ผลของวิตามินซีต่อการเปลี่ยนกลับการสูญเสียหน้าที่ของเอนโดทีเลียม ที่เกิดจากการเหนี่ยวนำด้วยเบาหวานในหนูแรท: บทบาทของ อนุมูลอิสระ เตตระไฮโดรไบออพเทอริน และไนตริกออกไซด์

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บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
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REVERSAL EFFECT OF VITAMIN C ON DIABETES INDUCED ENDOTHELIAL DYSFUNCTION IN RATS: ROLES OF REACTIVE OXYGEN SPECIES, TETRAHYDROBIOPTERIN, AND NITRIC OXIDE

Miss Pattarin Sridulyakul

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By	Miss Pattarin Sridulyakul
Field of study	Physiology
Thesis Advisor	Assosiate Professor Suthiluk Patumraj, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements of the Doctoral Degree

.....Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

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.....Member
(Associate Professor Narisa Futrakul, M.D., Ph.D.)

ภัทริน ศรีดุลยกุลย์: ผลของวิตามินซีต่อการเปลี่ยนกลับการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากการเหนี่ยวนำด้วยเบาหวานในหนูแรท: บทบาทของอนุมูลอิสระ เตตระไฮโดรไบออพเทอริน และไนตริกออกไซด์ (REVERSAL EFFECT OF VITAMIN C ON DIABETES INDUCED ENDOTHELIAL DYSFUNCTION IN RATS: ROLES OF REACTIVE OXYGEN SPECIES, TETRAHYDROBIOPTERIN, AND NITRIC OXIDE.)

อ.ที่ปรึกษา: รศ.ดร.สุทธิลักษณ์ ปทุมราช; 160 หน้า

การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาบทบาทของการให้วิตามินซีเสริมต่อการเปลี่ยนกลับการสูญเสียหน้าที่ของเอนโดทีเลียมในภาวะเบาหวาน หนูขาวเพศผู้สายพันธุ์ เสปรย์-ดอลเลย์ น้ำหนัก 200- 250 กรัม แบ่งออกเป็น 4 กลุ่ม คือ กลุ่มควบคุม (CON) กลุ่มเบาหวาน (DMโดยวิธีฉีดสารสเตรปโตโซโตซิน (STZ) เข้าทางหลอดเลือดดำในขนาดความเข้มข้น 50 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว) กลุ่มเบาหวานที่ได้รับวิตามินซีเสริมหลังจากฉีด STZ 6 สัปดาห์ (DM+Vit.C6wks) และกลุ่มเบาหวานที่ได้รับวิตามินซีเสริมหลังจากหลังจากฉีด STZ 2 วัน (DM+Vit.Cday2) สำหรับกลุ่ม DM+Vit.C6wks และ DM+Vit.Cday2 จะได้รับวิตามินซีในขนาดความเข้มข้น 1 กรัมต่อลิตรในสัปดาห์ที่ 6 และ วันที่ 2 หลังจากฉีดสาร STZ ตามลำดับ จากนั้นทำการศึกษาการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากการเหนี่ยวนำด้วยเบาหวานซึ่งจำแนกได้จากการลดลงของการตอบสนองของหลอดเลือดแดงรองในลำไส้ต่อสารอะเซทิลโคลีน (Ach) และจากการเพิ่มขึ้นของการเกาะติดของเม็ดเลือดขาวต่อเซลล์เอนโดทีเลียม ภาวะผิดปกตินี้สามารถแก้ไขได้โดยการชะลอการได้รับวิตามินซีเสริมหลังการสูญเสียหน้าที่ของเอนโดทีเลียม (DM+Vit.C6wks) และสามารถป้องกันได้โดยการได้รับวิตามินซีเสริมตั้งแต่แรกก่อนการสูญเสียหน้าที่ของเอนโดทีเลียม (DM+Vit.Cday2) ส่วนที่สำคัญจากผลการทดลองนี้ชี้ให้เห็นว่าการให้วิตามินซีเสริมสามารถเปลี่ยนกลับการสูญเสียหน้าที่ของเอนโดทีเลียมในหลอดเลือดขนาดเล็กในลำไส้ที่ถูกเหนี่ยวนำให้เป็นเบาหวานได้

หลังจากนั้นคณะผู้วิจัยมีวัตถุประสงค์เพื่อศึกษากลไกการทำงานในเชิงลึกของผลของวิตามินซีต่อการเปลี่ยนกลับ สมมติฐานแรกเชื่อว่าการที่วิตามินซีสามารถเปลี่ยนกลับหน้าที่ของเอนโดทีเลียมได้เนื่องมาจากการที่วิตามินซีสามารถกำจัดอนุมูลอิสระ (ROS) ได้โดยตรง เพื่อเป็นการทดสอบสมมติฐานนี้คณะผู้วิจัยจึงทำการวัดไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ซึ่งเป็น ROS ชนิดหนึ่งที่มีความเสถียรด้วยสารฟลูออเรสเซนต์ที่มีความจำเพาะต่อ H_2O_2 ที่มีชื่อเรียกว่า dihydrorhodamine 123 (DHR-123) ซึ่งทำการวัดตามเวลาที่เกิดขึ้นจริงโดยใช้ intravital fluorescence videomicroscopy ผลการวิจัยพบว่าความเข้มของการเรืองแสงที่สัมพันธ์กับ H_2O_2 เพิ่มขึ้นอย่างมากในกลุ่ม DM ที่ 6 สัปดาห์ เมื่อเทียบกับกลุ่ม CON ที่ 6 สัปดาห์ แต่เป็นที่น่าสนใจว่าทั้งสองกลุ่มที่ได้รับวิตามินซี (DM+Vit.C6wksและ DM+Vit.Cday2) แสดงการลดระดับของความเข้มของการเรืองแสงที่สัมพันธ์กับ H_2O_2 ในระดับใกล้เคียงกัน เนื่องมาจากผลของวิตามินซีต่อการกำจัด ROS ได้โดยตรง ดังนั้นคณะผู้วิจัยจึงทำการวัดไนตริกออกไซด์ (NO) ตามเวลาที่เกิดขึ้นจริง เนื่องมาจากความคิดที่ว่า NO ควรจะเพิ่มขึ้นเมื่อ ROS ถูกกำจัด โดยการใช้สี 4,5-diaminofluorescein- diacetate (DAF-2DA) ความเข้มของการเรืองแสงที่สัมพันธ์กับ NO จะถูกวัดในแต่ละกลุ่มการทดลอง ได้แก่ กลุ่ม CON กลุ่ม DM กลุ่ม CON ที่ได้รับวิตามินซีความเข้มข้น 2.6 มิลลิโมลาร์ โดยการหยดเป็นเวลา 20 นาที (CON +2.6 mM Vit.C) และ กลุ่ม DM ที่ได้รับวิตามินซีความเข้มข้น 2.6 มิลลิโมลาร์ โดยการหยดเป็นเวลา 20 นาที (DM+2.6 mM Vit.C) ผลการศึกษาวิจัยพบว่า ปริมาณ NO ลดลงอย่างมีนัยสำคัญทางสถิติในกลุ่ม DM ที่ 6 สัปดาห์ เมื่อเทียบกับ กลุ่ม CON ที่ 6 สัปดาห์ อย่างไรก็ตาม การให้วิตามินซีสามารถเพิ่มปริมาณ NO อย่างมีนัยสำคัญทางสถิติทั้งในกลุ่ม CON และกลุ่ม DM จากผลการทดลองนี้ชี้ให้เห็นว่าวิตามินซีสามารถกำจัด ROS ส่งผลให้ปลดปล่อย NO เพิ่มขึ้น

สมมติฐานที่สองของวิตามินซีต่อการเปลี่ยนกลับเชื่อว่ามาจากการที่วิตามินซีสามารถรักษาการทำงานของเตตระไฮโดรไบออพเทอริน (BH_4) ได้ การทดลองนี้หนูกลุ่ม CON ที่ 6 สัปดาห์ และ กลุ่ม DM ที่ 6 สัปดาห์ จะถูกแบ่งออกเป็น 6 กลุ่มย่อยคือ 1) กลุ่มเปรียบเทียบซึ่งหยด Krebs-Ringer solution 30 นาที (Vehicle) 2) กลุ่มเปรียบเทียบที่ได้รับวิตามินซีความเข้มข้น 2.6 มิลลิโมลาร์ 1 นาที หลังจากหยด Krebs-Ringer solution 30 นาที (Veh+Vit.C) 3) กลุ่มยับยั้งการสร้าง BH_4ซึ่งหยด DAHP ความเข้มข้น 20 มิลลิโมลาร์ 30 นาที (DAHP) 4) กลุ่มยับยั้งการสร้าง BH_4ที่ได้รับวิตามินซี 1 นาที หลังจากหยด DAHP 30 นาที (DAHP+Vit.C) 5) กลุ่มยับยั้งการสร้าง BH_4ที่ได้รับ BH_4 20 นาที หลังจากหยด DAHP 10 นาที (DAHP+BH_4) และ 6) กลุ่มยับยั้งการสร้าง BH_4ซึ่งหยด BH_4 20 นาที ตามด้วยวิตามินซี 1 นาที หลังจากหยด DAHP 10 นาที (DAHP+BH_4+Vit.C) ผลการศึกษาวิจัยพบว่าภาวะพร่อง BH_4 ที่เกิดจากการใช้สาร DAHP ทำให้เกิดการลดลงของการขยายตัวของหลอดเลือดต่อ Ach ทั้งในกลุ่ม CON และ กลุ่ม DM แต่เป็นที่น่าสนใจว่าการเปลี่ยนแปลงที่ผิดปกติดังกล่าวสามารถแก้ไขได้โดยการให้วิตามินซี การให้ BH_4 และ การให้วิตามินซีร่วมกับ BH_4 นอกจากนั้นยังพบว่าความเข้มของการเรืองแสงที่สัมพันธ์กับ H_2O_2 เพิ่มขึ้นอย่างมากเมื่อมีการยับยั้งการสร้าง BH_4 โดยการใช้สาร DAHP แต่เป็นที่น่าสนใจมากว่าการเปลี่ยนแปลงที่ผิดปกตินี้สามารถลดลงได้โดยการให้วิตามินซี การให้ BH_4 และ การให้วิตามินซีร่วมกับ BH_4 นอกจากนี้ควรที่จะให้ความสำคัญถึงผลของการให้วิตามินซีที่สามารถแก้ไขความผิดปกติของการลดลงของการขยายตัวของหลอดเลือดต่อ Ach ที่เกิดจากการใช้สาร DAHP ดังนั้นอาจจะสรุปได้ว่าวิตามินซีมีความสามารถเพิ่มการสร้าง BH_4 ขึ้นใหม่ส่งผลให้กระตุ้นการทำงานของเอนไซม์เอนโดทีเลียมไนตริกออกไซด์ซินเทสที่อยู่ในรูปแบบที่เอื้อต่อการสร้าง NO

โดยสรุปจากผลการศึกษาแสดงให้เห็นว่าวิตามินซีสามารถเปลี่ยนกลับการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากภาวะเบาหวานระยะเริ่มต้น ที่ 6 สัปดาห์ โดยผ่านทางกลไกการทำงานของวิตามินซีในการกำจัด ROS อย่างมีประสิทธิภาพ และความสามารถในการเพิ่มการทำงานของ BH_4 ดังนั้นกล่าวได้ว่าวิตามินซีสามารถเพิ่มการทำงานของ NO และวิตามินซีอาจนำมาใช้ในการดูแลผู้ป่วยเบาหวานในฐานะเป็นสารที่ใช้ในการรักษาได้

สหสาขาวิชา.....**สรีรวิทยา**..... **ลายมือชื่อนิสิต**.....

สาขาวิชา.....**สรีรวิทยา**..... **ลายมือชื่ออาจารย์ที่ปรึกษา**.....

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KEYWORD: REVERSAL EFFECT/ VITAMIN C/ DIABETIC RAT/ ENDOTHELIAL DYSFUNCTION/ TETRAHYDROBIOPTERIN

PATTARIN SRIDULYAKUL: REVERSAL EFFECT OF VITAMIN C ON DIABETES INDUCED ENDOTHELIAL DYSFUNCTION IN RATS: ROLES OF REACTIVE OXYGEN SPECIES, TETRAHYDROBIOPTERIN, AND NITRIC OXIDE. THESIS ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., 160 pp.

This study aimed to examine the effects of vitamin C on reversing endothelial dysfunction in diabetes mellitus. Male Spraque-Dawley rats weighing 200-250 g were divided into four groups: control (CON), diabetes (DM, using iv. injection of streptozotocin (STZ); 50 mg/kg BW), DM+Vit.C6wks, and DM+Vit.Cday2. For DM+Vit.C6wks, and DM+Vit.Cday2, they received vitamin C 1g/L on week 6th and on day 2 after STZ-injection, respectively. The diabetes-induced endothelial dysfunction was demonstrated by the impairment of mesenteric arteriolar acetylcholine (Ach)-induced vasodilation and the increase in leukocyte-endothelial cell interaction. These abnormalities were restored by delayed treatment of vitamin C (DM+Vit.C6wks) and prevented by early vitamin C supplementation (DM+Vit.Cday2). The importance of this present study was indicated that vitamin C supplementation could reverse diabetes-induced endothelial cell dysfunction in mesenteric microcirculation.

Next, we aimed to examine the underlying mechanism(s) of reversal effect of vitamin C. Base on the first hypothesis, it is believed that vitamin C could restore endothelial function by its direct scavenging reactive oxygen species (ROS). In order to prove this hypothesis, therefore, we monitored hydrogen peroxide (H_2O_2), one stable form of ROS, by using the specific H_2O_2 associated fluorescent dye called dihydrorhodamine 123 (DHR-123) with real time intravital fluorescence videomicroscopy. The results indicated that the H_2O_2-associated fluorescent intensity was greatly increased in 6wk-DM group compared to 6wk-CON. Interestingly, both vitamin C supplementation groups (DM+Vit.C6wks and DM+Vit.Cday2) have shown less H_2O_2-associated fluorescent intensity in the same manner. Together with this direct scavenging effect of vitamin C, therefore, we made a direct detection for nitric oxide (NO), since the idea was that NO should increase when ROS was scavenged. By using 4,5-diaminofluorescein-diacetate (DAF-2DA), the NO-associated fluorescent intensity was determined for each group; CON, DM, CON +2.6 mM Vit.C, and DM+2.6 mM Vit.C at 20-min vitamin C perfusion. The results showed that NO content was significantly decreased in 6wk-DM compared to 6wk-CON. However, vitamin C application could significantly increase NO contents in both CON and DM groups. Based on our finding, we indicated that vitamin C was able to scavenging ROS, as a consequence, it released NO.

The second hypothesis for reversal effect of vitamin C was addressed by its possibly acting on preserving tetrahydrobiopterin (BH_4) bioavailability. The experiments were conducted by dividing 6wk-CON and 6wk-DM into six groups: 1) Vehicle; 30-min Krebs-Ringer perfusion, 2) Veh+Vit.C; 1-min 2.6 mM vitamin C administration after 30-min Krebs-Ringer perfusion, 3) DAHP; 30-min BH_4 antagonist (20 mM, 2,4-diamino-6-hydroxypyrimidine (DAHP), 4) DAHP+Vit.C; 1-min 2.6 mM vitamin C administration after 30 min DAHP, 5) DAHP+BH_4; 20-min BH_4 donor after 10-min DAHP, and 6) DAHP+BH_4+Vit.C ; 10-min DAHP followed by 20-min BH_4 donor and 1-min vitamin C. The results indicated that the DAHP-mediated BH_4 deficiency significantly caused the reduction of Ach-induced vasodilatation in both CON and DM. Interestingly, this abnormality was improved by administration with vitamin C, or BH_4, or the combined vitamin C and BH_4. Furthermore, the H_2O_2-associated fluorescent intensity was greatly increased when BH_4 synthesis was blocked by DAHP. Interestingly, this abnormality could be attenuated by the administration of vitamin C, or BH_4, or the combined vitamin C and BH_4. Beside, it should be noted that the DAHP-induced the reduction of Ach-induced vasodilatation could be improved partially by vitamin C administration. Therefore, it may be concluded that vitamin C could regenerate BH_4 and consequently increase active form of coupled-eNOS.

