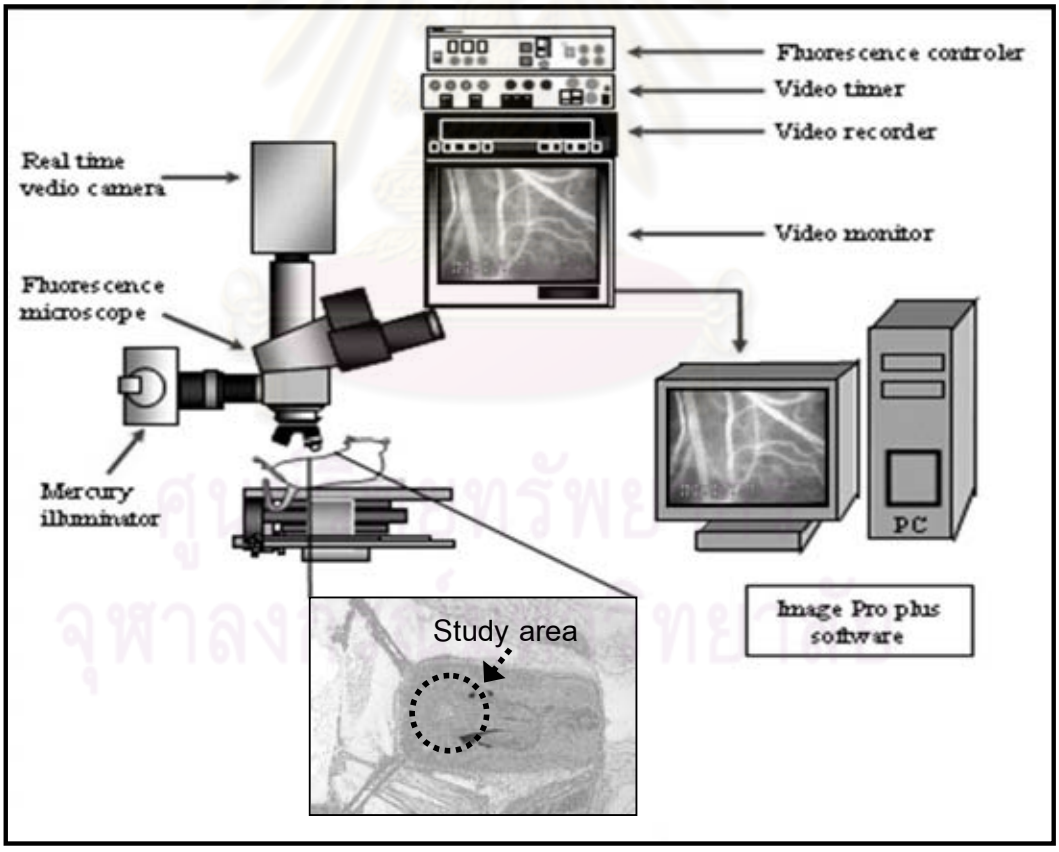
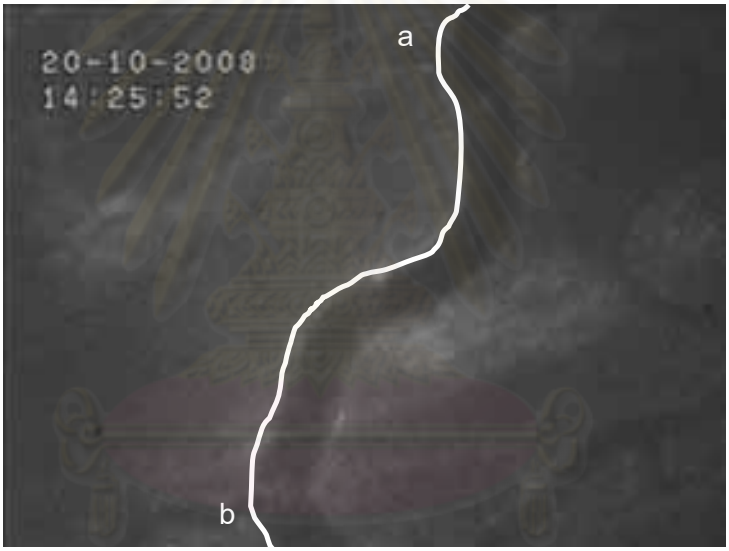


Figure 3.6 Schematic of setup for intravital microscopy of the gingival microvasculature in the rat

The numbers of adherent leukocytes were analyzed off-line during video playback. The leukocytes remained stationary on the endothelium of postcapillary venule for $\geq$30 seconds were defined and the observed venule length was measured by using a digital image processing software "Image Pro" (Plus Software Media Cybernatics, Inc, USA). The numbers of adherent leukocytes were manually counted and reported in the number of cell per 100 μm of vessel length (cell/100 μm venular length), showing in (Figure 3.7) (Sridulyakul et al., 2006: 315-321). And then, we used the data to calculate for the average numbers of adherent leukocytes from three observation areas.



$$\text{Numbers of adherent leukocytes} = \frac{\text{number of leukocyte adhere to the vessel x 100}}{\text{Length of vessel (ab)}} \quad \cdots\cdots \text{Equation 1}$$

Figure 3.7 Method for measurement numbers of adherent leukocytes

ELISA Technique for Measurement TNF-α in Serum

Principle of the essay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rat TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat TNF-α bound in the initial step. The sample values are then read off the standard curve.

Sample collection and storage

To determine serum TNF-α levels, blood sample obtained from the abdominal aorta was collected in a centrifuge tube and allowed to clot for 2 hours at room temperature before centrifuging at approximately 1000 Xg for 20 minutes. Remove serum and stored at -20°C until analysis for TNF-α performed.

Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, working standards, control, and samples as (Appendix).

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

3. Add 50 µL of Assay Diluent RD1-41 to each well.

4. Add 50 µL of Standard, Control, or sample to each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

6. Add 100 µL of rat TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Standard TNF-α concentration (pg/mL)	Absorbance (OD)
0	0.056
12.5	0.112
25	0.162
50	0.279
100	0.493
200	0.919
400	1.615
800	2.373

Table 3.1 The optical density of serial TNF-α concentration

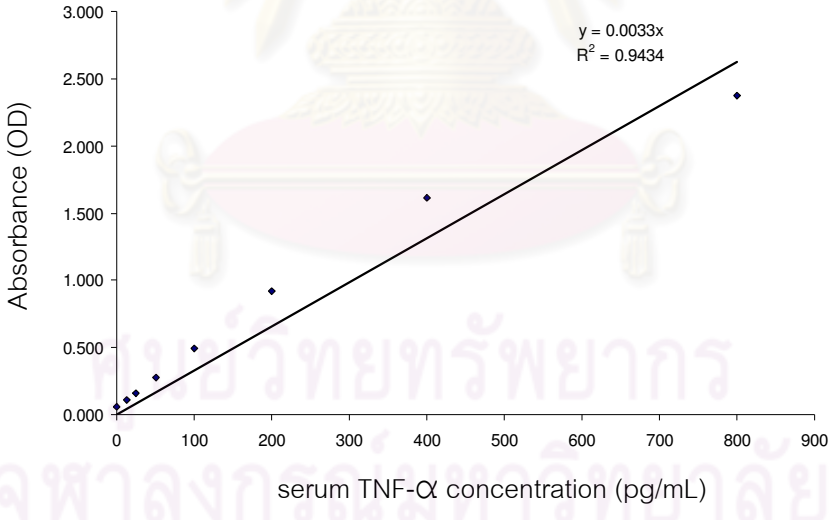


Figure 3.8 The standard curve of serum TNF-α level

Malondialdehyde measurement

Principle of the essay

Oxidation of polyunsaturated fatty acids leads to numerous peroxidic and aldehydic compounds, in particular the volatile low molecular weight aldehyde, MDA. The chemical composition of the end products of peroxidation will depend on the fatty acid composition of the lipid substrate used and upon what metal ion are present. Thus copper and ions give different end-product distribution as measured by the thiobarbituric acid (TBA) test. This is one of the most commonly used methods for detecting and measuring lipid peroxidation. The lipid material is simply heated with TBA at low pH, and the formation of a pink chromogen is measure at or close to 532 nm. The chromogen is form by reaction of one molecule of MDA with two molecules of TBA (Ohkawa, Ohishi, and Yagi, 1979: 351-358).

Sample collection and storage

Gingival tissues were obtained from rat mandible and stored at -20 °C until analysis for MDA performed.

Assay procedure

1. Prepare reagents, standards, and samples as directed (Appendix).

2. After washing the isolated tissues in ice-cold 0.9% (w/v) NaCl, the gingival tissue is prepared by homogenizing each gram of wet tissue in 9 mL of 1.15% KCl.