In conclusion, our results indicated that vitamin C could reverse diabetes-induced endothelial dysfunction at early stage of 6-wk diabetic induction. The possible mechanisms of vitamin C were worked through its potential on direct ROS scavenging and on enhancing BH_4 bioavailability. Therefore, it implied that vitamin C could increase NO bioavailability. And it may be used as a good therapeutic agent recommended for diabetic care.

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Student's signature Pattarin Sridulyakul
Advisor's signature

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LIST OF ABBREVIATIONS

Ach	=	Acetylcholine
AGEs	=	Advanced glycosylation end-products
AMPK	=	AMP-activated protein kinase
AP-1	=	Activator protein-1
AR	=	Aldose reductase
BBd	=	Diabetic BioBreeding
BG	=	Blood glucose
BH_2	=	Dihydrobiopterin
BH_4	=	Tetrahydrobiopterin
6R-BH_4	=	6R-5,6,7,8-tetrahydro-L-biopterin dihydrochloride
BK	=	Bradykinin
BW	=	Body weight
Ca^{2+}	=	Calcium
CaM	=	Calmodulin
CaM KII	=	CaM-dependent protein kinase II
CAT	=	Catalase
cGMP	=	Cyclic guanosine 3', 5'-monophosphate
cm	=	Centrimeter
cNOS	=	Constitutive nitric oxide synthase
CON	=	Control rats
DAF-2DA	=	4,5-diaminofluorescein-diacetate
DAF-2T	=	4,5-diaminofluorescein triazolofluorescein
DAFs	=	Diaminofluoresceins
DAHP	=	2,4-diamino-6-hydroxypyrimidine
DBP	=	Diastolic blood pressure
DHA	=	Dehydroascarbic acid

LIST OF ABBREVIATIONS (Continue)

DHFR	=	Dihydrofolate reductase
DHR 123	=	Dihydrorhodamine 123
dL	=	Deciliter
DM	=	Diabetic rats
e^-	=	Electrons
EDR	=	Endothelium-dependent relaxation
EIDR	=	Endothelium-independent relaxation
eNOS	=	Endothelial nitric oxide synthase
EPCs	=	Endothelial progenitor cells
ET	=	Endothelin
ET1	=	Endothelin-1
FAD	=	Flavin adenine dinucleotide
Fe	=	Ferric
FITC-Dextran	=	Fluorescein isothiocyanate labeled dextran
FMN	=	Flavin mononucleotide
GC	=	Guanylate cyclase
γ-GCS	=	γ-glutamyl cysteine synthetase
GLUT1	=	Glucose transporter 1
GR	=	Glutathione reductase
GSH	=	Glutathione
GSHPx	=	Glutathione peroxidase
GSSG	=	Oxidized glutathione reductase
GTP	=	Guanosine triphosphate
GTP-CH 1	=	Guanosine triphosphate cyclohydrolase 1
HbA1c	=	Glycosylated hemoglobin A
H_2O_2	=	Hydrogen peroxide

LIST OF ABBREVIATIONS (Continue)

hr	=	Hour
Hsp	=	Heat shock protein
IDDM	=	Insulin dependent diabetes mellitus
ICAM-1	=	Intracellular adhesion molecule-1
i.e.	=	Id est (that is)
iNOS	=	Inducible nitric oxide synthase
kg	=	Kilogram
L-NAME	=	N^{ω}-nitro-L-arginine methyl ester
$LOO^{\bullet}$	=	Peroxyl radical
MAP	=	Mean arterial blood pressure
MAPK	=	Mitogen-activated protein kinase
mg	=	Milligram
min	=	Minutes
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
μg	=	Microgram
μM	=	Micromolar
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NADH	=	Nicotinamide adenine dinucleotide
NE	=	Norepinephrine
nm	=	Nanometer
nNOS	=	Neuronal nitric oxide synthase
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NOSIP	=	eNOS-interacting protein

LIST OF ABBREVIATIONS (Continue)

NOSTRIN	=	eNOS traffic inducer
O_2	=	Oxygen
$O_2^{\bullet}$	=	Superoxide radical
$OH^{\bullet}$	=	Hydroxyl radical
$ONOO^{\bullet}$	=	Peroxynitrite
PKA	=	Protein kinase A
PKC	=	Protein kinase C
PTPS	=	6-pyrovoyl-tetrahydrobiopterin synthase
R^{-}	=	A radical
RAGEs	=	Glycosylation end-products receptor
RH	=	a component in the cell
ROS	=	Reactive oxygen species
SBP	=	Systolic blood pressure
SD	=	Standard deviation
Ser	=	Serine
SNP	=	Sodium nitroprusside
SOD	=	Superoxide dismutase
SR	=	Sorbitol dehydrogenase
STZ	=	Streptozotocin
TCA cycle	=	Trichloroacetic acid cycle
Thr	=	Threonine
TPR	=	Total peripheral resistance
Wks	=	Weeks
VCAM-1	=	Vascular adhesion molecule-1
VEGF	=	Vascular growth factor

CHAPTER I

INTRODUCTION

Diabetes mellitus is the syndrome of disturbed energy homeostasis, caused by an abnormal metabolism of carbohydrates, protein and fats. It is the most common endocrine-metabolic disorder of childhood and adolescent with important consequences on physical and emotional development (Foster 1998). The most devastating manifestations of diabetes are vascular complications. Persistent hyperglycemia (increase in blood glucose concentration in plasma) leads to deformation of specific glycoproteins, which may cause pathologic changes in the microvessels of vital organs (Werline 1996). Chronic vascular complications represent the main causes of morbidity and mortality in patients with diabetes, and occur in both micro and macrovasculatures (Sharpe et al., 1998).

Increasing evidences have suggested that altered endothelial function may play a major role (Palmer 1998) as an underlying cause of the pathogenesis of diabetic vasculopathy and microcirculatory disorder. In addition, previous studies in the aorta, mesenteric, cerebral and coronary arteries of animals with streptozotocin (STZ)-induced diabetes have shown an impaired relaxation in response to nitric oxide (NO)-mediated agonist, such as acetylcholine (Ach) (Fukao et al., 1997; De Vriese et al., 2000; Jariyapongskul et al., 2002; Chakraphan et al., 2005). Besides, in human with type 1 diabetes, the impaired responses of large arteries (Zenere et al., 1995) as well as the overexpression of leukocyte-endothelial interaction (Yang et al., 1996; Jariyapongskul et al., 2002) have been observed. Aforementioned studies suggest that alterations in the NO bioavailability play a central role in endothelial dysfunction induced by diabetes. This may be of major importance because NO can substantially inhibit several components of the atherogenic process, such as vascular smooth muscle cells contraction and proliferation, platelet aggregation, and leukocyte adhesion (De Caterina, *et al*., 1995 and Khan, *et al*., 1996).

Although various factors may engage in the pathogenesis of diabetic vasculopathy, many studies suggest a role for hyperglycemia-induced oxidative stress (Baynes 1991). Hyperglycemia may increase the production of reactive oxygen species (ROS) through several pathways, i.e. polyol pathway, nonenzymatic

glycosylation, glucose autooxidation and protein kinase C pathway (Du, et al., 2000; Nishikawa, et al., 2000; Brownlee. 2001). For example, hyperglycemia leads to increased cytosolic NADH/NAD^+ via the sorbital pathway which creates a redox imbalance (Williamson et al.,1993). Additionally, it has been reported that potential mechanisms that contributing in endothelial dysfunction is inactivation of NO by ROS, due to due to the rapid interaction between the NO and superoxide redical (O_2^-), leading to loss of NO bioavailability and increased production of peroxynitrite ($ONOO^-$), another ROS with potentially toxic effects. The principal of vascular ROS production by oxidase enzyme in the vascular wall include the reduced nicotinamide adenine dinucleotide/ reduced nicotinamide adenine dinucleotide phosephate (NADH/NADPH) oxidase and xanthine/xanthine oxidase, mitochondrial electron transport. Moreover, recent studies have found that increased eNOS uncoupling linking to a reduced tetrahydrobiopterin (BH_4) bioavailability is accompanied not only by reduced NO production but also by a direct contribution to vascular ROS production (Fridovich 1978; Bauersachs and Schafer 2005).

Oxidative stress is controlled by a variety of antioxidant cellular defense mechanisms consisting of enzymatic and nonenzymatic scavengers (Aydin A.,2001). Antioxidants are classified generally as endogenous antioxidants, such as, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)/oxidized glutathione reductase (GSSG-RD), those produced internally by animals and humans, and as exogenous antioxidants, i.e. glutathione, vitamin C and E, those which the body is not able to produce and which must be provided from external sources.

Antioxidant which have been studied in clinical DM are glutathione, vitamin C, vitamin E, SOD, allopurinol and hydrosoluble coenzyme Q10 (Frei 1994; Butler et al., 2000; Singh et al., 1999). Indeed, vitamin C is wildly regarded as an essential antioxidant--a strong reducing agent--due to its antioxidant properties (Wolinsky and Driskell, 1997). The plasma and tissue concentration of vitamin C have been reported to decrease in diabetic animals and human (Lindsay, et al., 1995; Kashiba 2000 and 2002; Jariyapongskul, et al., 2002; Backman, et al., 2003; Chakraphan, et al., 2005). Therefore, it has been suggested that the imbalance of oxidant and antioxidant system may be a crucial factor contributing to diabetes-induced endothelial dysfunction.

It has been shown by several studies that vitamin C reduced vascular oxidative stress (Nunes, *et al*, 1997, El-Missiry, 1999, Nazirogly, *et al*., 1999 and Chen, *et al*, 2001) and increased NO-mediated endothelium-dependent relaxations (Ting, *et al*.,

1996 and Jariyapongskul, *et al*., 2002). Vitamin C effectively prevents peroxidative damage of human plasma lipids by scavenging ROS, and sparing other endogenous antioxidants from consumption (Frei 1991). The effect of vitamin C in these studies has largely been attributed to scavenging of ROS. Therefore, the ROS-scavenging may be a major mechanism for improving the endothelium-dependent relaxation. However, the mechanism of the action of vitamin C on enhancing endothelial NO synthesis *in vivo* remains incompletely understood; it is believed that vitamin C could enhance generation of NO through diminished production of ROS by scavenging O_2^- to prevent the producing $OONO^-$. However, more recent studies have found evidence that induction with STZ in rats decreased BH_4 concentration. BH_4 which is an essential cofactor required for eNOS activity, was decreased due to the decrease in GTP cyclohydrolase expression (Meninger, *et al*., 2000 and Kohli, *et al*., 2004). Without adequate BH_4, NO synthesis by eNOS is impaired. Thus, *in vitro* studies showed that molecular effect of vitamin C might involve in stabilizing BH_4 (Cosentino and Luscher, 1999, Werner-Felmayer, *et al*., 2002, and Channon, 2004). In particular, the study performed in cultured endothelial cells has indicated that vitamin C could increase intracellular BH_4 (Heller, *et al*., 1999, 2001 and Huang, *et al*., 2000). Therefore, the second effect is believed that vitamin C could preserve eNOS cofactor BH_4, leading to enhance eNOS activity.

Such a beneficial effect of vitamin C, our previous study performed by Chakraphan., 2002 and Jariyapongsakul et al., 2002 have been revived the evidence that supplementation with vitamin C (1 g/ L daily) prevented endothelial from dysfunction in 12-wk diabetic rats with supported by several studies that working on high glucose or diabetic model (Sinclair et al, 1992; Eriksson and Kohvakka 1995; Nagano et al., 1996; Ting et al., 1996). Moreover, our previous study in the year 2003, Sridulyakul et al., indicated that the role of supplementation of vitamin C enhanced eNOS protein levels at 12-wk diabetic rats. However, the direct study working on effect of vitamin C supplementation even in animal model of diabetes for its treatment of endothelial dysfunction has not been clearly addressed.

Therefore, with this point of view, the present study aims to study the effect of vitamin C supplementation whether it can reverse endothelial cell from dysfunction in STZ-induced type 1 diabetic rats and further attempts to define its underlying mechanism(s) involving ROS scavenging and enhancing BH_4 bioavailability.

Research questions

Whether vitamin C supplementation could be able to reverse the diabetes-induced endothelial cell dysfunction or not? If so, what is the molecular mechanism underlying the reversal effect of vitamin C?

Objectives

1. To determine the effect of vitamin C supplementation whether it can reverse diabetes-induced endothelial dysfunction
2. To explore the molecular mechanism(s) underlying the reversal effect of vitamin C on endothelial dysfunction.

Hypothesis

Vitamin C supplementation can reverse diabetes-induced endothelial dysfunction. The molecular mechanisms of the reversal effect of vitamin C are involved by both ROS-scavenging and preserved BH_4 availability.

CHAPTER II

REVIEW LITERATURE

Diabetes Mellitus

Diabetes mellitus is a heterogeneous primary disorder of carbohydrate metabolism with multiple factors that generally involve absolute or relative insulin deficiency—type 1 diabetes, or insulin resistant—type 2 diabetes. All cause of diabetes ultimately lead to hyperglycemia. A defective or deficient insulin secretary response underlines the enhanced blood glucose concentration. Fat and protein metabolisms are also commonly affected (Atkinson and Maclaren, 1994 and Defronzo, 1998).

Diabetic complications include acute metabolic complications such as diabetic ketoacidosis (Carroll and Matz, 1983 and Kitabchi, 1989), hyperosmolar non-ketotic coma, lactic acidosis, hypoglycemia, and chronic complications such as macroangiophathy and microangiopathy (Ramsay, *et al*., 1988 and Sequist, *et al*., 1989).

Type 1 diabetes mellitus

Type I diabetes mellitus (formerly known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes) in which the beta cells of pancreas fail to produce the insulin because of it has been attacked and destroyed (Atkinson and Maclaren 1994; Defronzo 1998). Three interlocking mechanisms are responsible for the beta cells destruction: genetic susceptibility (Kyvink *et al.,* 1995), autoimmunity (Bach 1994) and an environmental insult (Verge *et al.,* 1996; Corad *et al.,*1997) as showed in Figure 1.1.

The destruction of beta cells results in deficiency and finally total loss of insulin secretion. What factors may trigger this autoimmune islet damage is a controversy concerning. Increasing evidences suggest that free radicals play as one of

factors in the beta cells damage. Streptozotocin (STZ) (Turk 1993) and alloxan (Oberley 1988) are two most commonly used drugs in developing the diabetes animal madels due to their ability to selectively damage the insulin-secreting beta cells of pancreatic islets and induce impairment of islet glucose oxidation and glucose-induce insulin secretion.

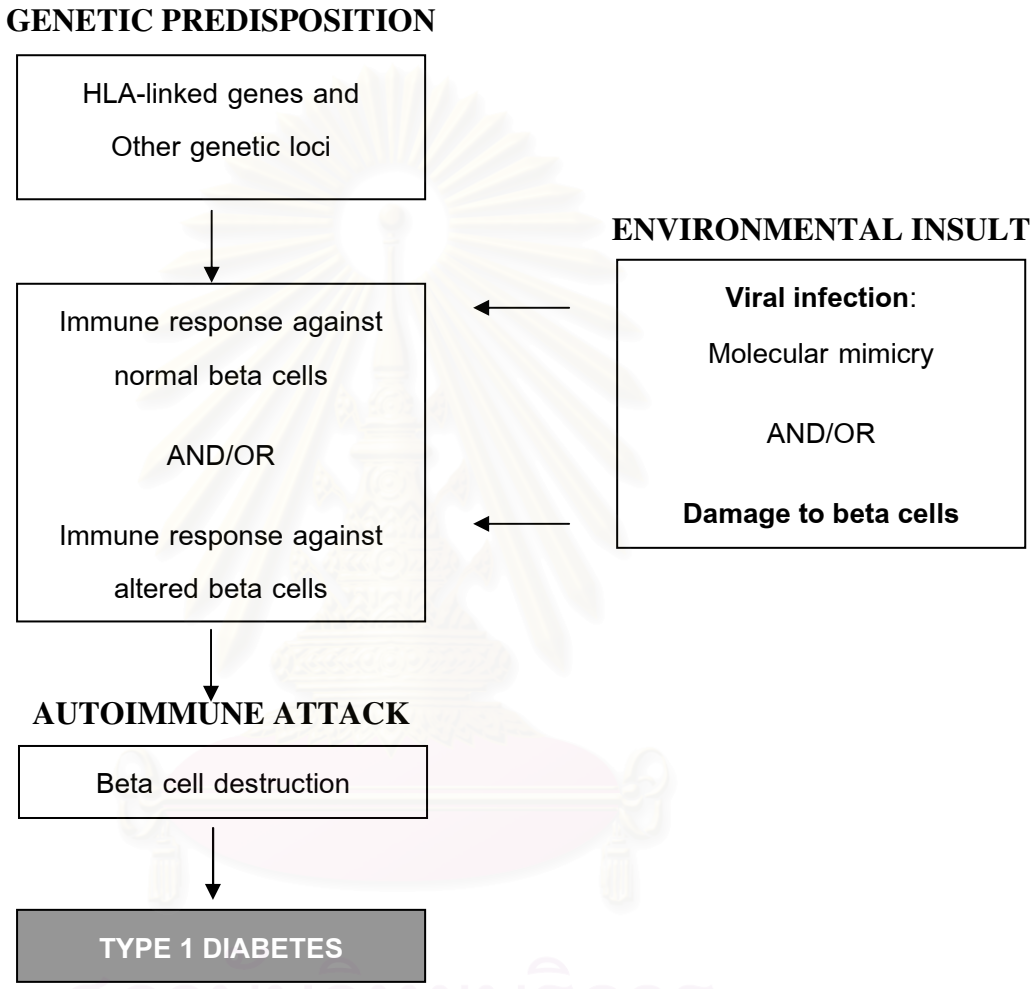


Figure 1.1 A simplified scheme to show pathways of beta cell destruction leading to type 1 diabetes mellitus

This abnormality is resulting in increased concentration of glucose in the plasma; hyperglycemia. In this type of diabetes develops most frequently in children, but is being increasingly noted later in life. The symptoms are excessive secretion of urine (polyuria), thirst (polydipsia), weight loss and tiredness (Cotran 1999). Type 1 diabetic patients require exogenous insulin administration in order to restore a normal metabolic state. For correct dosing insulin, the patient usually measures blood glucose

several times per day. For long-term evaluation of the treament HbA1c, a glycated form of haemoglobin A, is used to estimate the mean blood glucose level during the two to six weeks preceding sampling. However the patient with uncontrolled or long-term hyperglycemia can damage many of the body's system, in particular the blood vessels that also involve in the vascular disorder, which are microangiopathy and macroangiopathy, and caused primarily by hyperglycemia (Atkinson and Maclaren 1994; Defronzo 1998; Dorman 1984; Fuller 1983; Head 1990). A large literature had established that diabetes and hyperglycemia increase oxidative stress (Wolff 1991).

Oxidative stress

Recently, increasing evidence suggests that, in diabetes, oxidative stress play a key role in the pathogenesis of microvascular complication (Giugliano *et al.,* 1996). The oxidative stress is significantly increased in diabetes because prolong exposure to hyperglycemia increases the generation of the production of reactive oxygen species (ROS) and reduces capacities of antioxidant defense system (The Diabetes Control and Complications Trials (DCCT) Research Group 1993). Therefore, oxidative stress has been defined as a disturbance in the balance between the production ROS and antioxidant defense.

1) Reactive Oxyen Species (ROS)

A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell 1994). ROS include free radicals, as well as other oxygen-related reactive compounds (Halliwell and Gutteridge 1989). The ROS are the superoxide radical (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2).

The unpaired electron of O_2^- imparts high reactivity, unstable and short lived. O_2^- is water soluble and can act either as an oxidizing agent, where it is reduced to H_2O_2, or as an reducing agent, where it donates its extra electron to form peroxynitrite ($ONOO^-$) with nitric oxide (NO) (Touyz 2003; Halliwell B. 1991). Under physiological conditions in aqueous solutions at a neutral pH, its preferred reaction is the dismutation reaction that yields H_2O_2. However, when NO is produced in excess, a significant amount of O_2^- reacts with NO to produced $OONO^-$ (Touyz 2003). O_2^- is membrane impermeable but can cross cell membranes via anion channels.

H_2O_2 is generated through nonenzymatic or enzymatic dismutation of O_2^- (Halliwell 1991). Humans consume approximately 250 g of oxygen (O_2) per day and of this 3-5% is converted to O_2^- and other ROS (Touyz 2003). A typical human cell metabolizes about 10^{12} molecules of O_2 per day and generates approximately 3 x 10^9 molecule of H_2O_2 per hour. H_2O_2 is lipid soluble, can cross cell membranes and is stable under physiological conditions.

OH^- is a potent oxidant that can be produced directly from water of H_2O_2. It is extremely reactive and therefore has a very short half-life and dose not travel more than a few molecular diameters from its site of formation (Figure 1.2) (Touyz 2003;Halliwell 1991).

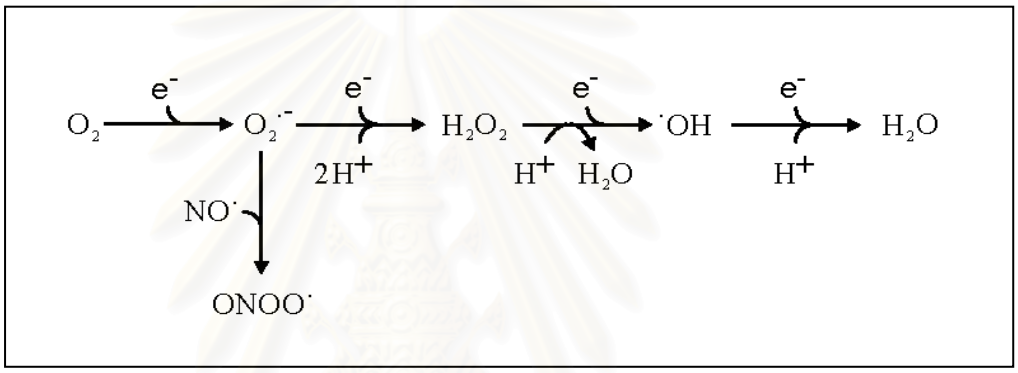


Figure 1.2 The formation of reactive oxygen species
(Modified from Chabot et al. 1998)

Moreover peroxyl radical (LOO^-), the one of ROS can be generated in the process of lipidperoxidation, which is initiated by the abstraction of hydrogen atom from polyunsaturated fatty acid (Figure 1.3) (Stahl and Sies 1997)

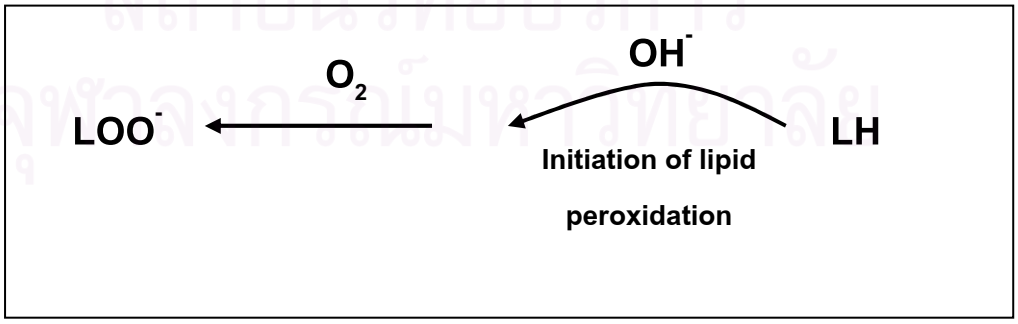


Figure 1.3 The formation of peroxyl radical
(Modified from Stahl and Sies 1997)

2) Antioxidant defenses

Since formation of ROS is a great threat to aerobic cells, the antioxidative defense systems are essential. There are a number of antioxidants present in the body and derived from diet. Based on the location, they can be divided into intracellular and extracellular antioxidants (Evans and Halliwell 2001). Intracellular enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx) (Evans and Halliwell 2001).

SOD (a CuZn- or Mn-containing enzyme) removes O_2^- by converting it into H_2O_2. There are very large amounts of SOD in the arteries of interstitium (Touyz 2003).

Catalase catalyses the direct composition of H_2O_2 to water and O_2 (Touyz 2003).

GSHPx is the other main enzyme neutralizing H_2O_2 into water and O_2. There is a family of GSHPx, which are the enzyme γ-glutamyl cysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione (GSH) synthesis, and glutathione reductase (GR) in which to reduce the oxidized form of glutathione (GSSG) back to GSH (Evans and Halliwell 2001). GSHPx is the major enzyme that protects the cell membrane against lipid peroxidation, since GSH donates protons to the membrane lipids maintaining them in a reduced state (Touyz 2003).

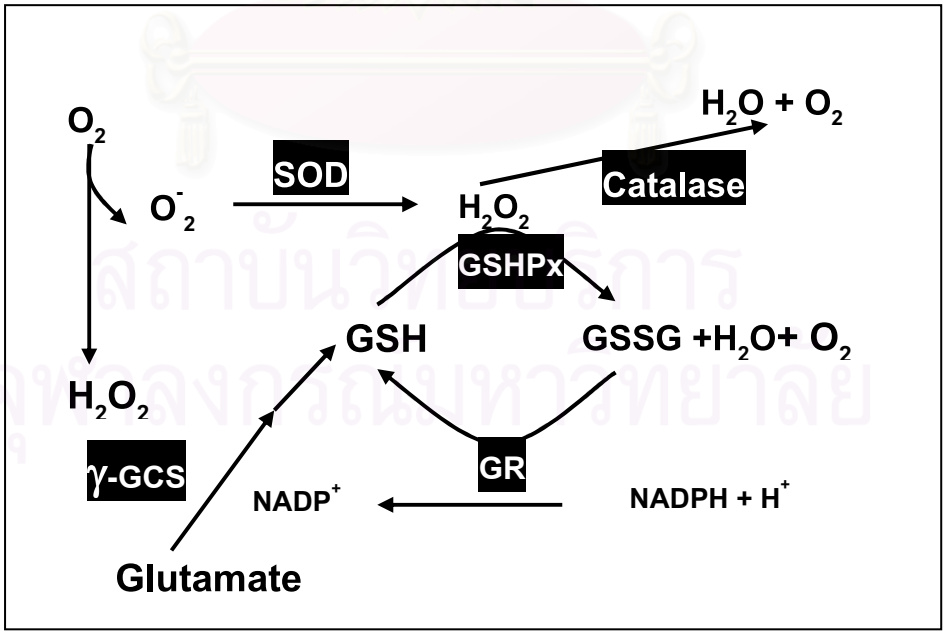


Figure 1.4 Co-operation of intracellular enzymatic antioxidants

Several extracellular antioxidants are the metal-bind proteins such as hemoglobin, myoglobin, transferring, lactoferrin, ferritin and ceruloplasmin are

involved in reducing OH^- formation and the proteins that directly scavenge ROS which are albumin, bilirubin and urate (Touyz 2003; Evans and Halliwell 2001).

In addition to endogenous enzyme antioxidants, numerous nonenzymatic antioxidants are also found in biological systems. These include ascorbic acid (vitamins C), α-tocopherol (vitamin E), flavonoids, carotenoids, bilirubin and thiols (Touyz 2003; Evans and Halliwell 2001).

Endothelial dysfunction in diabetes mellitus

Endothelial cell is crucial in the control of vascular tone, platelet and leukocyte adhesion to the endothelial surface, and smooth muscle cell proliferation (Luscher and Vanhoutte, 1990). In diabetes, hyperglycemia could damage endothlial cells due to the severe prolonged oxidative stress (The Diabetes Control and Complications Trials (DCCT) Research Group, 1993).

1) Endothelial cell; role of endothelium in control of vascular function

The endothelial cell layer participates in the control of vascular tone and blood pressure by producing relaxing and contracting factors, thus modulating tone of the underlying smooth muscle (Furchgott and Zawadzki 1980). In 1980, Furchgott and Zawadzki demonstrated that stimulation of the endothelium with acetylcholine (Ach) caused the release of a substance they named endothelium-derived relaxing factor (Furchgott and Zawadzki 1980). Until now, it also known as nitric oxide (NO) (Palmer *et al.,* 1987).

NO is a lipophilic gas which is formed enzymatically from its precursor L-arginine by three isoforms of NO synthase (NOS) follow by, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Stuehr 1999). NO diffuses to the vascular smooth muscle cells and activates guanylate cyclase (GC), which leads to the increased production of cyclic guanosine 3',5'-monophosphate (cGMP). Thus, in turn, activates kinase resulting in decline in intracellular ionized calcium in the vascular smooth muscle leading to relaxation and vasodilation (Moncada *et al.,* 1991). The NO production can be upregulated by physical forces, such as shear stress, as well as by several receptor-operating transmitters and

hormones such as Ach, bradykinin (BK), estrogen, substance P, serotonin, adrenaline and noradrenaline, adenosine and thromboxane (Moncada *et al.,* 1991) (Figure 1.5).

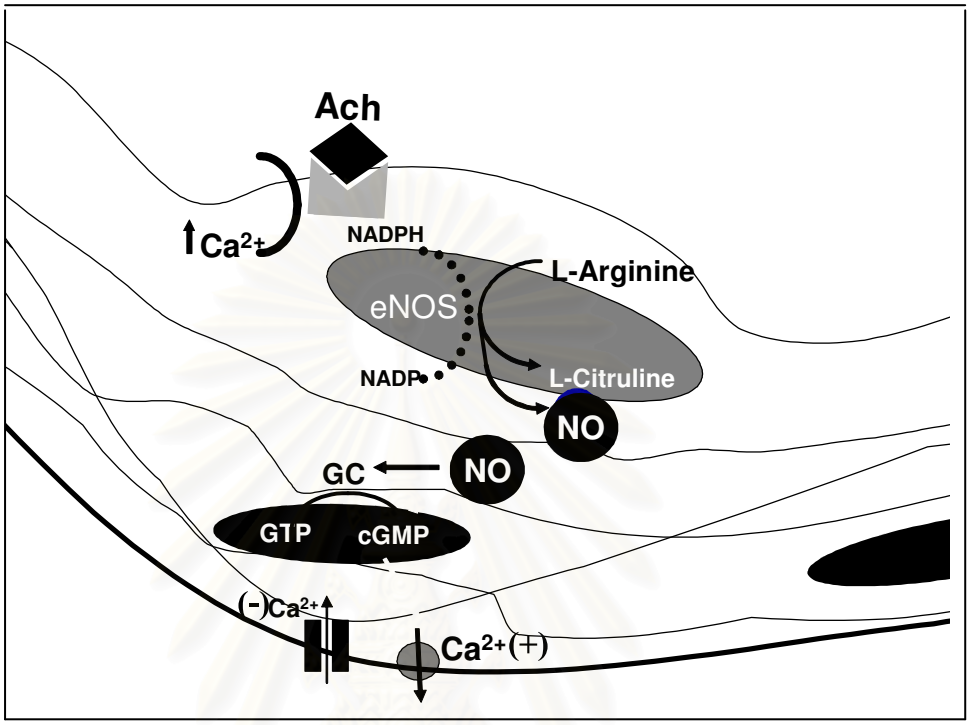


Figure 1.5 Schematic of pathway for nitric oxide production by vascular endothelium, which occurs in response to receptor activation by acetylcholine

2) Endothelial cell dysfunction

Especially to focus on vascular endothelial cells, the hyperglycemic could damage this targeted cells due to the severe prolonged oxidative stress (The Diabetes Control and Complications Trials (DCCT) Research Group 1993). Therefore, the endothelial dysfunction was commonly characterized by a decrease in endothelium-dependent relaxation resulting in the loss of NO bioactivity in the vessel wall (Vriese, *et al*., 2000). Several diabetic animal model studies have shown impaired relaxation in response to agonist such as Ach or BK, which was used to test the vasoactive function of endothelium, especially in mesenteric artery of STZ-induced diabetic rats as summarized in Table 1.1 (Taylor, *et al*., 1992, Diederich, *et al*., 1994, Heygate, *et al*., 1995, Fukao, *et al*., 1997, Palmer, *et al*., 1998 and Chakraphan, *et al*., 2005).

Table 1.1 Experimental studies in rats demonstrated the impaired endothelium-dependent relaxation according to diabetes.

Reference	Type1 Diabetes model	Vessel	EDR	EIDR
Taylor et al., 1992	STZ, 5-6 w	Mesenteric artery	Ach ↓	SNP ↔
Diederich et al., 1994	STZ, 6-24 w	Mesenteric artery	Ach ↓	SNP↑ 6w, ↔ 12- 24w
Heygate et al., 1995	BBd, 6-8 w	Mesenteric artery	Ach↓, BK↓	SNP ↔
Fukao et al., 1997	STZ, 8-12 w	Mesenteric artery	Ach ↓	Pinacidil ↔
Palmer et al., 1998	STZ, 4-5w	Mesenteric artery	Ach ↓	SNP ↔

(EDR, endothelium-dependent relaxation;EIDR, endothelium-independentrelaxation; BBd, diabetic biobreed; SNP, sodium nitroprusside) (↔ and ↓ were indicated for unchanged and decreased, respectively.)