3. Pipette the following solutions into a series of glass tubes with screw cap:

Solution	Blank (mL)	Standard (mL)	Unknown (mL)
Sample	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH 3.5)	1.5	1.5	1.5
0.8 % TBA	1.5	1.5	1.5
TMP stock standard	-	0.2	-
Distilled water	0.8	0.6	0.6

4. Heated the tube in the water-bath at 95°C for 60 minutes.

5. After cooling the tubes by immersion with tab water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1 (v/v)) are added and shaken vigorously (at least 1 minute).

6. After centrifugation at 4,000 rpm for 10 minutes, the organic layer is removed and its absorbance at 532 nm is measured.

7. The content of lipid peroxide is expressed in term of nmole MDA/g wet wt., converting the OD reading using the data generated as the calibration curve, below.

Calibation curve

Prepare a series of tube containing TMP stock standarded in water in the following concentrations: 4, 8, 12, 16, 20, and 24 nmole/mL. Perform the procedure as in step 2, above. Determine the absorbance at 532 nm. Then plot the optical density versus nmole of MDA of tissue homogenate (Figure 3.9).

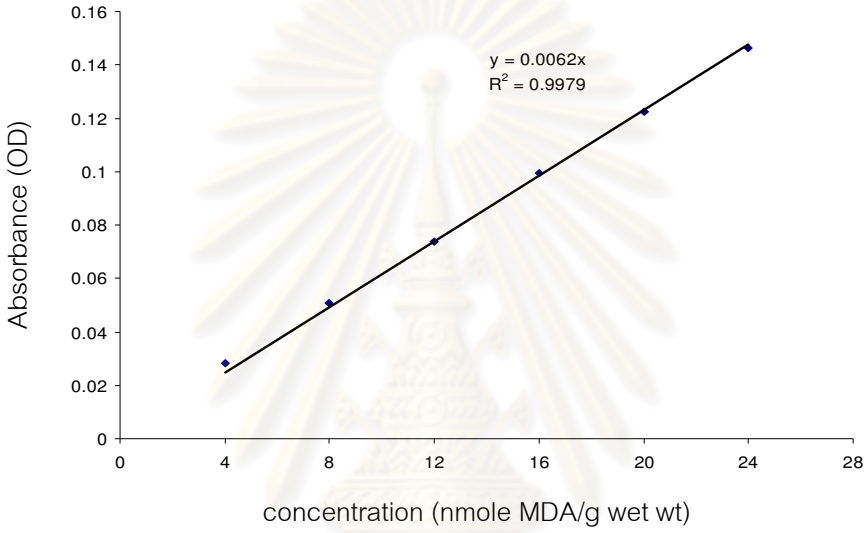


Figure 3.9 The sample of standard curve for assay MDA level

Measurement of metabolic parameters

At the end of experiment, the blood sample was collected from the abdominal aorta. Blood glucose and HbA1c were measured using the enzymatic method by BRIA Laboratory Co, Ltd. (Bangkok, Thailand).

Data analysis

All data were presented as means ± standard errors of mean (SEM). For comparison among groups of animals, one way analysis of variance (one-way ANOVA) was used. Tukey's test was employed to compare the difference in pairs of means between diabetes and control, and between diabetes and treated diabetes. The statistical probability (*p*-value) less than or equal to 0.05 were considered to indicate statistical significance.

CHAPTER IV

RESULTS

This chapter of results composed of five major parts which were supported to investigate the effect of curcumin and THC supplementation on prevention of gingival microvascular dysfunction in STZ-induced diabetic rats. These five major parts were listed in followings ;

Part 1. The effects of curcumin and THC supplementation on metabolic changes

- blood glucose

- glycosylated hemoglobin

- body weight

Part 2. The effects of curcumin and THC supplementation on serum TNF-α levels

Part 3. The effects of curcumin and THC supplementation on leukocyte-endothelial interaction

Part 4. The effects of curcumin and THC supplementation on gingival blood flow

Part 5. The effects of curcumin and THC supplementation on gingival oxidative stress

Part 1. The effects of curcumin and THC supplementation on metabolic changes

Wistar rats body weight 200-250 g were injected STZ 55 mg/kg/BW into tail vein, resulted in polyphagia, polyuria, polydipsia and hyperglycemia within 48 hours and showed persistent hyperglycemia throughout the experiment. The criterion used for diabetic rats was the blood glucose level that had to be higher than 200 mg/dL in this study.

Eight weeks after STZ injection, body weight were lower in STZ, STZ+CUR and STZ+THC rats (264.33±9.82, 263.67±6.15 and 284.17±12.61 g; respectively) compared with CON, CON+CUR and CON+THC rats (431.83±5.00, 416.67±8.99 and 407.50±21.97 g; p=0.00) (Table 4.1). Blood glucose levels (BG) were significantly elevated in STZ, STZ+CUR and STZ+THC rats (479.50±29.56, 455.00±16.94 and 441.00±38.30 mg/dL; respectively) compared with CON, CON+CUR and CON+THC rats (180.00±20.73, 171.83±15.47 and 178.83±17.41 mg/dL; p=0.00) (Table 4.1). HbA1c were elevated in STZ, STZ+CUR and STZ+THC rats (9.00±0.24, 8.75±0.44 and 8.53±0.27 %; respectively) compared with CON, CON+CUR and CON+THC rats (4.40±0.05, 4.35±0.02 and 4.17±0.11 %; p=0.00) (Table 4.1).

Part 2. The effects of curcumin and THC supplementation on serum TNF-α levels

TNF-α is one of the major pro-inflammatory cytokine involved in inflammation of diabetic microvascular. In the present study, serum TNF-α levels were determined by ELISA kit.

Serum TNF-α levels was significantly elevated in STZ rats (407.64±125.07 pg/mL) compared with CON (90.45±11.54 pg/mL; p=0.028). The TNF-α levels in STZ+CUR rats (73.53±14.01 pg/mL) was reduced significantly compared to STZ rats (p=0.01). Even through in THC supplementation of STZ+THC rat (116.24±82.59 pg/mL) has tend to lower TNF-α levels than STZ rats, but not significantly at p<0.05 (Table 4.2).

Part 3. The effects of curcumin and THC supplementation on leukocyte-endothelial interaction

The leukocyte that was counted as adherent one has to remain stationary for equal to or longer than 30 seconds in postcapillary venule (Sridulyakul et al., 2006: 315-321). The numbers of leukocytes adherence were counted per 100 μm of vessel length.

In the present video microscopic visualization showed image of leukocytes of leukocytes adhering to the endothelium in control, diabetic, curcumin and THC treated rats (Figure 4.2). The numbers of leukocyte adherence was increased significantly in STZ rats (1.31±0.19 cells/100 μm) compared with CON, CON+CUR and CON+THC rats (0.28±0.03, 0.34±0.02 and 0.41±0.06 cells/100 μm; p=0.00). Interestingly, the numbers of leukocytes adherence in STZ+CUR and STZ+THC rats (0.49±0.02 and 0.43±0.04 cells/100 μm) were significantly lower than in STZ rats (p=0.00). The result showed that STZ+CUR rats could reduce leukocyte adherence in the same manner as STZ+THC rats (Table 4.3 and Figure 4.3).

Part 4. The effects of curcumin and THC supplementation on gingival blood flow

By using laser Doppler flowmetry the GBF was assessed from lower interdental papilla of the rat mandible. GBF of the STZ rats (432.30±111.69 AU) was decreased significantly compared to CON rats (872.00±17.90 AU; p=0.03). Curcumin and THC supplementation had effect to increase GBF in STZ+CUR and STZ-THC rats (486.70±173.29 and 640.18±92.16 AU; respectively). However, STZ+CUR and STZ-THC rats still decreased GBF compared with CON rats (Table 4.4 and Figure 4.4).

Part 5. The effects of curcumin and THC supplementation on gingival oxidative stress

In this present study MDA, product of lipidperoxidation was used as indicator of oxygen free radical. The MDA levels were higher in STZ rats (27.21±3.02 nM/g wet wt) compared with CON, CON+CUR and CON+THC rats (13.67±0.62, 15.26±0.92 and 15.47±1.16 nM/g wet wt; p=0.00). Interestingly, MDA levels in STZ+CUR and STZ+THC (19.09±1.09 and 19.30±1.07 nM/g wet wt) were significantly lower than in STZ rats (p=0.01). Additionally, the result shows that STZ+CUR can lower the MDA levels in the same manner as STZ+THC (Table 4.5 and Figure 4.5).

Linear regression was performed to establish the relationship between the gingival MDA levels and number of leukocyte adhesion (Figure 4.6), and between the gingival MDA levels and GBF (Figure 4.7) for the mean of CON, CON+CUR, CON+THC, STZ, STZ+CUR, and STZ+THC groups. Apparently, the measured gingival MDA levels were correlated with number of leukocyte adhesion and GBF (y=0.0743x-0.8184, R^2=0.90, p=0.001 and y=-35.086x+1327, R^2=0.79, p=0.002; respectively).