Furthermore, the impaired endothelium-dependent relaxation resulting in the decrease of NO bioactivity could cause the pathophysiology of leukocyte-endothelial interaction since activity of NO produced by eNOS inhibits leukocyte adhesion and transmigration (Moncada , *et al*., 1991). The process of leukocyte traveling on endothelium is composed of 1. migration 2. rolling 3. activation and adhesion 4. transmigration (Figure 1.6). Several studies showed that enhanced leukocyte adhesion was demonstrated in diabetes mellitus (Hadcock, *et al*., 1991, Yang, *et al*., 1996 and Jariyapongskul, *et al*., 2002).

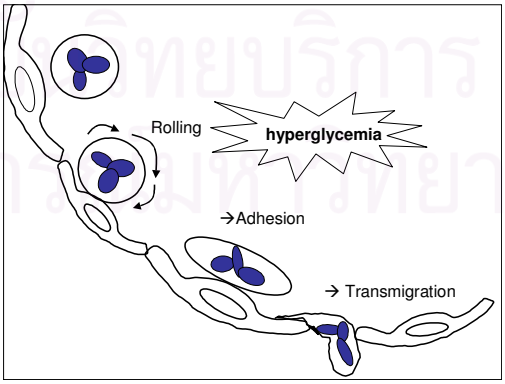


Figure 1.6 Three major steps of leukocyte-endothelium interaction are rolling, adhesion, and transmigration.

In particular, it has been well documented that hyperglycemia could generate the increase in ROS through several pathways including polyol pathway, nonenzymatic glycosylation, glucose autoxidation, and protein kinase C (Du, *et al*., 2000 , Nishikawa, *et al*., 2000 and Brownlee, 2001).

3) Generation of ROS in type 1 diabetes

A large literature had established that diabetes and hyperglycemia increase oxidative stress (Wolff 1991), but neither the understanding mechanism nor the consequences on other pathways of hyperglycemia damage were known. In 2000, Brownlee *et al*., discovered the underlying mechanism by which hyperglycemia causes oxidative stress in cells which are susceptible to diabetic complication, that is in cells which do not down regulate glucose transport in the presence of hyperglycemia. This mechanism involves overproduction of O_2^- by the mitochondrial electron-transport chain, as a consequence of increased electron donor (NADH and $FADH_2$) generation by increased glucose oxidation in the Trichloroacetic acid cycle (TCA cycle). This increases the electrochemical potential difference across the mitochondrial membrane above the threshold value required for increased O_2^- production (Figure 1.7).

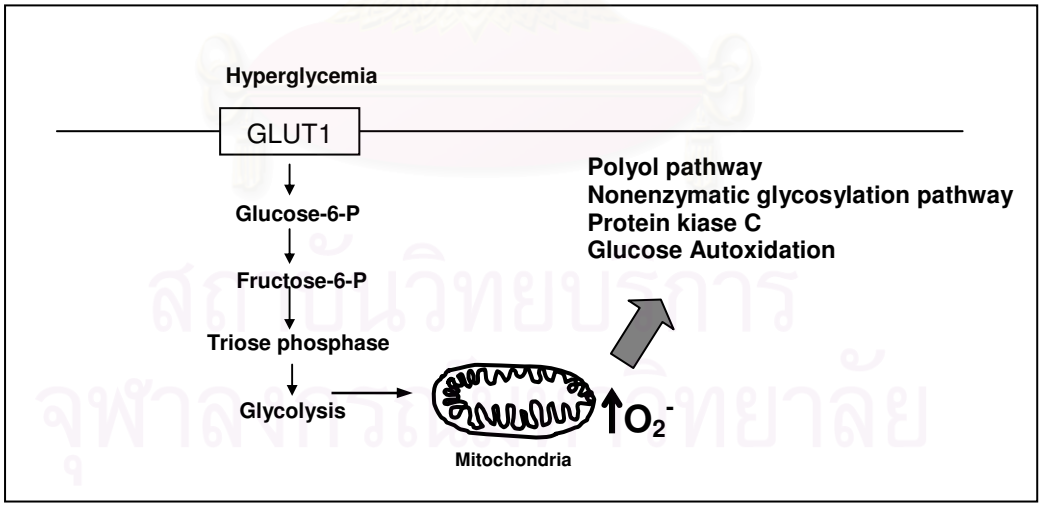


Figure 1.7 In endothelial cells, glucose can pass freely through the cell membrane in an insulin-independent manner via glucose transporter 1 (GLUT1) under intracellular hyperglycemia induced overproduction of O_2^- at mitochondria level.

The consequences of this O_2^- production are to activate all of the major mechanisms of hyperglycemic damage as follow, flux through the polyol pathway, increased nonenzymatic glycosylation, flux through the hexosamine pathway, protein kinase C activation and glucose autoxidation (Nishikawa *et al.,* 2000; Brownlee 2001; Du *et al.,* 2000)

Polyol pathway

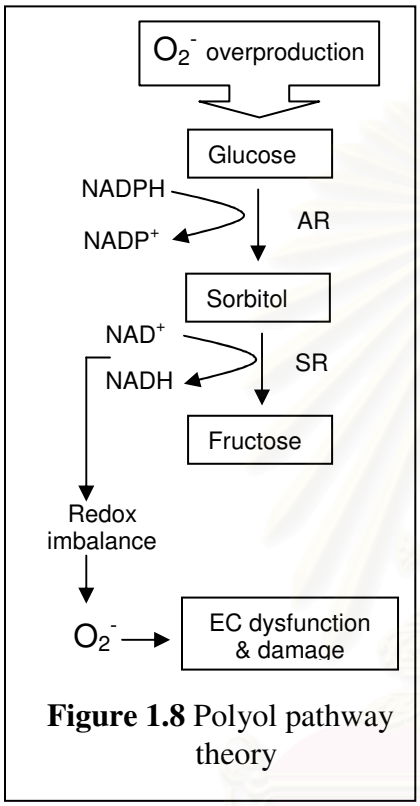


Figure 1.8 Polyol pathway theory

O_2^- overproduction can also induce an increased flux through the polyol pathway (Baynes 1991). Originally it was thought that increased intracellular sorbitol and fructose content due to aldose reductase (AR) and sorbitol dehydrogenase (SR) activity. Oxidation of sorbitol to fructose is coupled to reduction of NAD^+ to NADH leading to redox imbalance (NADH /NAD^+ ratio). An increase in cytosolic NADH /NAD^+ ratio is linked to O_2^- formation (Figure 1.8). Due to a high level of expression of AR in the lens compared with other tissues, an increased level of sorbitol is believed to contribute to the development of cataract in the pathogenesis of diabetic retinopathy (Kioshita. 1974).

The depletion in cytoslolic cellular NADPH caused by increase in AR flux may decrease the generation of NO in endothelial cells (Tesfanarian 1994). Because NADPH is required for generation of NO form arginine (Figure 1.9). Therefore this pathway contribute to secondary complications of endothelial cells dysfunction and damage in diabetes.

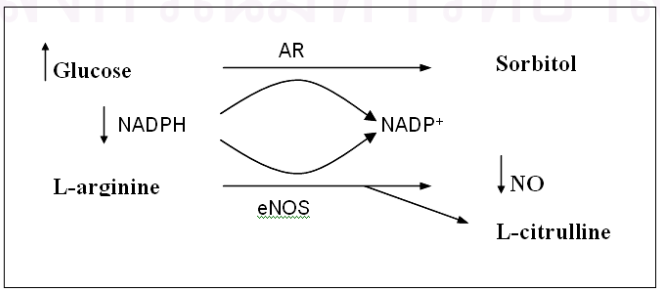


Figure 1.9 The polyol pathway and eNOS pathway that required NADPH as a cofactor

Nonenzymatic glycosylation pathway

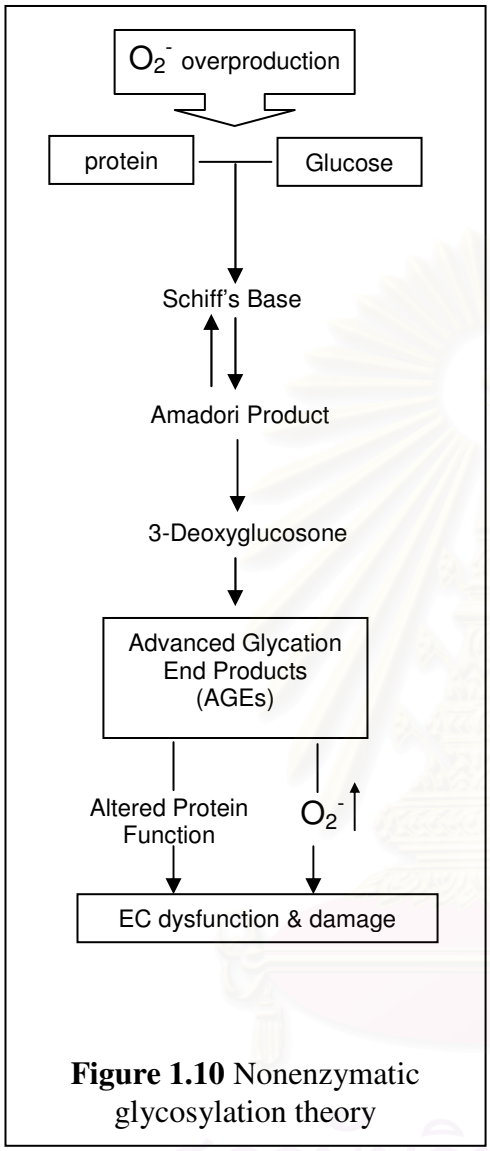


Figure 1.10 Nonenzymatic glycosylation theory

Nonenzymatic glycosylation of proteins under hyperglycemia conditions is accompanied by the O_2^- production. Initially, glucose reaction with the amino group of protein without the aid of enzyme to form the schiff's base, reversible glycosylation product. It subsequently undergoes an amadori product, which is also reversible. Further reactions, rearrangments, dehydration and cleavage irreversibly result in the formation of crosslinking complexes called advanced glycated end product (AGEs) (Figure 1.10.) (Wolff *et al.,* 1991). Formation of AGEs may damage cells by impairing the function of a wide range of protein (Brownlee *et al.,* 1984) including modifications of extracellular structural proteins such as collagen and intracellular protein (Giardio *et al.,* 1994; Chibber *et al.,* 1999).

This pathway also involved in the production of $O_2^{-\cdot}$. As when AGEs binds to its receptor (RAGEs), intracellular production of O_2^- occurs leading to increased expression of cell adhesion molecules (Dandona *et al.,* 2000) and increased expression of monocyte tissue factor (Maggi *et al.,* 1996) resulting in the progression of endothelial cells dysfunction and damage.

Protein kinase C

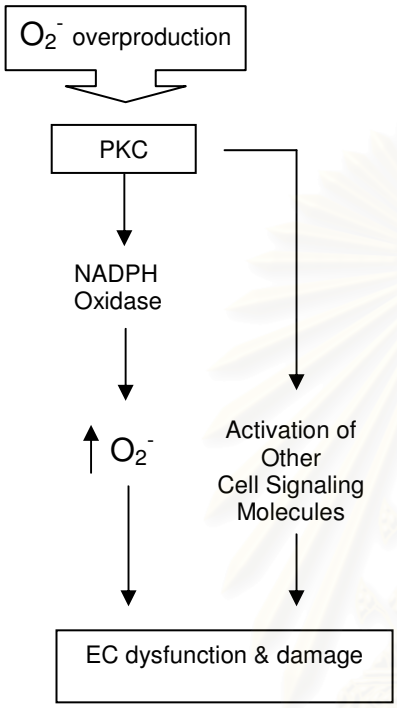


Figure 1.11
Protein kinase C theory

The activation of protein kinase C (PKC), due to O_2^- overproduction, induces a *de novo* synthesis of the enzyme NADPH oxidase, which significantly contributes to produce more O_2^- (Figure 1.11) (Bohlen and Nase 2001). In addition, PKC activation can also impact other signaling pathways such as mitogen-activated protein kinase (MAPK) or nuclear transcription factor (Tonlindon 1999). Activation of PKC in endothelial cells of the retina, aorta, heart, glomeruli and nerves can produce vascular endothelial cells damage that induces increased permeability , alteration in blood flow (Shiba *et al.,* 1993), increased leukocyte adhesion and decreased NO bioactivity (Kuboki *et al.,* 2000; Bohlen and Nase 2001).

PKC is a phospholipid dependent serine/ theronine kinase composed of at least 12 isoforms (Mellor and Parker 1998). The PKC-β isoforms are activated in the aorta and heart of diabetic rats, while PKC-α, β and ε are all activated in the retina with diabetes (Shiba *et al.,* 1993). In the glomeruli of rat with diabetes, the α, β, δ, ε and ζ isoforms of PKC have all been shown to be activated. Recently, it has been reported that a PKC-β isoform-specific inhibitor (LY 333531) has been developed and its usefulness in inhibiting the progression of diabetic complication has been demonstrated (Inoguchi *et al.,* 1992).

The overall mechanisms of hyperglycemia-induced ROS can be summarized by Figure 1.12.

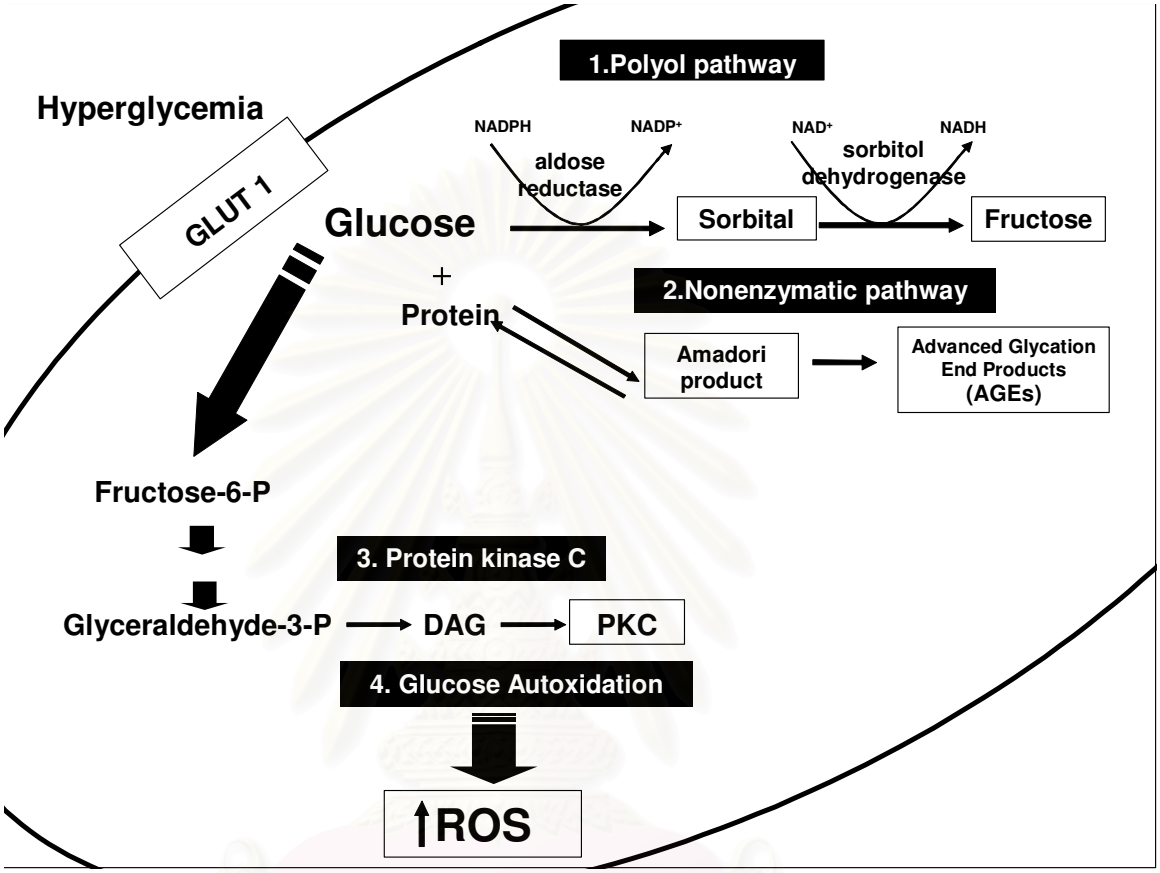


Figure 1.12 The major mechanisms of hyperglycemic damage

Moreover, the severity of high glucose induced ROS could be extended in diabetes because the decreased capacity of antioxidant defense system, including enzymatic antioxidants such as, SOD, catalase and GSHPx, and non-enzymatic or exogenous antioxidants such as, carotenoids, vitamin E and vitamin C (Giugliano, *et al*.,1996 and Vriese, *et al*., 2000).

Vitamin C

1) General properties and structure

Vitamin C (ascorbic acid) is a water-soluble antioxidant vitamin and essential cofactor for many enzymes. It is synthesized from glucose required by most animals, but not by humans, primates, guinea pigs, and some other animals which lack the enzyme L-gulonolactone oxidase; this makes these animals rely on dietary vitamin C (Padayatty, *et al*., 2003). Therefore, humans require vitamin C in their diet. Fruits and vegetables are rich in vitamin C. While prolonged storage of unprocessed fruits and vegetables leads to the loss of vitamin C, most means of food processing (boiling, steaming, freezing, canning) preserve vitamin C to a great extent (Bsoul and Terezhalmy 2004).

Vitamin C exists in humans in two biologically active forms, including ascorbic acid, and its oxidized form—dehydroascorbic acid (DHA), that give its antioxidant capabilities (Wolinsky and Driskell, 1997) (Figture 1.13). Ascorbic acid is easily oxidized to DHA, which can in turn be reduced again to ascorbic acid (Wolinsky and Driskell, 1997).

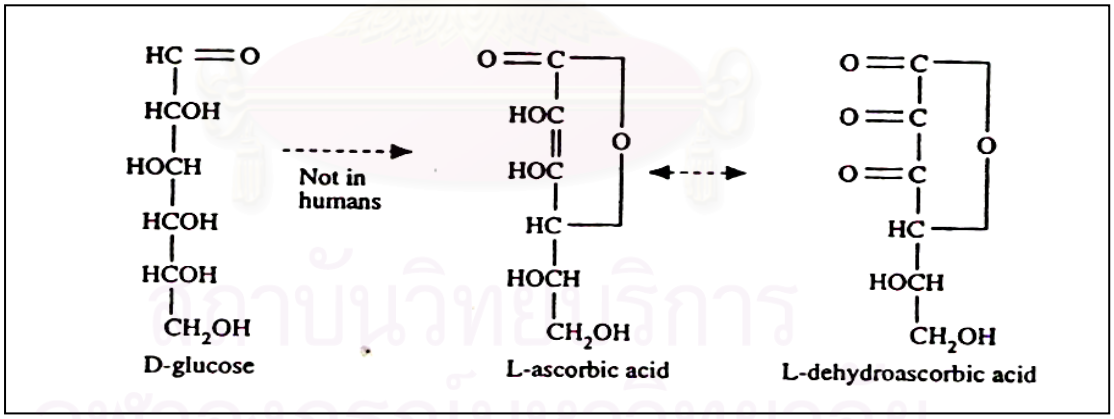


Figure 1.13 The structure and inter-conversion of glucose, ascorbic acid, and dehydroascorbic acid

Vitamin C is absorbed in the upper small intestine at physiological dietary intakes (50 to 200 mg/day or 80-90% efficient). This efficiency rate, however, declines with increased intake. Vitamin C is actively co-transported with sodium against an electrochemical gradient into intestinal (small bowel) epithelial cells. Once

in the cells, a concentration gradient is created by both brush border absorption and intracellular reduction of DHA to ascorbic acid. Facilitated diffusion of ascorbic acid into the circulation is sodium independent and follows a concentration and electrochemical gradient. A similar transport mechanism is responsible for the near complete reabsorption of ascorbic acid in the kidneys. Large intakes (gram doses) of vitamin C may be absorbed by passive diffusion (Wolinsky and Driskell 1997). After absorbtion, vitamin C rapidly equilibrates in intra- and extracellular compartments. Although no particular organ acts as a storage reservoir for the vitamin, there are several tissues and plasma distribution of vitamin C in an adult human in descending order as follows, pituitary gland, adrenal gland, eye, lens, brain, liver, spleen, kidneys, heart muscle, lungs, skeletal muscle, testes, thyroid, leukocytes, and plasma (Basu and Schorah 1982).

Ascorbic acid and, significantly, one of its metabolites, oxalate, are found in the urine only in excess states. The latter is pertinent, as it accounts for one of the few potential clinical toxicities of systemic vitamin C supplementation, oxalic acid renal stones (Bsoul and Terezhalmy. 2004).

2) Antioxidant properties

The biological actions of vitamin C are attributed to its antioxidant properties. Vitamin C sequentially donates electrons (e^-) from the double bond between the second and third carbons on the 6-carbon molecule, becoming oxidized to the ascorbyl radical in the process. The ascorbyl radical is generally much less reactive than the free radical that was quenched. The ascorbyl radical can also be recycled back to vitamin C through three separate enzymatic pathways as well as by reducing compounds such as glutathione. Vitamin C can also aid in the recycling of other antioxidants, most notably vitamin E (Halliwell, 1996 and Padayatty, *et al*., 2003).

Compounds which can be reduced by vitamin C include ROS such as, O_2^-, OH^-, H_2O_2 and many others (Brody 1994 and Padayatty, *et al*., 2003) (Figure 1.14).

$$\text{HO-ASC-OH} + OH^- \longrightarrow \text{HO-ASC=O} + H_2O$$
$$\text{HO-ASC-OH} + O_2^- \longrightarrow \text{O=ASC=O} + H_2O_2$$
$$\text{HO-ASC-OH} + H_2O_2 \longrightarrow \text{O=ASC=O} + 2\ H_2O$$

Figure 1.14 Possible reactions between vitamin C (HO-ASC-OH) and ROS

Moreover, vitamin C may be involved in reducing damage to the cell from radicals. A simplified mechanism (Figure 1.15) shows the OH^- reacting with a component in the cell (RH), abstracting (pulling off) a hydrogen radical (H^-). The product is a radical (R^-), but it is one that is more stable than OH^-. Vitamin C may donate a H^- to this product, thus repairing it before further deterioration can occur (Brody 1994).

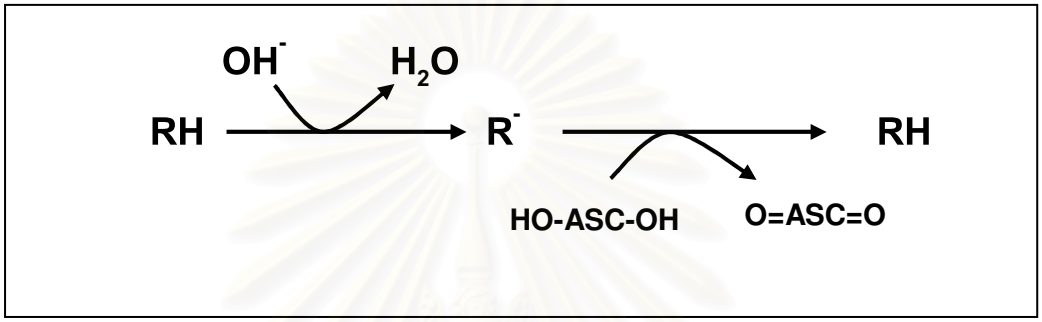


Figure 1.15 Possible use of vitamin C (HO-ASC-OH) in reducing damage from radical (Modified from Brody 1994)

Vitamin C is also thought to be involved in recycling the α-tocopheryl radical back to α-tocopherol (Halliwell 1996). Moreovere, vitamin C acts as an electron donor for eight known enzymes. Out of these, three participate in collagen hydroxylation, which increases the stability of the collagen structure, two participate in the synthesis of carnitine, one participates in the biosynthesis of norepinephrine from dopamine, one adds amide groups to peptide hormones to increase their stability, and one modulates tyrosine metabolism (Padayatty, *et al*., 2003).

Vitamin C and diabetes mellitus

It has been reported that plasma concentration of vitamin C are generally believed to reflect dietary intake. Therefore, the studies in those with type1 diabetes showed that plasma vitamin C concentration were lower than animals and humans without diabetes (Lysy, *et al*., 1992, Lindsay, *et al*., 1995, Jariyapongskul, *et al*., 2002 Backman, *et al*., 2003, and Chakraphan, *et al*., 2005). These findings are summarized in table 1.2.

Table 1.2 Plasma vitamin C in type 1 diabetes mellitus

Reference	Type1 diabetic model	Plasma vitamin C levels
Lysy J, et al., 1992	Patients	↓
Backman JA, et al., 2003	Patients	↓
Lindsay RM, et al., 1995	STZ-rats and BBd-rats	↓
Jariyapongskul A, et al., 2002	STZ-rats	↓

(BBd rats, diabetic BioBreeding rats)(↓ was indicated for decreased.)

Vitamin C and endothelial dysfunction in diabetes mellitus

Given the involvement of oxidative stress in diabetes endothelial dysfunction, supplementation with vitamin C could be interesting, by allowing a delay in the appearance or in the development of vascular endothelial dysfunction (Bonnefont-Rousselot, *et a*l., 2000). Of the three studies that examined blood glucose concentrations (Som, *et al*., 1981, Sinclair, *et al*., 1991 and Jariyapongskul, *et al*., 2002) two reported that plasma glucose remained unchanged following shot-term supplementation with vitamin C (Som, *et al*., 1981 and Sinclair, *et al*., 1991) and one reported that plasma glucose reduced after long-term supplementation with vitamin C for 36 wks (Jariyapongskul, *et al*., 2002). Moreover, another study found that infusion of vitamin C into the brachial artery improved forearm blood flow in diabetic patients (Ting, *et al*., 1996). Recently, *in vivo* studies using intravital fluorescence microscopy in STZ rats have demonstrated an important role of vitamin C supplementation not only in reduced leukocyte endothelial cell interactions but also in enhancing endothelial nitric oxide synthase (eNOS) protein levels in heart and aorta (Jariyapongskul, *et al*., 2002; Sridulyakul, *et al*., 2004) These findings are summarized in table 1.3.

Table 1.3 Experimental studies examining vitamin C supplementation and its impact on various health outcomes

Reference	Diabetic model	Vitamin C supplementation	Outcome
Som et al., 1981	Patients	0.5 g/day for 15 days	Plasma glucose ↔
Sinclair et al., 1991	Patients	1 g/day for 6 weeks	Plasma glucose ↔
Jariyapongskul et al., 2002	STZ-rats	1 g/L/day for 36 weeks	Plasma glucose ↓
Ting et al., 1996	Patients	24 mg/ minute for 10 minutes	Forearm blood flow ↑
Jariyapongskul et al., 2002	STZ-rats	1 g/L/day for12, 24 and 36 weeks	Leukocyte adhesion ↓
Sridulyakul et al., 2004	STZ-rats	1 g/L/day for12, 24 weeks	eNOS protein levels in heart and aorta ↑

(↔, ↓, and ↑ were indicated for unchanged, decreased, and increased, respectively.)

As an overall conclusion, Schematic A (Figure 1.16) has summarized the possible antioxidant effect of vitamin C on preserving vascular endothelium in diabetes mellitus.

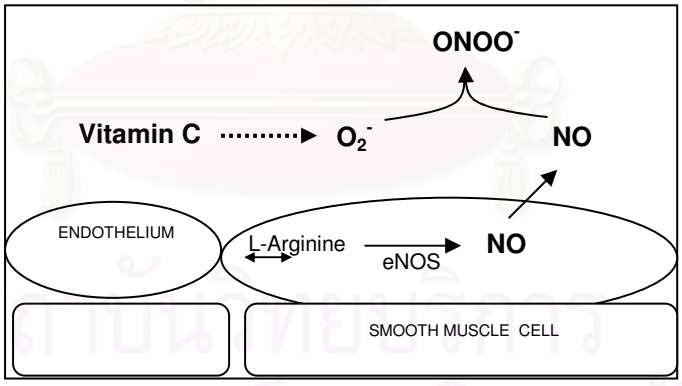


Figure 1.16 Schematic A: Potential mechanism by which vitamin C preserves the biological activity of endothelium-derived NO

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNOS) is the main source of NO in vasculature. Deregulation of eNOS is involved in pathogenesis of many cardiovascular diseases especially in type 1 diabetes (Vanhoutte 1997; Harrison 1997).

In addition, three isoform of NOS were identified in mammalian system: nNOS, iNOS and eNOS. They are encoding by different genes and have different tissue distribution and regulation. Therefore the main feature of eNOS isoform is summarized in table 1.4 (Andrew and Mayer 1999).

Table 1.4 The main feature of eNOS isoform

Cellular localization	Tissue distribution	Regulation	Expression	Gene structure	Chromosome location
Membrane bound	Vascular endothelium, pulmonary epithelium	Ca^{2+}/CaM, shear stress	Constitutive	26exons, 25 introns	7q35-36 of Chromosome7

Structure of eNOS

eNOS is a very complex enzyme that contains several cofactors in the domain structure (Alderton *et al.,* 2001). (Figure 1.17)

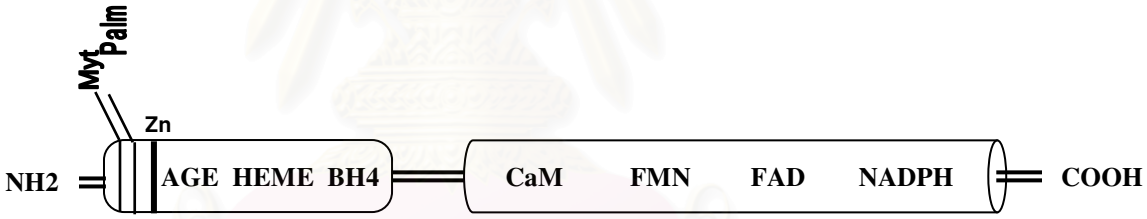


Figure 1.17 Domain structure of eNOS

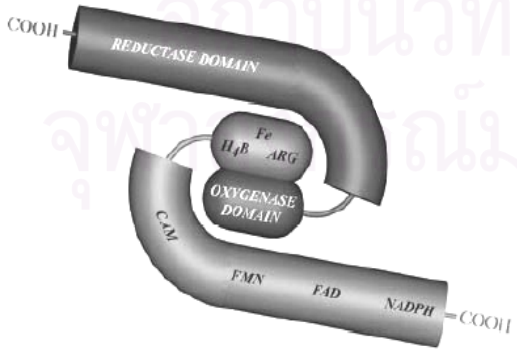


Figure 1.18 Domain strcure of eNOS dimer

Active eNOS has a dimeric form (Figure 1.18). Each monomer has two domains that are a C-termial reductase domain and an N-terminal oxygenase domain. The former contains binding sites for the redox cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin (CaM), and the latter binds heme group,

L-arginine (AGE), and tetrahydrobiopterin (BH_4). NADPH acts as a two-electron (e^-) donor. FAD and FMN serve as an e^- storage pool and transfer agent (Alderton *et al.*, 2001).

Catalytical mechanism of eNOS

Biosynthesis of NO involves a two-step of eNOS-catalysed reaction for the conversion of L-arginine to L-citrulline and produced NO production. The first step is the hydroxylation of the amide nitrogen atom of L-arginine. The second step is conversion of N^G-Hydroxy-L-Arginine to L-citrulline and NO. NADPH serves as an electron (e^-) donor for both reactions (Andrew and Mayer 1999) (Figure 1.19).