Table 4.1 Mean±SEM of body weight (g), Blood glucose levels (mg/dL) and Glycosylated hemoglobin level (%) of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC).

Group (n=6)	Body weight (g)	Blood glucose (mg/dL)	HbA1c (%)
CON	431.83±5.00	180.00±20.73	4.40±0.05
CON+CUR	416.67±8.99	171.83±15.47	4.35±0.02
CON+THC	407.50±21.97	178.83±17.41	4.17±0.11
STZ	264.33±9.82*	479.50±29.56*	9.00±0.24*
STZ+CUR	263.67±6.15*	455.00±16.94*	8.75±0.44*
STZ+THC	284.17±12.61*	441.00±38.30*	8.53±0.27*

* Significantly different as compared to CON ($p<0.05$)

Table 4.2 Mean±SEM of serum TNF-α levels (pg/mL) of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC).

Group	TNF-α levels (pg/mL)
CON (n=5)	90.45±11.54
CON+CUR (n=4)	371.59±70.35
CON+THC (n=4)	185.12±39.61
STZ (n=5)	407.64±125.07*
STZ+CUR (n=7)	73.53±14.01#
STZ+THC (n=5)	116.24±82.59ns

* Significantly different as compared to CON ($p<0.05$)

\# Significantly different as compared to STZ ($p<0.05$)

ns No Significant different as compared to STZ

Table 4.3 Mean±SEM of leukocyte adhesion of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC).

Group (n=6)	Numbers of leukocyte adhesion (per 100 μm of vessel length)
CON	0.28±0.03
CON+CUR	0.34±0.02
CON+THC	0.41±0.06
STZ	1.31±0.19*
STZ+CUR	0.49±0.02#
STZ+THC	0.43±0.04#

* Significantly different as compared to CON ($p<0.05$)

\# Significantly different as compared to STZ ($p<0.05$)

Table 4.4 Mean±SEM of MAP (Mean arterial blood pressure) and GBF (Arbitary Unit; AU) of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC).

Group (n=6)	MAP (mmHg)	GBF (AU)
CON	112.77±5.28	872.00±17.90
CON+CUR	105.38±7.45	819.48±51.41
CON+THC	108.96±10.3	851.82±32.91
STZ	115.84±11.63	432.30±111.69*
STZ+CUR	113.98±12.7	486.70±173.29^{ns}
STZ+THC	110.62±9.92	640.18±92.16^{ns}

* Significantly different as compared to CON ($p<0.05$)

ns No significant different as compared to STZ

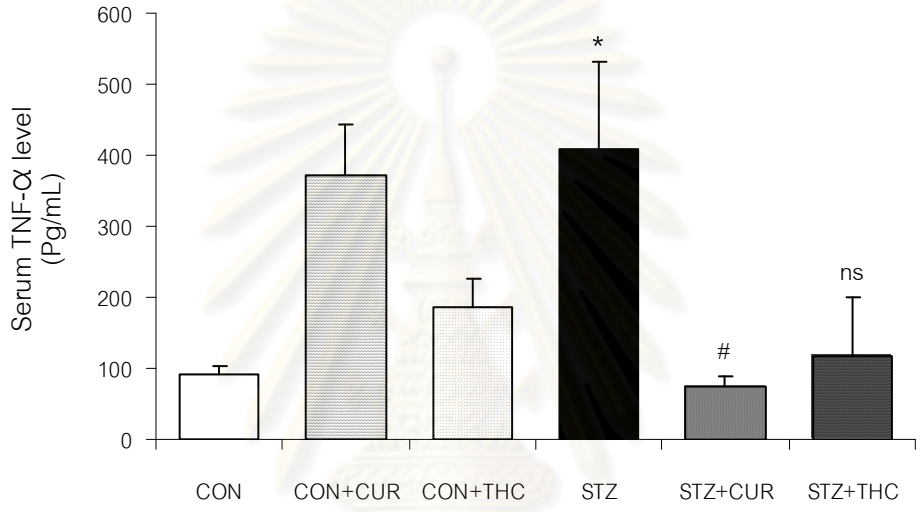
Table 4.5 Mean±SEM of gingival MDA levels (nM/g wet weight) of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC).

Group (n=6)	MDA (nM/g wet weight)
CON	13.67±0.62
CON+CUR	15.26±0.92
CON+THC	15.47±1.16
STZ	27.21±3.02*
STZ+CUR	19.09±1.09#
STZ+THC	19.30±1.07#

* Significantly different as compared to CON ($p<0.05$)

\# Significantly different as compared to STZ ($p<0.05$)

Figure 4.1 Mean±SEM of serum TNF-α levels (pg/mL) of control rats treated with NSS (CON; n=5), control rats treated with curcumin (CON+CUR; n=4), control rats treated with THC (CON+THC; n=4), diabetic rats treated with NSS (STZ; n=5), diabetic rats treated with curcumin (STZ+CUR; n=7), and diabetic rats treated with tetrahydrocurcumin (STZ+THC; n=5).



Values: mean±SEM

* Significantly different as compared to CON (p<0.05)

\# Significantly different as compared to STZ (p<0.05)

ns No significant different as compared to STZ

Figure 4.2 Intravitral microscopic demonstration of leukocyte adhesion in the postcapillary a) CON, b) CON+CUR, c) CON+THC, d) STZ, e) STZ+CUR, and f) STZ+THC. White dots represent leukocytes stained by intravenous injection of fluorescein marker, R6G.

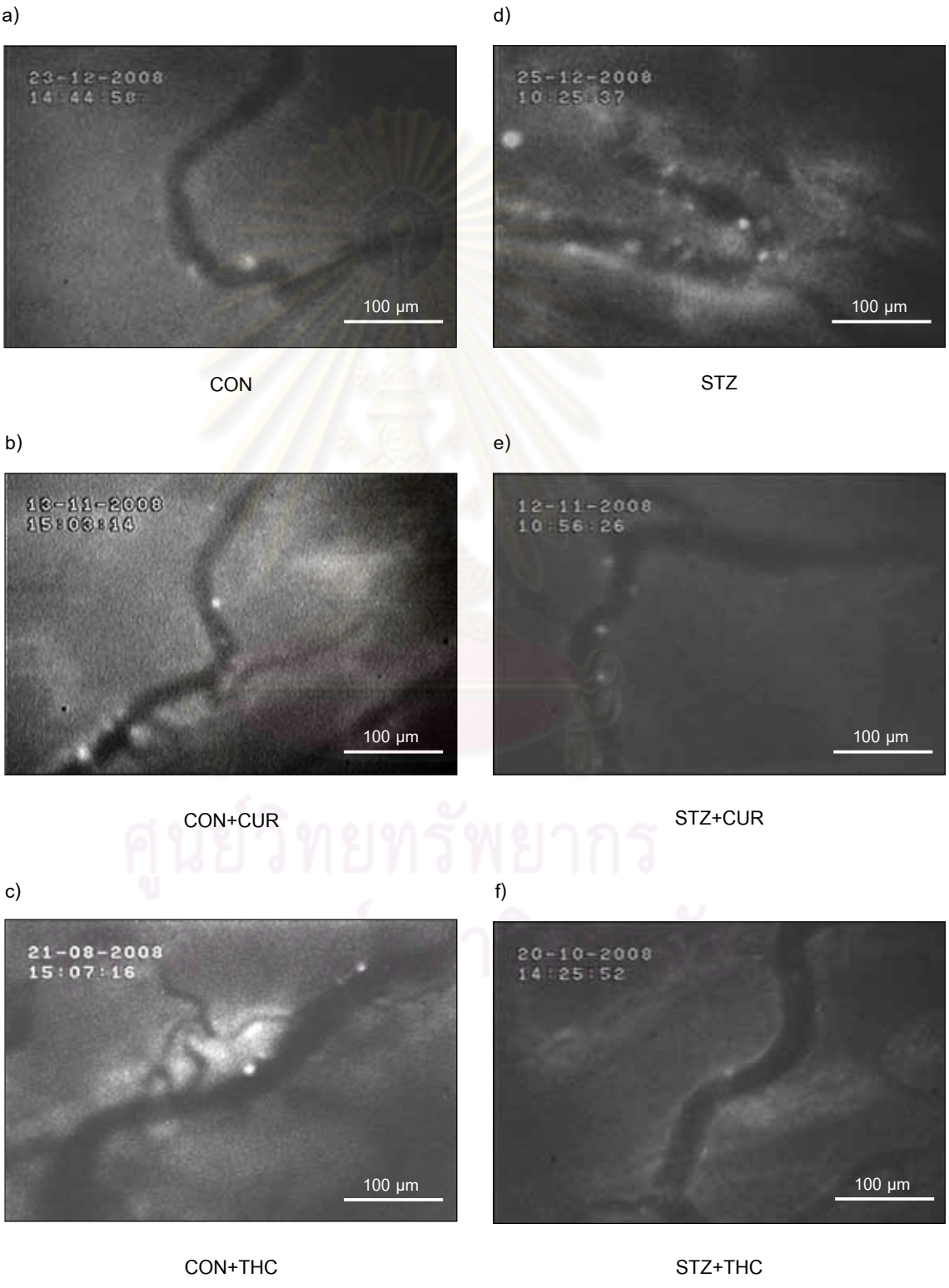
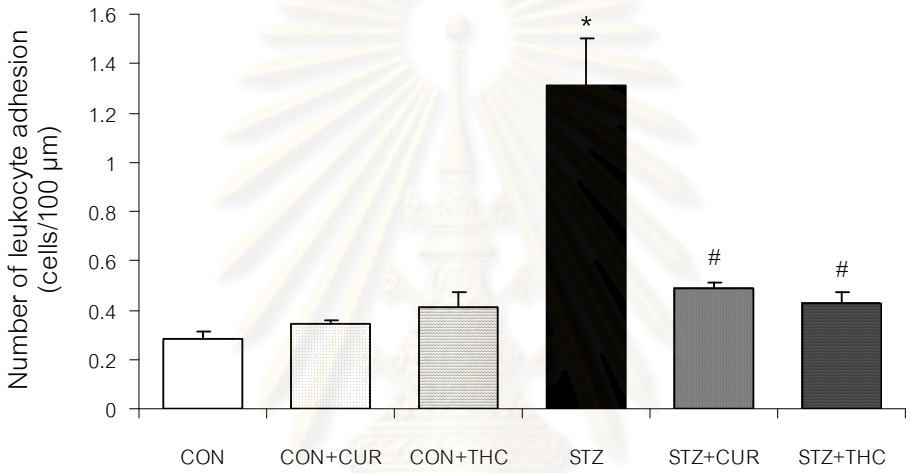