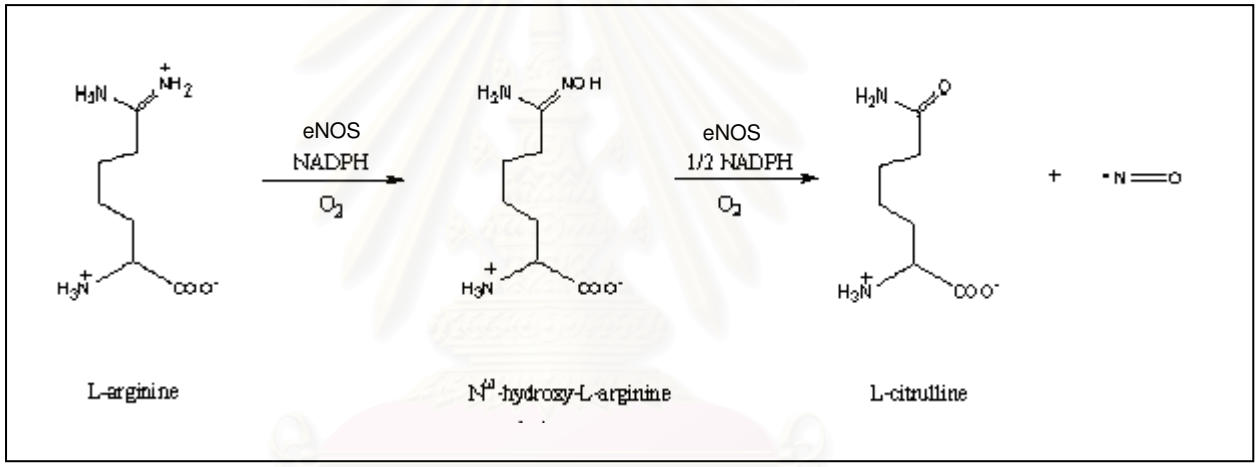


Figure 1.19 The eNOS reaction

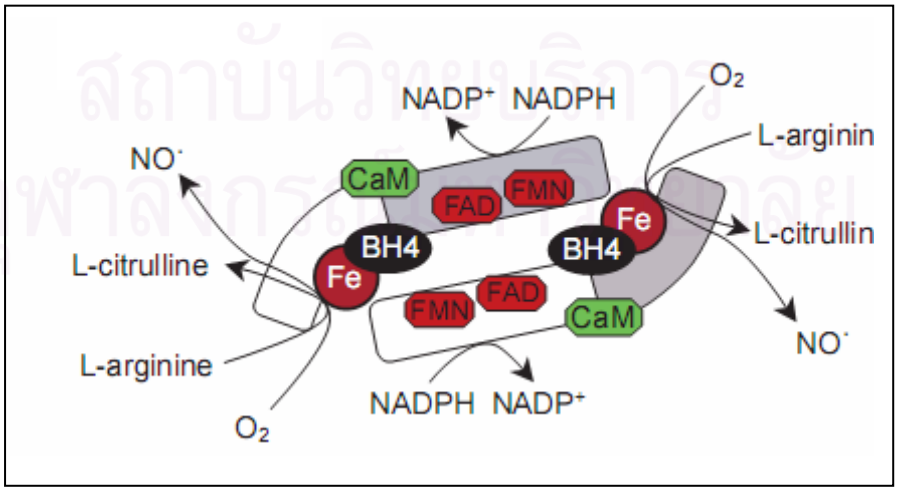
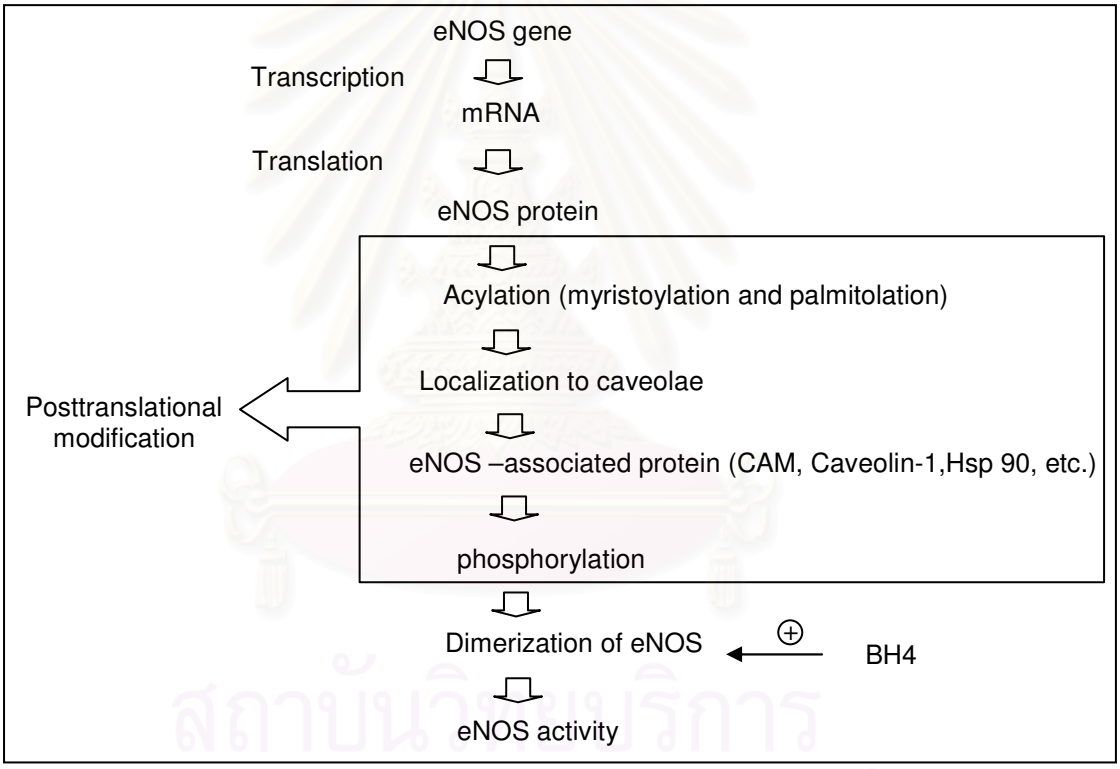


Figure 1.20 Mechanism of normal eNOS under optimal condition (Channon 2004)

Under optimal condition eNOS is a homodimeric oxidoreductase that catalyzes the production of NO from the L-arginine, using molecular O_2. Upon binding of Ca^{2+}/calmodulin, e^- is donated by NADPH from the reductase domain and proceeds via flavins FAD and FMN to the oxygenase domain. There they interact with the ferric (Fe) haem iron and BH_4 at the active site to catalyse the reaction of O_2 with L-arginine, generating citrulline and NO as products (Figure 1.20) (Channon 2004).

Different levels of eNOS regulation

In endothelial cells, eNOS activity is regulated through multiple integrated pathways that can be summarized by this bellowing diagram.



The regulation of eNOS starts with two stimulated pathways: a Ca^{2+}-dependent and a Ca^{2+}-independent pathway (Andrew and Mayer 1999; Dimmeler *et al.,* 1991). Firstly, eNOS gene transcription is activated by an increase in intracellular Ca^{2+} via hormones (e.g., catecholamine, estrogen and vasopressin), autacoids (e.g., BK, histamine and Ach), and platelet-derived mediators (e.g., serotonin and ADP) (Andrew and Mayer 1999). The latter pathway is activation of eNOS gene by mechanical forces including shear stress (Yeh *et al.,* 1999) and cyclic strain (Wung *et al.,* 2001). After that eNOS mRNA translates to eNOS protein. Interestingly, plasma

membrane invaginations composed of glycosphingolipid, cholesterol, and integral membrane protein caveolin. In addition, caveolae have been implicated in the transcytosis, pytocytosis, endocytosis, and the compartment relevantalization of signaling molecules. Interestingly, the other function is particularly control eNOS activity (Garcia-Cardena G *et al.,* 1996).

Next level of eNOS regulation is a posttranslational modification. N-termial myristate and palmitate of eNOS were dual acylation, lipid modification, into palmitolation and myristation. Interestingly, these sites are required for targeting of eNOS to plasma membrane caveolae and also found only in eNOS isoform (Figure 1.21)

(Feron *et al.,* 1998).

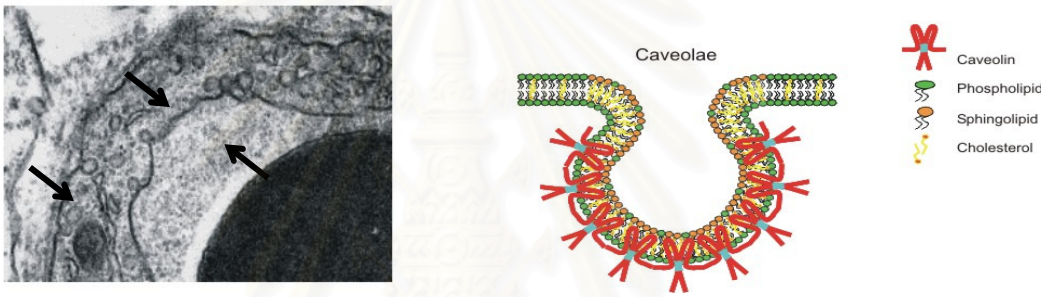


Figure 1.21 Caveolae, a unique "cellular organelle" with a unique "marker protein"

(From http://www.ruf.rice.edu/~rur/issue1_files/razani.html)

The regulatory interaction between caveole and acylation may influences with the eNOS-associateed protein. It now seem that eNOS activity is dependent on the protein-protein interaction (Busse and Fleming 2003), as the following below;

Caveolin-1 The binding of caveolin-1 to the reductase domain of eNOS compromises its ability to bind CaM and to donate e^- to the heme group, there by inhibiting eNOS activity within the caveolae (Figure1.21) (Ghosh *et al.,* 1998). Although eNOS and caveolin-1 are transported together from Golgi complex to caveolae via vesicles, the caveolin-1 within the Golgi complex is not regulating eNOS activity (Govers *et al.,* 2002).

Calmodulin (CaM) CaM was the first protein shown to interact with NOS and is necessary for the enzymatic activity of all three isoform. CaM binding to eNOS increases the rate of e^- transfer from the reductase domain to the heme center of oxygenase domain (Alderton *et al.,* 2001).

Dynamin The potential motor protein can associate with an increased eNOS by Ca^{2+} ionophores (Cao *et al.,* 2001).

G-protein-coupled receptors There have been reported that eNOS associates directly with G-protein-coupled receptors and specifically with the intracellular domain 4 of the bradykinin B2 receptor, the angiotensin II A1 receptor, endothelin-1 ETB receptor, and the ATP p2y2 receptor. The interaction of this protein to eNOS is reported to be associated in unsimulated endothelial cells, whereas dissociation of these proteins occurs in respond to endothelial cells stimulation (Marrero *et al.,* 1999).

Heat shock protein (Hsp) 90 Hsp 90 is involved in the regulation of eNOS activity by the agonist-stimulated tyrosine phosphorylation of Akt kinase at caveolae (Sato *et al.,* 2000)

Kinases Most of the kinases that are protein kinase A (PKA) (Fulton *et al.,* 1999), AMP-activated protein kinase (AMPK) (Chen *et al.,* 1999), and CaM-dependent protein kinase II (CaM KII) (Fleming *et al.,* 2001), shown to phosphorylate eNOS on serine or threonine residues physically associated with the enzyme directly or binding to an adaptor protein such as Akt binding with Hsp 90 (Sato *et al.,* 2000). Other kinases reported are the MAP kinase, ERK1 and ERK2, as well as the cyclic nucleotide dependent kinase PKA and PKC that mechanisms by which these kinases regulated eNOS activity are controversy (Bernie *et al.,* 2000; Bult *et al.,* 2000).

eNOS-interacting protein (NOSIP) NOSIP is a 34 kDa protein that binds to the C-terminal region of the eNOS oxygenase domain. It is reported to decrease eNOS activity and promoted the translocation of eNOS from the caveolae to intracellular compartments (Dedio *et al.,* 2001).

eNOS traffic inducer (NOSTRIN) NOSTRIN is a 58 kDa protein which binds to the C-terminal oxygenase domain of eNOS. It is reported to decrease eNOS activity similar to NOSIP by promote the translocation of eNOS from the caveolae to vesicle-like subcellular structures (Zimmermann *et al.,* 2002).

Lastly, the posttranslational modification is phosphorylation. eNOS can be phosphorylated on serine (Ser) or threonine (Thr) and tyrosine residues for regulating eNOS activity (Fleming *et al*., 1998). There are numerous potential phosphorylational sites, but the most is known about the functional consequences of phosphorylation of Ser residue (human eNOS sequence: Ser^{1177}; bovine eNOS sequence: Ser^{1179}) in the reductase domain (Boo *et al., 2002*) and a Thr residue (human eNOS sequence: Thr^{495}; bovine eNOS sequence: Thr^{497}) within the Ca^{2+}/calmodulin-binding reductase

domain (Fleming *et al.,* 2001;, Matasubara *et al.,* 2003). Other residue is Ser^{114} (bovine eNOS sequence: Ser^{116}) that localized with in the oxygenase domain, although this residue does not seem to play a crucial role in the regulation of eNOS activity. However, given the particular place of Ser^{114} to the BH_4 binding site, that this residue regulates the dimerization of eNOS by determining zinc binding or may acts as a phosphoryl switch determining whether eNOS generates NO or O_2^- ions (Kou *et al.,* 2001).

Table1.5 Summarizes the phosphorylation of eNOS residue that involved in various stimulators and associated with eNOS activity

Residue	Stimulator	Kinase	eNOS activity
Ser^{1177}	-Shear stress - Estrogen and VEGF -Insulin -Bradykinn	-Akt and PKA -Akt -Akt and AMPK -CaM KII	Activation
Thr^{495}	- Ca^{2+}-elevating agonist (e.g., bradykinin, histamine, and Ca^{2+} ionopore)	-PKC	Inhibition
Ser^{114}	- Shear stress, bradykinin and lipid signaling molecule lysophosphatidic acid	-MAP kinase, ERK1/2	Activation

Tetrahydrobiopterin

Terahydrobiopterin (BH_4) is known as an essential cofactor required for activity of all NOS isoforms. In addition, BH_4 binds close to the heme active site at N-terminal oxidase domain of NOS (Werner-Felmayer, *et al*., 2002, Cosentino and Luscher, 1999 and Channon, 2004). During activation of NOS, BH_4 is needed for allosteric and redox activation of its enzymatic activity (Raman, *et al*., 1998). BH_4

also plays a direct role in the multistep oxidation of arginine through the N-hydroxy-L-arginine intermediate and the subsequent generation of NO (Channon 2004).

BH_4 is synthesized via de novo pathway from guanosine triphosphate (GTP) by GTP cyclohydrolase I (GTPCH I), the first and rate-limiting enzyme; 6-pyrovoyl-tetrahydrobiopterin synthase (PTPS); and sepiapterin reductase (SR). *BH_4 is easily to be oxidized by ROS, forming dihydrobiopterin (BH_2) and finally biopterin.* Synthesis of BH_4 is also possible through salvage pathway from the synthetic pterin—sepiapterin, which is metabolized to BH_2 by SR and changed to BH_4 by dihydrofolate reductase (DHFR) when BH_2 levels are supraphysiologic (Werner-Felmayer, *et al*., 2002, Cosentino and Luscher, 1999 and Channon, 2004) (Figure 1.22).

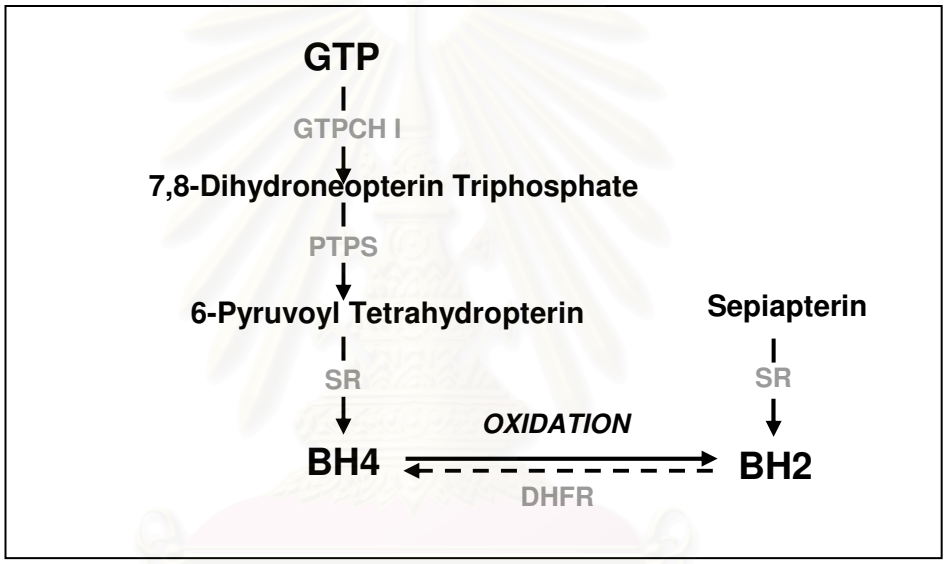


Figure 1.22 Tetrahydrobiopterin (BH_4) biosynthesis and metabolism

When BH_4 is limiting or absent, eNOS biochemistry is fundamentally altered in a number of ways, not merely by a loss of enzymatic activity. First, eNOS dimerization is destabilized, leading to a reduction in the relative proportion of eNOS dimmers versus monomers present in the cell. Second, eNOS catalytic activity becomes "uncoupled." In this situation, the stoichiometric coupling between the reductase domain and L-arginine oxidation at the active site is lost. However, e^- transfer from NADPH through the flavins to O_2 is not inhibited, but results in a formation of O_2^- radical and/or H_2O_2 (Figure 1.23) (Cannon 2004). eNOS uncoupling is regulated not just by BH_4 but also by other mechanisms, including oxidation status of the Zn^{2+}-thiolate center (Zou *et al*., 2002) and by availability of the substrate L-arginine. The relative contributions of BH_4 versus L-arginine availability appear to

vary in different cell types under different conditions and between different NOS isoforms. Nevertheless, BH_4 appears necessary, even if not always sufficient to prevent NOS uncoupling.