Figure 4.3 Leukocyte adhesion of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC) (n=6/group).

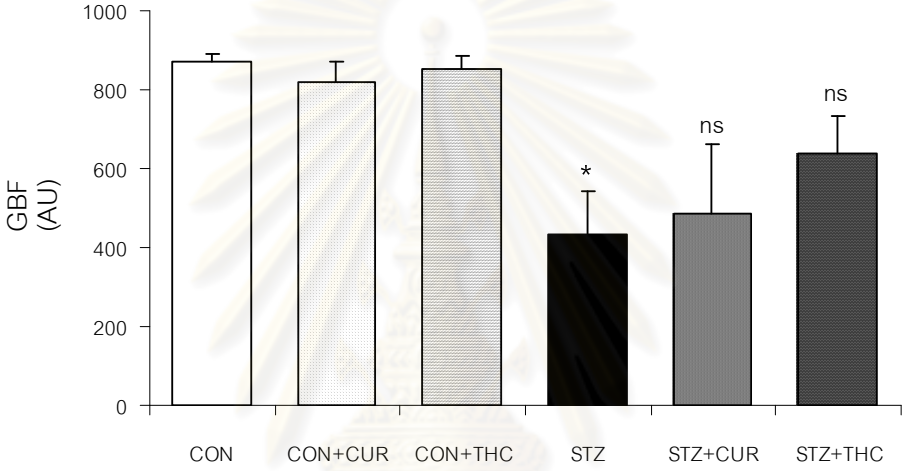


Values: mean±SEM

* Significantly different as compared to CON (p<0.05)

\# Significantly different as compared to STZ (p<0.05)

Figure 4.4 GBF of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC) (n=6/group).

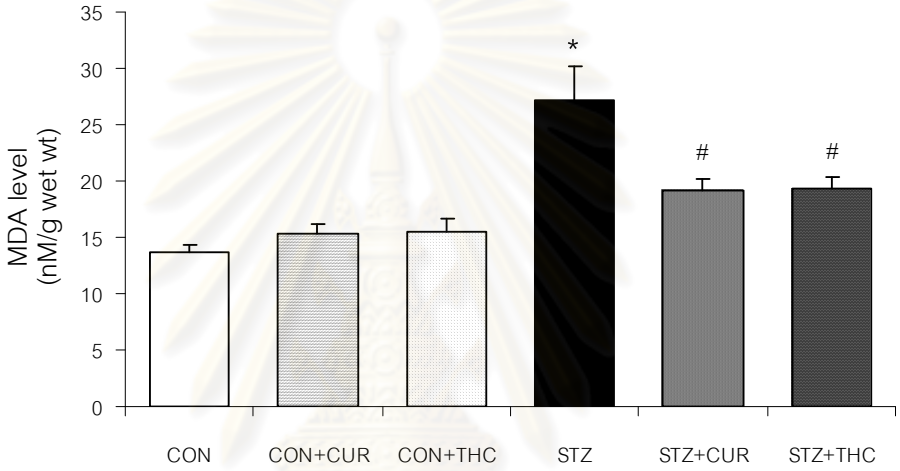


Values: mean±SEM

* Significantly different as compared to CON (p<0.05)

ns No significant different as compared to STZ

Figure 4.5 Gingival MDA levels of control rats treated with NSS (CON), control rats treated with curcumin (CON+CUR), control rats treated with THC (CON+THC), diabetic rats treated with NSS (STZ), diabetic rats treated with curcumin (STZ+CUR), and diabetic rats treated with tetrahydrocurcumin (STZ+THC) (n=6/group)..



Values: mean±SEM

* Significantly different as compared to CON (p<0.05)

\# Significantly different as compared to STZ (p<0.05)

Figure 4.6 The correlation between gingival MDA levels and number of leukocyte adhesion were examined by using Pearson's Correlation and the best-fitting linear regression in mean of each groups (CON, CON+CUR, CON+THC, STZ, STZ+CUR, and STZ+THC) (Pearson's Correlation=0.90, *p*=0.001).

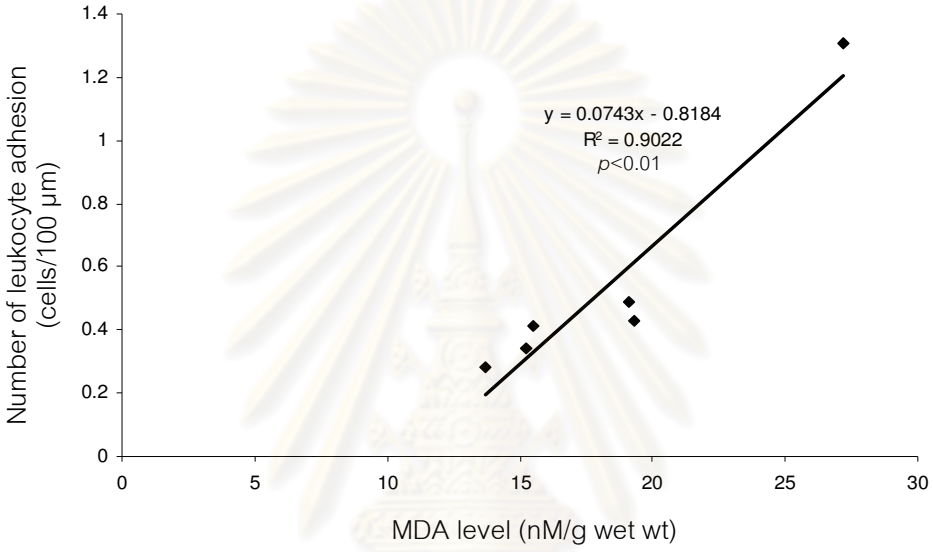
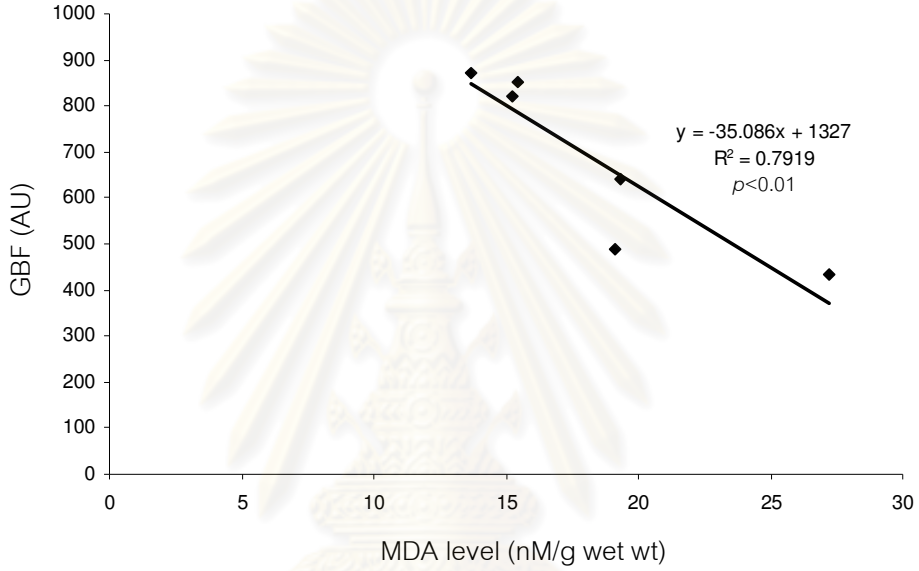


Figure 4.7 The correlation between gingival MDA levels and GBF were examined by using Pearson's Correlation and the best-fitting linear regression in mean of each groups (CON, CON+CUR, CON+THC, STZ, STZ+CUR, and STZ+THC) (Pearson's Correlation=0.79, $p<0.002$).



CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to investigate the effects of curcumin and THC supplementation on gingival microvascular dysfunction in diabetic rats. From the result, it could be discussed as follows:

I. The metabolic changes in diabetic model

Streptozotocin (STZ) has highly specific cytotoxic action on the β-cells of the Islets of Langerhans by generating radical species such as nitrous oxide that aggravated DNA damage (Kroncke et al., 1995: 179-185). STZ has been used extensively to induce the experimental model imitated type 1 diabetes mellitus in rat. Numerous studies are performed with using this model, providing its benefit in studying diabetic vascular complications (Wei et al., 2003: 44-50). STZ-induced β-cells destruction is accompanied by characteristic alteration in blood insulin and glucose concentration, which causes hyperglycemia and reduction in blood insulin level (Akbarzadeh et al., 2007: 60-64). In the present study, hyperglycemia (blood glucose $\geq$ 200 mg/dL) occurs within 48 hours after the single dose of STZ 55 mg/kg/BW intravenous administration and persists throughout the experiment. Diabetic symptoms, resulted from metabolic derangement including marked hyperglycemia, polyphagia, polydipsia, polyuria and weight loss were found at around 6-8 weeks of age. These symptoms can be described as follows.

Chronic hyperglycemia increases glucose concentration in the blood, therefore glucose reabsorption in the proximal renal tubuli is incomplete. Part of the glucose remains in the urine and induces an osmetic diuresis and thus polyuria. The renal and electrolytes loss trend to deplete intracellular water, causing dehydration which increased thirst (polydipsia) by triggering the osmoreceptors on the thirst centers of the brain. Insulin insufficiency promotes catabolism of proteins and fats which leads to elavating negative energy balance. Finally, the energy imbalance in turn, leads to

increasing appetite. Nevertheless, the increased appetite is occurred but catabolic effects predominate over, resulting in weight loss and muscle weakness (Cotran, 1999).

Blood glucose and glycosylated hemoglobin

In this study, blood glucose levels were significantly elevated in STZ, STZ+CUR and STZ+THC rats compared with CON, CON+CUR and CON+THC rats. HbA1c levels were elevated in STZ, STZ+CUR and STZ+THC rats compared with CON, CON+CUR and CON+THC rats similar to blood glucose. The result showed that the rats which were treated with STZ had hyperglycemia during experiment. Because a single evaluation of blood glucose is often insensitive indicator of metabolic control, we also measured HbA1c concentration which indicated plasma glucose concentration over a prolonged period of time and used as an index for how well of glycemic control (Cerami, Stevens, and Monnier, 1979: 431-437).

The supplementation of curcumin did not have lowering effect on blood glucose and HbA1c in this study. These findings agree with those reported by Suryanarayana et al. (2005: 2092-2099). In their experiment, they found that the curcumin treatment, without having an effect on hyperglycemic status, reduced the increased oxidant stress that causes cross-linking of skin collagen in STZ-induced diabetic rats, improved in metabolic status in terms of lipid peroxidation, and reduced urinary excretion of electrolytes. Curcumin by its free radical scavenging property reduced physiological dysfunction. However, there is controversy about the effect of curcumin on blood glucose levels. Some other studies have reported the reducing effect of curcumin on blood glucose levels. Hyperglycemia-induced oxidative stress that causes diabetes complications such as diabetic nephropathy was reduced in STZ-induced diabetes rats that were treated with curcumin (Sharma, Kulkarni, and Chopra, 2006: 940-945). Nevertheless, the mentioned study did not explain how the mechanism of curcumin work to reduced hyperglycemia in diabetic rats.

In this study, THC supplement tend to reduce blood glucose (441.00±38.30 mg/dL) and HbA1C (8.53±0.27 %) levels to a greater extent than curcumin (455.00±16.94 mg/dL and 8.75±0.44 %). However, the difference is not significant. Pari, Karthikesan, and Menon (2010: 109-117) have reported that a supplementation of THC

80 mg/kg BW reduced blood glucose levels and elevated plasma insulin levels in STZ–nicotinamide-induced diabetic rats. The similar results were reported by Murugan and Pari (2006: 122-127), they suggested that THC decreased blood glucose concentration in diabetes by triggering the proinsulin synthesis and insulin release. However, THC supplement concentration was different in the present study. THC 100 mg/kg BW might not have the ability to reduced blood glucose levels in STZ-induced diabetic rats.

Body weight

In present study, STZ, STZ+CUR and STZ+THC rats lost their body weight compared to the CON, CON+CUR and CON+THC rats. STZ has cytotoxic action on β-cells of the Islets of Langerhans that can cause reducing insulin reduction, leads to increase catabolism of proteins and fats and negative energy balance, resulting in weight loss.

In the experiment, we investigated the effect of curcumin and THC on blood glucose and HbA1C levels. These results showed that curcumin and THC supplementation did not have any effect to reduce hyperglycemia, which did not colloborate with the previous observation in efficacy of curcumin and THC in reduction of hyperglycemia (Patumraj et al., 2006: 481-489; Pari et al., 2010: 109-117). Since the hyperglycemia still existed in diabetic rats that were treated curcumin and THC, negative energy balance and weight loss continue to occur in them. This finding supports the previous study of Chanpoo et al. (2010: S152-S159).

II. The effects of curcumin and THC supplementation on serum TNF-α levels

In diabetes, chronic hyperglycemia produces oxidative stress and alters host response to infection. This impaires neutrophils recruitment, neutrophils phagocytic function, and exaggerates inflammatory response. These responses link to immoderate periodontal tissue destruction. Oxidative stress triggered inflammatory response, resulting in increasing production of pro-inflammatory mediators such as IL-1b, IL-6, and TNF-α (Lalla et al., 2000: 50-62; Nassar, Kantarci, and van Dyke, 2007: 233-244).

TNF-α is one of the very potent pro-inflammatory mediators. The TNF-α action is through bind with specific cell surface receptors such as an epithelial cell-type receptor (p55) and a myeloid cell-type receptor (p75). In the event that TNF-α has binded with its receptors, it activates numerous signal transduction pathways. As the result of this activation, several effects have occurred and two of which are the following. First, the expression of variety of transcription factors, cytokines, growth factors, receptors, cells adhesion molecules, mediators of inflammatory processes. The second effect is the alteration in the connective or extracellular matrix tissues. In our study, serum TNF-α levels were increased in STZ compared with CON, CON+CUR and CON+THC rats. The result supported that the hyperglycemia elevated TNF-α expression in serum, which refer to vascular inflammation. This finding conforms to the elevating TNF-α in many *in vitro* and *in vivo* studies of diabetes models. (Reddy et al., 2009: 63-74; Chan, Kanwar, and Kowluru, 2010: 55-63; Takano et al., 2010: 379-384). The mechanism of this event was demonstrated that hyperglycemia leads to enhance the ROS generation and the accumulation of AGEs. Altogether, these induce inflammation through transcription factor NF-ĸB, which is important transcription factor that plays a key role in cellular responses to stimuli pro-inflammatory cytokines. NF-ĸB has been reported to plays a key role in vascular diabetic complications including diabetic cardiomyopathy, retinopathy, and nephropathy (Patel and Santani, 2009: 595-603). The cause of pathogenesis was demonstrated by Bierhaus et al. (2001: 2792-2808) that persistent NF-ĸB activation participated in chronic diabetic complications through hyperglycemia induced ligand-RAGE interaction.

In addition, NF-ĸB has been reported to link with periodontal disease progression in diabetes. In physiological condition, NF-ĸB binds with a family of regulatory proteins, called inhibitors of NF-ĸB (IĸB) to form NF-ĸB dimers in the cytoplasm. In hyperglycemia the elevation of AGEs and oxidant stress stimulates phosphorylation and degradation of IĸBα and the subsequent. After that, NF-ĸB is released and translocates into the nucleus that result to increase pro-inflammatory cytokines such as TNF-α which cause gingival inflammation and destruction (Southerland, Taylor, and Offenbacher, 2005: 171-178).