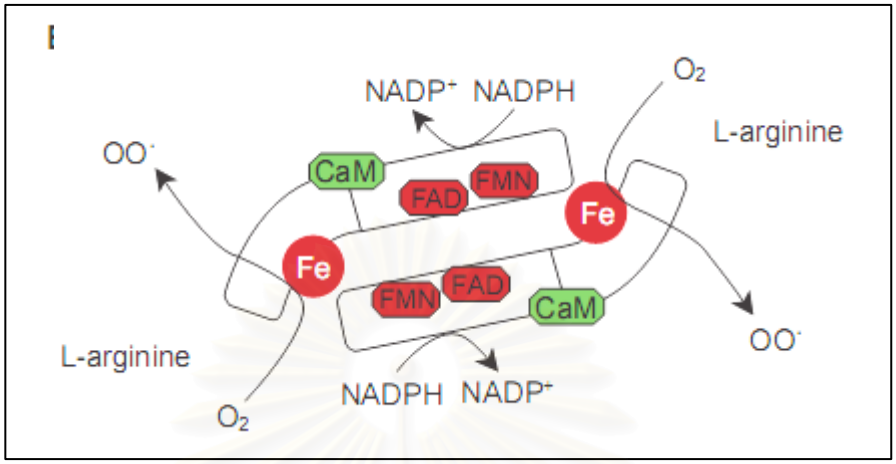


Figure 1.23 Mechanism of uncoupled eNOS in conditions of BH_4 dificiency (Channon 2004)

Tetrahydrobiopterin and diabetes mellitus

Varieties of different animal diabetic models as well as from clinical studies have shown a deficiency in eNOS cofactor BH_4. In addition, it has been reported that reduced intracellular BH_4 concentration was found in the fructose-fed insulin resistant rat model (Sinozaki, *et al*., 1999) as well as in endothelial cells of diabetic BioBreeding (BBd) rats and STZ-induced diabetic rats (Meninger, *et al*., 2000 and Kohli, *et al*., 2004).

Oxidation of BH_4 by reactive oxygen species (ROS) forming dihydrobiopterin (BH_2) that is inactive for eNOS cofactor function, raising the possibility that oxidative loss of BH_4 may mediate some of the observed effects of increased ROS production on endothelial function in diabetes (Alp, *et al*., 2003).

The underlying reason for the decreased availability of BH_4 has not yet been clarified. However, under physiological condition, GTPCH I is the rate limiting enzyme in the production of BH_4. Therefore, it can be speculated that decreased expression of GTPCH I protein may be involved in the pathophysiology of decreased generation of BH_4 in endothelial dysfunction as shown in coronary endothelial cells of diabetic BB rats, (Meninger , *et al*., 2000 and 2004).

Interestingly, an impairment of Ach-mediated endothelium-dependent vasorelaxation in isolated aorta from STZ-induced diabetic rat was improved by exogenous BH_4 administration (Piper, 1997). Recent study also demonstrated that acute incubation for 30 min with 10 μM sepiapterin, which raised BH_4 via the salvage pathway, significantly enhanced the sensitivity of the mesenteric arteriole to Ach-mediated endothelium-dependent vasorelaxation in diabetic mice (Pannirselvam, *et al*., 2002). They also reported that BH_4 had no effect on Ach-mediated relaxation in eNOS knockout mice, suggesting that the cellular basis for the action of BH_4 to improve endothelial dysfunction in their diabetic model is mediated through the eNOS pathway.

Thus, these findings suggested that BH_4 availability or synthesis is necessary for improving or normalizing the release of NO by modulating eNOS function.

Vitamin C and Tetrahydrobiopterin

It has been shown in several studies that antioxidant vitamin C reduced vascular oxidative stress (Nunes, *et al*., 1997, El-Missiry, 1999, Nazirogly, *et al*., 1999 and Chen, *et al.*, 2001) and increased NO-mediated endothelium-dependent relaxations (Ting, *et al*., 1996 and Jariyapongskul, *et al*., 2002).

As aforementioned studies, the antioxidant effects of vitamin C to scavenge ROS may be a mechanism for improvement of endothelial function. However, the molecular mechanisms underlying the *in vivo* antioxidant effects of vitamin C in enhancing endothelial NO synthesis are not fully understood.

Regarding another effect of vitamin C on the BH_4 availability, *in vitro* studies have been shown that vitamin C potentiates NO synthesis in a BH_4-dependent manner in endothelial cells obtained from human umbilical veins and coronary arteries (Heller, *et al*., 1999), and porcine aorta (Huang, *et al*., 2000). Similar effects have been observed in purified recombinant bovine eNOS (Huang, *et al*., 2000). Furthermore *in vivo* study by d'Uscio *et al*, 2003, demonstrated that long-term treatment with vitamin C had a beneficial effect on endothelial dysfunction of $apoE^{-/-}$ aorta by protection of BH_4. Therefore, vitamin C effectively increases endothelial NO synthesis and BH_4 levels (Heller, *et al*., 1999, Huang, et al., 2000, and d'Uscio *et al*., 2003). Moreover, vitamin C did not affect mRNA expression or activity of GTPCH

I, the rate-limiting enzyme for BH_4 synthesis (Heller, *et al*., 2001). Interestingly, treatment of endothelial cell with physiological concentrations of vitamin C lead to an increase in intracellular BH_4 levels and this effect of vitamin C can be discribed by a chemical stabilization of its cofactor, BH_4 (Baker, *et al*., 2001 and Heller, *et al*., 2001). Indeed, Toth, *et al*., 2002, have proposed the chemical stabilization of BH_4 by vitamin C as summarized in Schematic B (Figure 1.24). They demonstrated that BH_4 reacted faster with O_2^- than vitamin C. Moreover, their results have been indicated that H_2O_2 released from the auto-oxidation of BH_4 is an important, but not the only, factor leading to oxidation of vitamin C. Since in the absence of H_2O_2, using catalase, BH_4 still accelerated up to 2-fold the oxidation rate of ascorbic acid, besides H_2O_2, BH_2 was also able to be oxidized by vitamin C.

1. $BH_4 + O_2 \longrightarrow BH_2 + H_2O_2$
2. HO-ASC-OH + $O_2 \longrightarrow$ O=ASC=O + H_2O_2
3. HO-ASC-OH + 2 $H_2O_2 \longrightarrow$ 2 O=ASC=O + 4 H_2O
4. HO-ASC-OH + $BH_2 \longrightarrow$ BH_4 + O=ASC=O

Sum (1-4): 4 HO-ASC-OH + 2 O_2 = 4 O=ASC=O + 4 H_2O

Figure 1.24 Schematic B: Proposed catalytic effect of BH_4 on vitamin C (HO-ASC-OH) oxidation in the mixture of BH_4, vitamin C and O_2, an extensive oxidation of vitamin C takes place, whereas the concentration of BH_4 remains relatively unchanged (Toth *et al.,* 2002)

Thus, these molecular mechanisms of action of vitamin C may play a crucial role to improve endothelial dysfunction by preserving BH_4, cofactor of eNOS.

According to the crucial vascular effect of BH_4 and its close relationship to vitamin C as mentioned above, our study has the particular aim to assess whether vitamin C supplementation will have any contribution on vascular effect of BH_4 or not. Especially, the *in vivo* chemical stabilization of BH_4 by vitamin C in relevant to endothelial vascular reaction will be clarified.

CHAPTER III

MATERIALS AND METHODS

The diagram shown in Figure 3.1, indicated the conceptual framework of this study. The idea of this conceptual framework was based on an interested area--endothelial cells which were considered as a target for the effect of vitamin C.

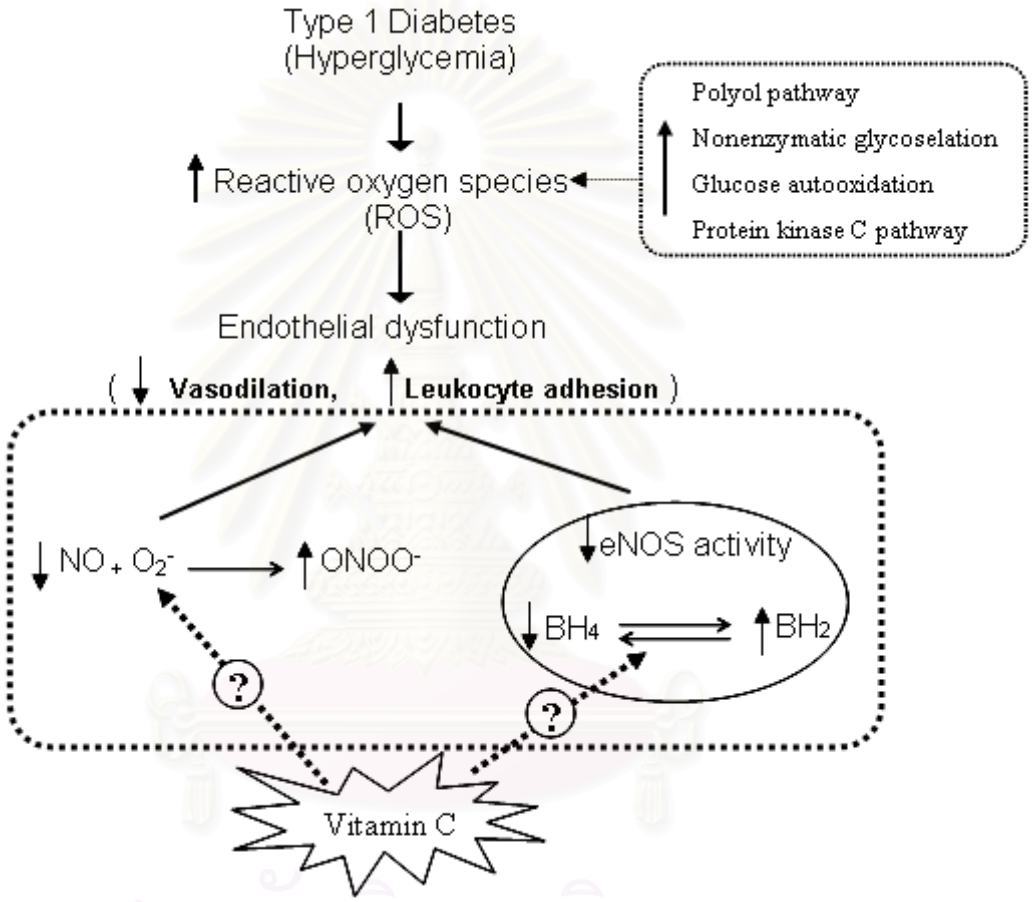


Figure 3.1 The conceptual framework of the present study showed when sustained blood glucose concentration (hyperglycemia) target area--endothelial cell, was effected by several mechanisms, ie, polyol pathway, nonenzymatic glycosylation, glucose autooxidation and protein kinese C pathway that enhanced reactive oxygen species (ROS) contents resulting in endothelial dysfunction. Therefore, the hypothesis of this study is that vitamin C can reverse endotheial cell from dysfunction in streptozotocin-induced type 1 diabetic rats by scavenging ROS contents and preserving BH_4 bioavailability.

Animal preparation

Male Spraque-Dawley rats weighing 200–250 g were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. They were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University before being used in the experiments and after that they were also be raised in this place. The animals were kept in a well-ventilated room temperature (25 ± 3°C) under controlled environmental condition of 12 hr light/dark period which provide darkness from 7.00 PM to 6.00 AM. Four animals were kept per cage (13"x 29"x7.5" stainless steel cage). They were fed with regular dry rat chow (Purina laboratory Chow, Premium Quality feed, Zuelig Gold Coin Mills Pte., Singapore) and tap water *ad libitum* throughout the experimental period.

Our experiments were performed following the "Guideline for experimental animals suggested by the National Research Council of Thailand (1999)" and approved by Ethical Committee, Faculty of Pharmacy, Chulalongkorn University.

Diabetic induction

The diabetic rats were induced by single injection of streptozotocin (STZ, Sigma-Aldrich Co, USA. 50 mg/kg bw), which is freshly prepared by dissolving in citrate buffer (pH 4.5, Sigma-Aldrich Co, USA) and immediately injected into the tail vein of 8-hr fasted rats. The diabetic condition will be defined as a blood glucose concentration ≥200 mg/dl and will be verified at 48-hr after the injection of STZ and prior to each experiment with glucometer (Advance Glucometer, Bochring Mannhem, Germany). If the STZ-treated rats did not meet this inclusion criterion, they will be excluded from the study. Control rats were injected by the same volume of citrate buffer (pH 4.5) instead.

Experimental design

According to our previous study performed by Chakraphan. 2002 and Jariyapongsakul *et al*., 2002 the results showed that supplementation with vitamin C with 1 g/ L L-ascorbic acid daily prevented endothelial from dysfunction in 12 weeks DM rats. Morover our previous study by Sridulyakul *et al*., 2004 indicated that the role of supplementation of vitamin C enhanced eNOS protein levels at 12 weeks DM. Although the beneficial effect of viamin C supplementation has suggested to be protecting diabetes-induced endothelial dysfunction, its reverse effect has not been studied. Therefore, in our experimental design, three experimental protocols were performed.

Experimental protocol 1, To study the effect of vitamin C supplementation to reverse diabetes-induced endothelial dysfunction, we have to know the time point of an occurring of mesenteric endothelial dysfunction in DM rats. Therefore, we would like to determine whether mesenteric endothelial dysfunction exists as early as week 6^{th} after STZ injection.

Experimental protocol 2, After confirming the endothelial dysfunction, we would like to examine whether the delayed vitamin C supplementation can reverse endothelial dysfunction. Moreover, we also aimed to confirm the preventive effect of vitamin C by early supplementation for 12 weeks before endothelium become dysfunction.

Experimental protocol 3, The underlying mechanisms of vitamin C on dibetes-induced endothelial dysfunction were then examined. The experimental design for protocol 3 was based on the hypothesis and the conceptual framework (Figure 3.1) of the present study as shown previously.

Protocol 1: To determine the occurrence of endothelial dysfunction at week 6th of diabetic induction

At week 6th of the experimental periods after STZ injection, the animals were divided randomly into two groups of 6 weeks control (6-wks CON (n=6)), and 6 weeks diabetes (6-wks DM (n=6)).

On the day of experiment, rats were anesthetized with intraperitonial injection of sodium pentobarbital (50 mg/kg bw, ip.) and a tracheostomy was performed. A catheter was inserted into a carotid artery for measuring arterial blood pressure using a pressure transducer (Nihon Koden, Japan) and collecting blood. A jugular vein was cannulated for injection of fluorescence tracers. The conditions of diabetes induced-endothelial dysfunction were characterized by: 1) the evaluation of leukocytes adhesion and 2) the viualization of vascular dilatation to acetylcholine (Ach).