In this study, the supplementation of curcumin was able to reduce serum TNF-α levels. In other study, oral curcumin supplementation in type 2 diabetic rats decreased hepatic expression of TNF-α by the reduction of NF-κB activity in liver tissue (Weisberg, Leibel, and Tortoriello, 2008: 3549-3558). This report agrees with our finding for the anti-inflammatory effect of curcumin. The potent antioxidant effect of curcumin has been reported to scavenge oxidative and nitrosative radicals that prevent oxidative stress in endothelial cells (Motterlini et al., 2000: 1303-1312). From this ability, curcumin have been suggested to prevent the expression of TNF-α induced by NF-κB in hyperglycemia. In addition, another studies reported that cell culture and diabetic mice treated with curcumin could reduce direct activation of NF-κB without antioxidant ability through inhibiting phosphorylation of IkBα (Weber et al., 2006: 2450-2461; Kanitkar et al., 2008: 702-713).

THC supplementation has tended to reduce serum TNF-α levels in this study. However, the data was not significant. This result agreed with the cell culture experiment of Sandur et al. (2007: 1765-1773). They reported that THC is less effective than curcumin to decrease TNF-induced NF-κB activation because THC lacks the conjugated bonds in the central seven-carbon chain. Therefore, THC was completely inactive for suppression of the transcription factor. However, the study by Leyon and Kuttan (2003: 77-83), has reported that THC could reduce serum TNF-α levels on angiogenesis-induced animals. In addition, many studies have reported the effect of THC in preventing oxidative stress induced inflammation on diabetes complications in various organs. Thus, the antioxidant ability to scavenge ROS of THC is important reason to prevent inflammation in diabetic rats (Murugan and Pari, 2006: 1720-1728; Pari and Murugan, 2007: 665-671).

III. The effects of curcumin and THC supplementation on leukocyte-endothelial interaction.

In this experiment, intravital fluorescence microscopy was used to measure the exhibition of leukocyte adhesion. Leukocyte was detected in camera by labeled with

R6G, this fluorescence dye has emission wavelength lies between 530 and 540 nm (Mempel et al., 2004: 406-417).

In the present study, we chose the gingival postcapillary venule with the diameter of 20-30 μm for observing the leukocyte-endothelial interaction using an intravital fluorescence microscope. Our results showed that the numbers of leukocyte adherence in STZ rats were significantly elevated compared with CON rats.

The polymorphonuclear leukocyte (PMN), which is one of the main inflammatory cells, shows abnormal properties in diabetic patients. This causes the alteration of integrin pattern, thus could affect the interaction between PMN and EC (Hopps, Camera, and Caimi, 2008: 197-202). Furthermore, the activation of endothelium promotes the over expression of the immunoglobulin family of adhesion molecules members such as ICAM-1 and VCAM-1. These molecules are important to activate endothelium by the adhesion of monocytes, lymphocytes and neutrophils (Carlos and Harlan, 1994: 2068-2101). The elevation of PMN-endothelial interaction in the small vessels that initiated abnormality in capillary. Finally, capillary was obstructed and occluded that lead to ischemic organ injury (Fisher and Meiselmann, 1994: S21-S34; Kim et al., 2005: 1534-1542). Moreover, the harmful occurrence is that contract of circulating leukocyte with the EC rises a cascade of event contributes to further leukocyte activation. Once activated, PMN causes abnormality in vascular by release ROS and mediator of proteolytic tissue degradation such as neutrophil elastase that contribute to oxidative stress, subsequent inflammation, and causing endothelial damage (Smedly et al., 1986: 1233-1243).

Several studies have indicated that both hyperglycemia (Haubner et al., 2007: 560-565; Piga et al., 2007: 328-334) and AGEs-RAGE interaction may enhance the expression of adhesion molecules and damage endothelium. (Schmidt et al., 1995: 1395-1403; Basta et al., 2002: 816-822). The study by Zhang et al. (2003: 472-478) suggested that diabetes increased NAD(P)H oxidase activity and oxidative stress which enhanced redox state by the upregulation of VCAM-1. And treatment with AGEs could induce the expression of VCAM-1 through activated ERK1/2 and JNK signaling pathways. For the result of the elevation in leukocyte-endothelial interaction in diabetic rats, they suggested that oxidative stress could lead to the formation of other potent leukocyte chemoattractants, such as PAF and LTB4 (Salas et al., 1999: 59-66). Recent

studies have demonstrated that antioxidant agents (such as probucol) and AGEs inhibitor (such as aminoguanidine) prevented the leukocyte rolling and migration into endothelium in diabetic rats (Sannomiya, Oliveira, and Fortes, 1997: 894-898; Zanardo et al., 2003: 211-219).

In the present study, curcumin supplementation had the effect to reduce the number of leukocyte adhesion to endothelium of postcapillary venule in STZ+CUR rats. The result is in agreement with the study by Patumraj et al. (2006: 481-489). Rajakrishnan et al. (2002: 171-173) showed the result of reduction in leukocytes attachment to collagen, the major component of the vessel wall subendothelium in ethanol-induced oxidative stress cells. In the study of Leclercq et al. (2004: 926-934), suggested that curcumin inhibited NF-κB which activated ICAM-1 mRNA expression in lipogenic methionine and choline deficient (MCD)-fed mice developed oxidative stress. These concluded that curcumin prevent leukocyte adhesion, which activated by NF-κB cascade in diabetic rats, through the antioxidant effect. The same result was shown in THC supplementation of STZ+THC rats. This result agrees with the previous study of Jariyapongskul, Patumraj, and Suksumrarn (2008: 151-155). They demonstrated that THC supplementation could prevent leukocyte adhesion in STZ-induced diabetes rats by antioxidant effect.

Moreover, we investigated correlation between gingival MDA levels and number of leukocyte adhesion. Our correlation results were significantly correlated and could be fitted by a linear line: y=0.0743x-0.8184, R^2=0.90, and *p*=0.001 (Figure 4.6). These findings suggested that the increase of leukocyte adhesion induced by oxidative stress could be prevented by curcumin and THC supplementation.

IV. The effects of curcumin and THC supplementation on gingival blood flow

Gingival blood flow (GBF) was assessed from lower interdental papilla of the rat mandible by laser Doppler flowmetry. The results showed that GBF in STZ rats were significantly reduced compared with CON rats. This result was supported by several *in vivo* and *in vitro* studies of diabetic model (Yagihashi et al., 1996: 793-799; Trachtman, Futterweit, and Crimmins, 1997: 1276-1282). NO, synthesized by eNOS, plays crucial

roles in regulation of blood flow. In hyperglycemia, O_2^- reacts with NO to produce $ONOO^-$, which is highly potent reactive oxygen and nitrogen species. This molecule reacts avidly with oxidized BH_4. Insufficiency of BH_4 resulting in the production of superoxide anions instead of NO that call “eNOS uncoupling” (Forstermann and Munzel, 2006: 1708-1714) and resulting in GBF reduction.

The reduction of eNOS expression and activity and/or NO availability caused vascular abnormality in diabetes. Those refer to increased vascular resistance in various organs and tissues. In the study by Erdos et al. (2004: 1352-1359), they reported that cerebral arteries of type 2 diabetic rats showed the impairment of endothelium-dependent relaxation after treated with ACh (endothelial-dependent vasoactive agent, is used to induce eNOS generate NO). Then administration of substrate for eNOS (L-arginine), diabetic rats were improved endothelium-dependent relaxation (Popov et al., 2002: 109-120). The reduction of NO production and availability also contributed to platelet aggregation and adhesion (Gresele et al., 2010: 1262-1268). Moreover, oxidative stress can increase potent vasoconstrictors such as ET-1 and ANG II. The over expression in vasoconstrictors have been correlated with increased vascular resistance, reduced blood flow, and predisposed atherogenesis in diabetes (Giacchetti et al., 2005: 120-126; Sethi et al., 2006: 175-183).

In our study, THC supplementation tended to increase GBF in STZ-induced diabetic rats. However the data was not significant. Our result demonstrated that the reduction of GBF could be partially prevented by THC. The effect on prevention of reduced blood flow was supported by the study of Jariyapongskul et al. (2008: 151-155), their results demonstrated that THC administration could improve cerebral blood flow in STZ-induced diabetic rats. The preventing effect correlated with antioxidant activity of THC to reduced oxidative stress in diabetic rats. The reduction of oxidative stress refers to enhance endothelium-dependent vasodilator. In the result of curcumin supplementation, GBF was tended to increase in STZ-induced diabetic rats but less than THC. Curcumin was reported to increase vasoconstrictor ET-1 levels in human microvascular endothelial cells (HMECs) culture treated with high glucose (Farhangkhoee et al., 2006: 1-8). The elevation of ET-1 might decrease the effectiveness of curcumin in preventing GBF reduction. However, the study by Awasthi et al. (2010:

87-94) has reported that curcumin could improve cerebral blood flow reduction in diabetic rats. It can be suggested that the effect of curcumin to prevent blood flow reduction in diabetes might be different in various tissues and species.

In addition, we investigated correlation between gingival MDA levels and number of leukocyte adhesion. Our correlation results were significantly negative correlated and could be fitted by a linear line: y=-35.086x+1327, R^2=0.79, *p*=0.002 (Figure 4.7). These findings suggested that the reduction of GBF induced by oxidative stress could be restored by curcumin and THC supplementation.

V. The effects of curcumin and THC supplementation on gingival oxidative stress

In oxidative stress, ROS interacts with fatty acid or fatty side chain to generate lipid peroxidation in an organism. Malondialdehyde (MDA) is one of the final products, which is generated by both lipid peroxidation and as a byproduct of prostaglandin and thromboxane synthesis. MDA interacts with low density lipoprotein (LDL) to produce oxidised-LDL (ox-LDL), which leads to pathologic effects including the induction of atherosclerosis, atherothrombosis, and plaque erosion (Nakhjavani et al., 2010: 582-585). MDA is elevated in plasma and various tissues and use as oxidative stress markers in diabetic models (Requena et al., 1996: 48-53; Piconi, Quagliaro, and Ceriello, 2003: 1144-1149). In our study, the MDA levels were significantly higher in gingival tissues of STZ rats when compared to CON rats. These concentrations were increased due to hyperglycemia augmented lipid peroxidation (Niskanen et al., 1995: 802-808).

The elevation of oxidative stress in hyperglycemia is contributed to the development of diabetic vascular complications. Increased ROS generation was found in both type 1 and type 2 diabetic patients. Oxidative stress leading to oxidize lipids in lipoproteins and cell membranes as well as the elevated oxidative modification of amino acids and DNA. ROS modulates a lot of transcription factors including NF-kB, peroxisome proliferator activated receptorgamma (PPARgamma), and pathways linked to apoptosis to induce gene expression which regulated endothelial function (Napoli, de Nigris, and Palinski, 2001: 674-682). The event causes pathogenesis in endothelium dysfunction including vascular resistance, leukocyte adhesion, platelet aggregation,

smooth muscle cell proliferation, and induction of apoptotic cell death in endothelial cell (Esper et al., 2008: 17-43; Xu et al., 2009: 167-175).

There are many suggestions that the oxidative stress is generated from hyperglycemia induced ROS production in metabolic pathway of mitochondria and glycated proteins to produce AGEs. From the results of our studies HbA1c which induced by hyperglycemia was found to increase in STZ-rats. The elevation of lipid peroxidation is associated with pathogenic implications. These were supported by the increasing of pro-inflammatory cytokine (TNF-α), numbers of leukocyte adhesion and the reduction of GBF. In normal physiology, ROS levels are controlled by endogenous antioxidant enzymes including, superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase and GSH reductase (Ulrich-Merzenich et al., 2009: 2-16). However, hyperglycemia causes augmentation of ROS generation which refers to insufficiency of endogenous antioxidant. Thus the replacement of exogenous antioxidant might improve the vascular complication in diabetes.

Interestingly, curcumin supplementation can reduce gingival MDA levels in STZ+CUR rats compared with STZ-rats. The result agrees with the reduction of liver MDA levels in STZ-induced diabetic rats after treated with curcumin in the study by Patumraj et al. (2006: 481-489). Curcumin alleviates oxidative stress through the antioxidant effect. This effect was explained by the structure of curcumin, H-atom from β-diketone and/or the phenolic OH^- group is transferred to ROS and convert it to ineffective molecule (Hatcher et al., 2008: 1631-1652). Moreover, some studies were reported that curcumin could alleviate oxidative stress by increasing endogenous antioxidant enzymes. It up regulates heme oxygenase-1 (HO-1), a redox stress-inducible protein, known to protect cells against various types of stress through activation of the hormetic nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway (Pugazhenthi et al., 2007: E645-E655; Mandal et al., 2009: 672-679). As a consequence, curcumin might prevent oxidative stress in vascular diabetic rats by free radical scavenging ability and elevation endogenous antioxidant.

Similar result was also shown in THC supplementation group. THC reduced MDA level of diabetic rats. This result was supported by the study of Wongeakin et al. (2009: 259-265), they demonstrated that administration of THC could reduce liver MDA

levels in STZ-induced diabetic rats. Interestingly, THC has antioxidant effect as well as curcumin. It conflicted with the study by Osawa et al. (1995: 1609-1612). They reported that THC has strong antioxidant activity among all curcuminoids in rabbit erythrocyte membrane ghost and rat liver microsome. However, the study by Nakamura et al. (1998: 361-370) demonstrated that THC has the inhibitory activity weaker than curcumin in tetradecanoylphorbol acetate (TPA) - induced O_2^- generation in differentiated HL-60 cells. This conflict was implicated that the antioxidant ability between curcumin and THC were difference in cell type and tissues.

VI. Comparison of the protective effects of curcumin and tetrahydrocurcumin on gingival microvascular dysfunction

In our study, the protective effects between curcumin and tetrahydrocurcumin supplementation on the elevation of gingival MDA levels were not difference. These result agreed with the study of Wongeakin et al. (2009: 259-265). They reported that curcumin supplementation could prevent the elevation of liver MDA as well as THC in STZ-induced diabetic rats. These result suggested that the antioxidant effect of curcumin and THC were equal in this study. That referred to the same prevention effect of curcumin and THC on oxidative stress induced gingival microvascular dysfunction including, the elevation of serum TNF-α levels, leukocyte-endothelial interaction, and the reduction of GBF. However, the study of Pali and Murugan (2007: 323-329) reported that the effect of THC for protection against lipid peroxidation-induced membrane damage was greater than curcumin. This result correlated with the study of Murugan and Pali (2007: 241-245) which reported that THC have a better protective effect than curcumin on oxidative stress induced elevation of hepatic and renal markers in type 2 diabetic rats because of the greater antioxidant effect.

Diagram shown in Figure 5.1 represents the proposed mechanisms for the effect of curcumin and THC on the gingival microvascular dysfunction.

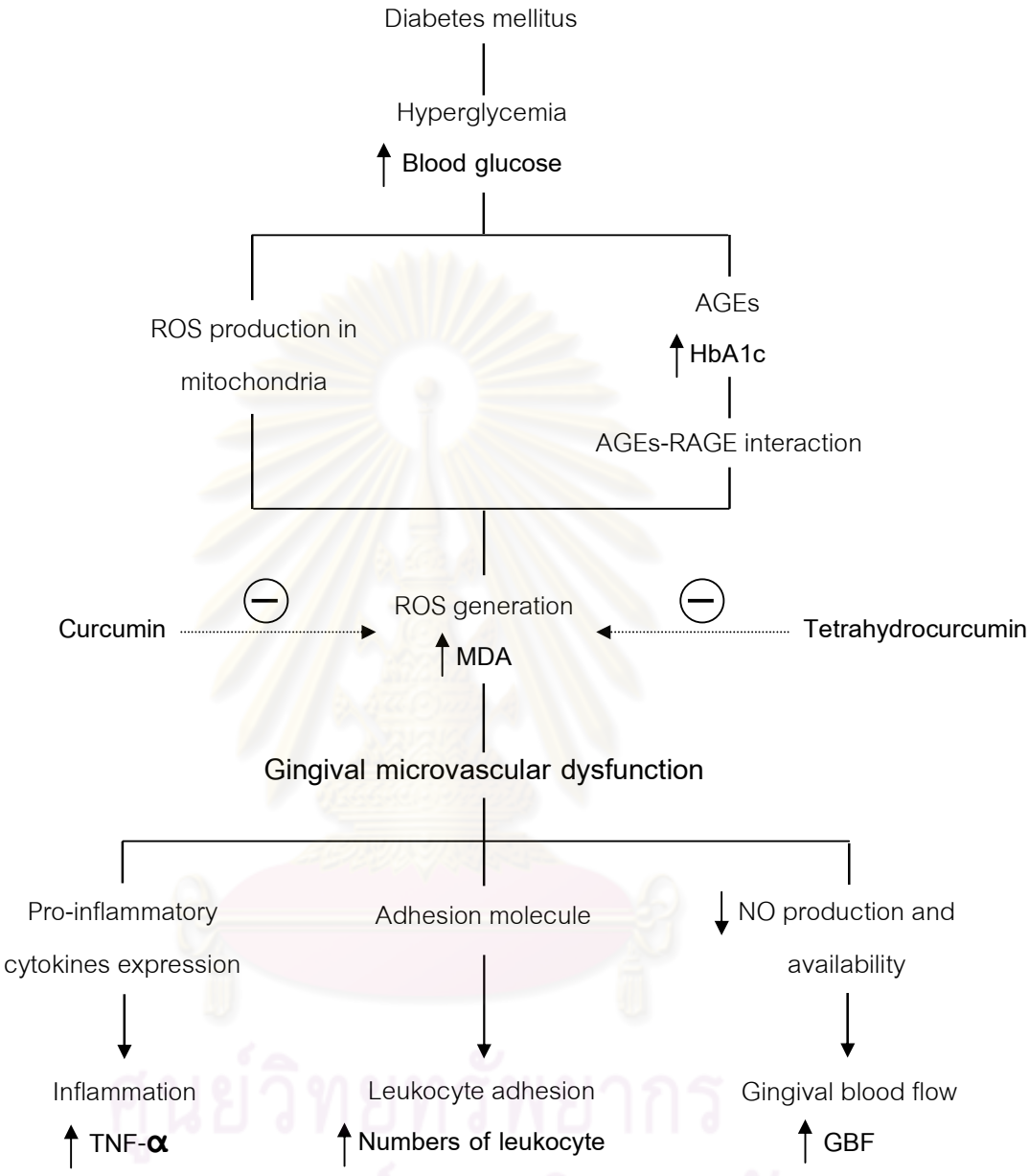


Figure 5.1 Proposed mechanism for the effect of curcumin and THC on the gingival microvascular dysfunction.

CHAPTER VI

CONCLUSION

In the present study, the effects of curcumin and THC supplementation on diabetic induced gingival microvascular dysfunction were studied by using intravital fluorescence microscopy, laser Doppler flowmetry and ELISA techniques. The following are the conclusion of our findings.

1. The elevation of blood glucose, HbA1c and the reduction of body weight were demonstrated in STZ, STZ+CUR, and STZ+THC rats compared with CON rats. These results were implicated that curcumin and THC supplementation did not have effect to decrease hyperglycemia, protein glycation, and metabolic derangement in diabetic rats.

2. Plasma TNF-α levels were elevated in STZ rats compared with CON rats. Interestingly, curcumin and THC supplementation could reduce plasma TNF-α levels. Consequently, curcumin and THC prevented inflammation in diabetic rats.

3. Using intravital fluorescence microscopic study, STZ rats had a significant increasing leukocytes adhesion to endothelial lining of gingival postcapillary venules compared with CON rats. Interestingly, the leukocytes adhesion were prevented by curcumin and THC supplementation.

4. The reduction of gingival blood flow was observed in STZ rats by laser Doppler flowmeter. Curcumin and THC have tended to increase gingival blood flow but data were not significant. Therefore, curcumin and THC have partial prevention of gingival blood flow reduction in diabetic rats.

5. The MDA levels were significantly elevated in STZ rats compared with CON rats. Interestingly the significant decrease of MDA levels were obtained in STZ+CUR and STZ+THC rats compared with STZ rats.

6. The comparison for all parameters of prevention effect in microvascular dysfunction between curcumin and THC supplementation were not different. The result were suggested that the supplementation of both curcuminoid in 100 mg/kg BW in diabetic rats were similar expressed the prevention effect in these dose.

7. The results showed that the supplementation of curcumin and THC reduced MDA levels in diabetic rats. However, both curcuminoid did not have effects to reduce blood glucose and HbA1c levels. Therefore, the antioxidant ability of curcumin and THC could reduce oxidative stress and prevent gingival microvascular dysfunction in diabetic rats without hypoglycemic effect.

8. Finally, it is suggested that curcumin and THC supplementation could prevent gingival microvascular complication in diabetic rat. They might be beneficial for diabetic patients in order to prevent gingival diabetic complication.

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APPENDIX

I. Curcumin Chemistry and Biology

Curcumin occurs naturally in the rhizome of *Curcuma longa Linn*, which is grown commercially and sold as turmeric. Curcumin is a yellow–orange powder that is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide, and acetone. Curcumin has a melting point of 183 °C, molecular formula of $C_{21}H_{20}O_6$, and molecular weight of 368.37 g/mol. Curcumin is free-radical scavenging activity, which contains a phenolic group. This is superb electron donor by donating the H-atom from the phenolic group to free radical molecule for neutralizing free radicals forming stable products. Another way, the antioxidant activity of curcumin could be mediated through antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Aggarwal et al., 2006).

II. Tetrahydrocurcumin Chemistry and Biology

THC is one of the major colourless metabolites of curcumin, which is derived from curcumin by hydrogenation. THC is soluble in water, molecular formula of $C_{21}H_{20}O_6$, and molecular weight of 377.2 g/mol. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin. Tetrahydrocurcumin has been reported to the strongest antioxidant activity among all curcuminoids. Several studies in experimental animals indicate that tetrahydrocurcumin also prevent cancer as well as a protective agent against inflammation atherosclerotic lesions (Pari and Murugan, 2003: 481-486).

III. Streptozotocin-induced diabetic rat model

The animal model of type 1 diabetes mellitus that is used in this study is induced by a single intravenous injection with the dose of 55 mg/kg BW streptozotocin (Sridulyakul P., et al. 2006).

In this study, we used STZ-treated rat model as an type 1 diabetes mellitus because this model closely resemble to vascular complications in type 1 diabetes mellitus in human. The dose of 55 mg/kg BW is used by a single intravenous injection.

IV. ELISA Technique for Measurement TNF-α in Serum

Reagent preparation

Bring all reagents to room temperature before use.

1. Rat TNF-α Kit Control

Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

2. Wash Buffer

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

3. Substrate Solution

Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

4. Rat TNF-α Standard

Reconstitute the rat TNF-α Standard with 2.0 mL of Calibrator Diluent RD5-17. Do not substitute other diluents. This reconstitution produces a stock solution of 800 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

5. Use polypropylene tubes

Pipette 200 µL of Calibrator Diluent RD5-17 into each tube. Use the stock solution to produce a dilution series (Figure A). Mix each tube thoroughly before the next transfer. The undiluted rat TNF-α Standard serves as the high standard (800 pg/mL). Calibrator Diluent RD5-17 serves as the zero standard (0 pg/mL).

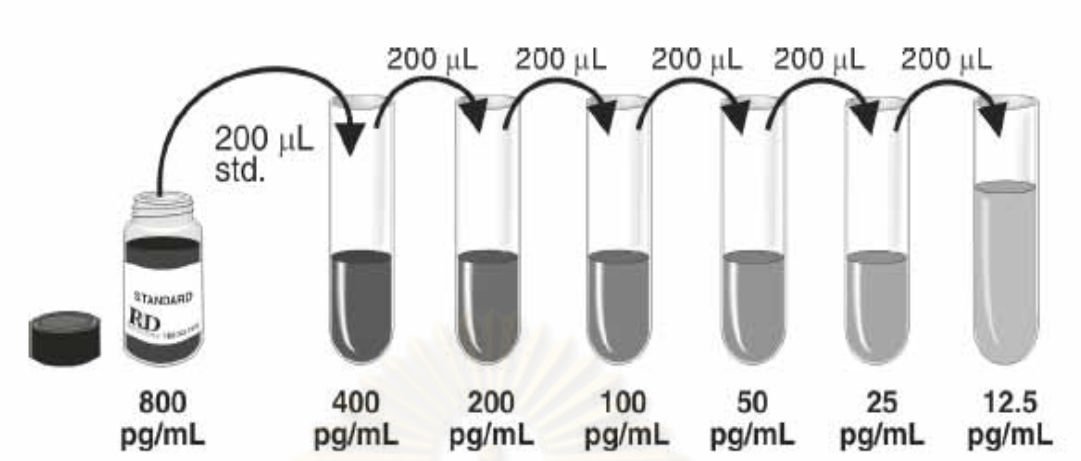

Figure A Serial dilution of TNF-α Standard

V. Malondialdehyde (MDA) assay

Reagent preparation

1. 8.1% (w/v) Sodium docecyl sulfate (SDS)

Dissolve SDS 8.1 g in distilled water and allow to stand overnight at room temperature until it is dissolved. Then make up to 100 mL. Do not shake because this solution will produce a lot of bubbles.

2. 20% (v/v) of acetic acid solution (pH 3.5)

Pipette 200 ml of 37 % HCl into a 1 liter volumetric flask and make up to 1,000 mL with distilled water.

3. 0.8% (w/v) Thiobarbituric acid (TBA)

Weigh TBA 0.8 g, then add distilled water to make this solution up to 100 mL and mix, heat and stir until it is dissolved.

4. 1,1,3,3-Tetramethoxypropane (TMP) or malondialdehyde bis solution

TMP is used as a external standard. The level of lipid peroxide is expressed as nmole of MDA. Prepared stock 10^3 nmole TMP with distilled water, then pipette 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 mL of this stock TMP solution. These will give the following concentration of standard TMP: 4, 8, 12, 16, 20, and 24 nmole/mL. Prepare stock TMP fresh.

5. 1.15% (w/v) KCl

Dissolved KCL 11.50 g in 1,000 mL of distilled water and mix thoroughly.

BIOGRAPHY

Mr. Dusit Promrug was born on May 24, 1982 in Mahasarakham, Thailand. He graduated Bachelor of Nursing Science from Faculty of Nursing, Thammasat university in 2005.