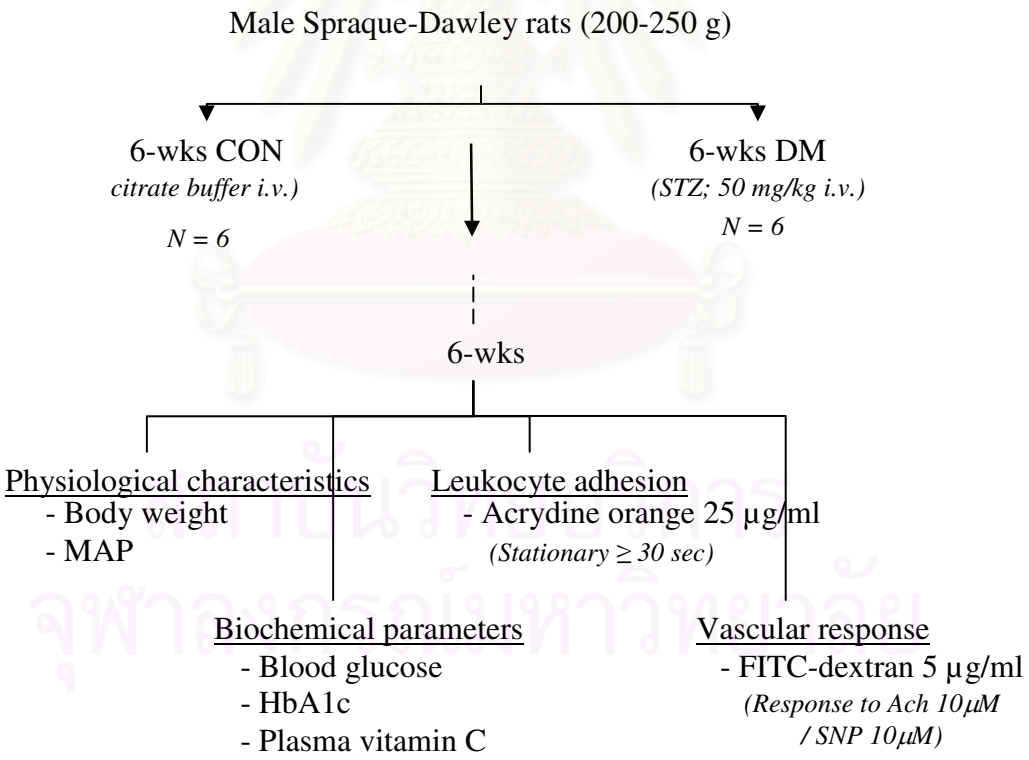


Figure 3.2 Experimental design to determine the occurrence of endothelial dysfunction at week 6th of diabetic induction

1.1 Measurement of physiological characteristics and biochemical parameters

In the present study, the physiological characteristics were determined including: body weight (BW) and mean arterial blood pressure (MAP). Arterial blood pressure was measured via a canular inserted into the carotid artery using a pressure transducer (Nihon Koden, Japan). Arterial pressure was reported in terms of mean arterial blood pressure (MAP) calculated by MAP = 1/3 (systolic blood pressure + 2 diastolic blood pressure).

Besides, the biochemical parameters of blood glucose, plasma vitamin C, and glycosylated hemoglobin A (HbA1c) levels were also determined. All these parameters were determined at the end of each experiment in arterial blood withdrawn from the carotid artery via catheter. The blood glucose was determined using a glucometer (Advance Glucometer, Boehringer Mannheim, Germany). The HbA1c was analyzed from whole blood using colorimetric method (Bangkok RIA lab Co, Thailand). The plasma vitamin C levels were measured using enzyme-assisted spectrophotometric method (Research Center, Ramathibodi Hospital, Mahidol University, Thailand).

1.2 Evaluation of leukocyte adhesion

On the day of experiment, after anesthetization with sodium pentobarbital, rats were prepared for studying mesenteric microcirculation using intravital fluorescent videomicroscopy as described previously by Chakraphan *et al*, 2002. The abdomen was opened via midline incision, and ileocecal portion of the mesentery was carefully removed from the abdomen and positioned on a Plexiglas chamber. The exposed mesentery was covered with a piece of plastic warp to prevent drying of the tissue and the mesentery was also be perfused continuously with Krebs–Ringer solution (pH 7.4) maintaining at 37◦C to avoid dehydration throughout the experiments (Booth et al., 2001; Schaffler et al., 1998; Wood et al, 1999.). Leukocytes in mesenteric After 20-30 min equilibration, the 0.5 ml acrydine orange (25 µg/ml; Sigma-Aldrich Co, USA) was bolus injected intravenously for visualizing the behavior of leukocytes in mesenteric microvasculature (Chakraphan. *et al*, 2002, Jariyapongskul, *et al*., 2002). The epi-illumination of acrydine orange labeled microcirculation was then observed

and recorded real time by using a set of intravital fluorescent microscopic equipment. The equipment set is consisted of video microscopy (Nikon, Tokyo, Japan) with 20x and 10x objective and eyepieces lens, respectively (Nikon, Japan), SIT videocamera (Hamamatsu Photonics, Japan), camera controller (Hamamatsu C2400, Japan), video timer (For A company limited VTG-33, Japan) and video recorder (Sharp multi system VC-S5, Japan) (Figure 3.3).

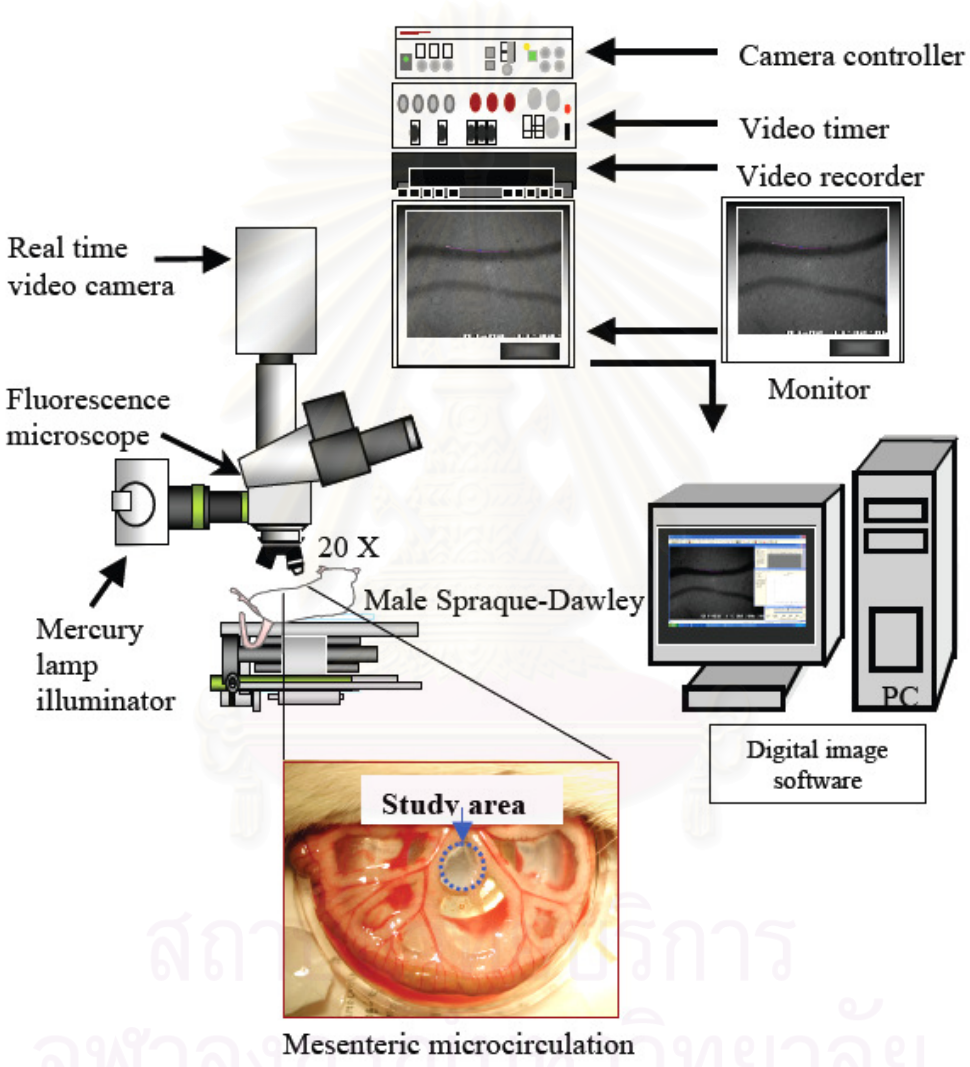


Figure 3.3 An experimental setup for *in vivo* studying mesenteric microcirculation using intravital fluorescence video-microscopic technique

During the experiment, the real time video image of studied area in mesenteric microcirculation was observed on monitor (Sony SLV x311) and recorded by using video recorder (Sony SVT-124P). The videotape of each expriment was then analyzed off-line using digital image processing software.

Twenty minutes after the acrydine orange injection, a single unbranched mesenteric postcapillary venule (20 to 30 µm diameter, >150 µm length) was chosen for observation of leukocyte adherence (Kubes et al., 1991; Salas et al., 1998).

The numbers of leukocytes adherence were analyzed off-line during video-playback. Leukocytes were considered to be adherent to mesenteric postcapillary venule if it remained stationary for ≥30 seconds and was counted by using a digital image software (GLOBAL LAB Image II; Data Translation, MA, USA). The numbers of leukocytes adherence were expressed as the number of cells per 100 µm of vessel length (cells/100-μm venular length).

1.3 Responses of mesenteric arterioles to vasodilators

To visualize the vascular dilatation to vasodilators, a fluorescein isothiocyanate labeled dextran (FITC-Dextran; MW 250,000, Sigma-Aldrich Co, USA) at 5 µg/ml was administered intravenously. The second-order mesenteric arterioles (15 to 30 µm) were chosen for studying the vascular reactivity to two different vasodilators, endothelium-depentdent vasodilator acetylcholine (Ach) and endothelium-indepentdent vasodilator sodium nitroprusside (SNP).

Before topical application of Ach (10µM; 1 ml/5min), the selected arterioles were preconstricted with topical application of norepinephrine (NE, 10µM; 0.1 ml). Video images of the stdied area were taken at one minute after NE and 5 min after Ach application. After that the selected arteriole was rinsed by Krebs–Ringer solution (pH 7.4) until its diameter was return to normal. With the same protocol, to ensure that the vascular smooth muscle function was not involved in diabetic condition, SNP (10µM; 1ml/5min), which produces vasodilatation indepentdent of NOS, was applied topically on the arterioles after preconstriction with NE.

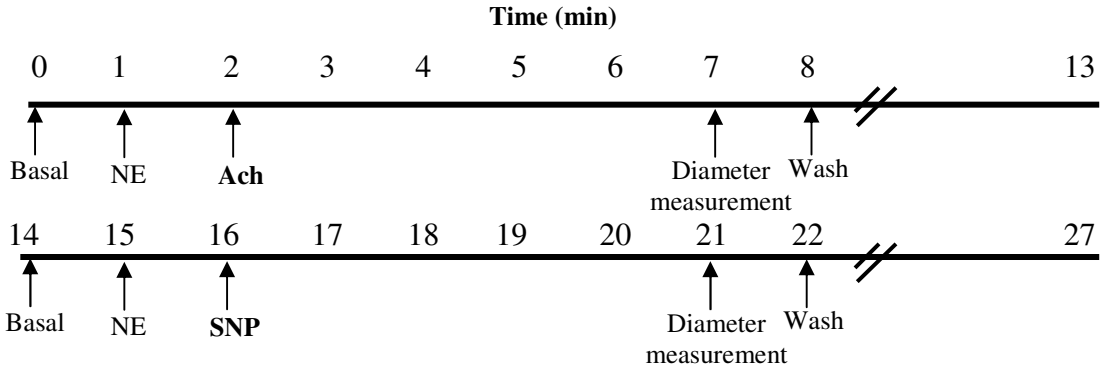
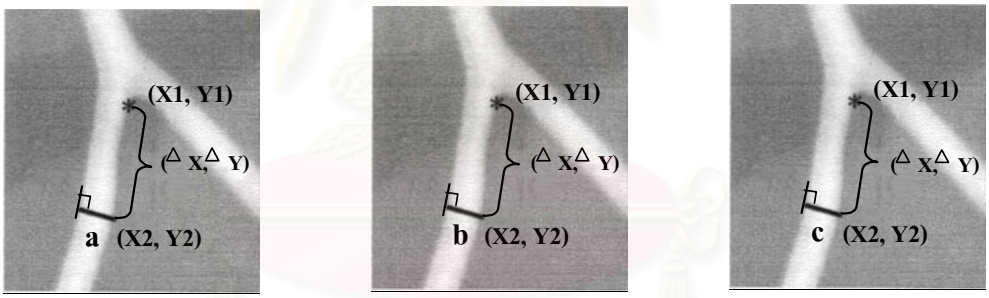


Figure 3.4 Experimental design for visualization vascular reactivity to two different vasodilators

Mesenteric microvascular diameters were measured from FITC-videoimages, using the digital image software (GLOBAL LAB Image II; Data Translation, MA, USA). The arteriolar diameter in micrometer (μm) was calculated as the mean of triple measurements from three video frames by using the same reference point as a marker for measuring at the same position in each frame as shown in (Figure 3.5).



$$\text{mean arteriolar diameter} = \frac{a + b + c}{3}$$

Figure 3.5 Method for measurement of arteriolar diameter

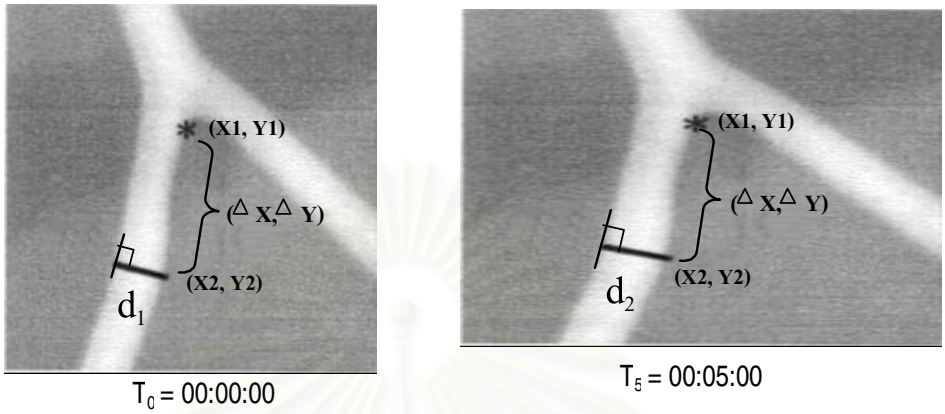
(x_1 , y_1) : The position at the vessel branch

(*) : A reference point for locating the (x_2 , y_2) where the diameter was measured.

The diameter was obtained by mean of three frames:

$$d = (a+b+c)/3$$

Vasodilation responses were expressed as the percentage (%) changes of arteriolar diameters from the baseline values (NE-preconstricted diameter) and the calculation method was shown in (Figure 3.6).



$$\% \text{ changes of arteriolar diameter from baseline} = \frac{d_2 - d_1}{d_1} \times 100$$

Figure 3.6 The percentage of arteriolar diameters was calculated from the difference between baseline diameter (d_1) and diameter at 5 min after the application of each vasodilator

Protocol 2 To examine the effect of vitamin C supplementation reverses the diabetes-induced endothelial dysfunction.

The animals were divided randomly into two groups of 12 weeks control (12-wks CON (N=6)), and 12 weeks diabetes (12-wks DM (N=18)). Each group was then subdivided into four subgroups of:

1) Control rats (**12-wks CON**) (n=6)
2) Diabetic rats (**12-wks DM**) (n=6)
3) Diabetic rats with vitamin C treated after 6 weeks STZ-injection (**12-wks DM+VitC$_{6wks}$**) (n=6)
   [This delayed phase of vitamin C supplementation group was designed to study the reverse effect of vitamin C.]
4) Diabetic rats with vitamin C treated since day 2th after the STZ-injection (**12-wks DM+VitC$_{day2}$**) (n=6)
   [This early phase of vitamin C supplementation group was designed to study the preventive effect of vitamin C.]

Vitamin C supplementation

Vitamin C solution (L-ascorbic acid, 99%, Sigma-Aldrich, Co, USA) was prepared freshly every day by dissolving in drinking water at the concentration of 1 g/ L (Chakraphan., 2002; Dai and McNeill., 1995; Jariyapongsakul *et al*., 2002). The solution was daily supplemented *ad libitum* to the rats.

At week 12th of experimental period after STZ injection, to exammine whether vitamin C supplementation can reverse the diabetes-induced endothelial cell dysfunction, the intravital fluorescence videomicroscopy was performed similar to the 1st protocol where *in vivo* studies of endothelial dysfunction in mesenteric microcirculation was characterized by 1) the evaluation of leukocyte adhesion and evaluation of leukocytes adhesion and 2) reduced vascular response to Ach (10μM).

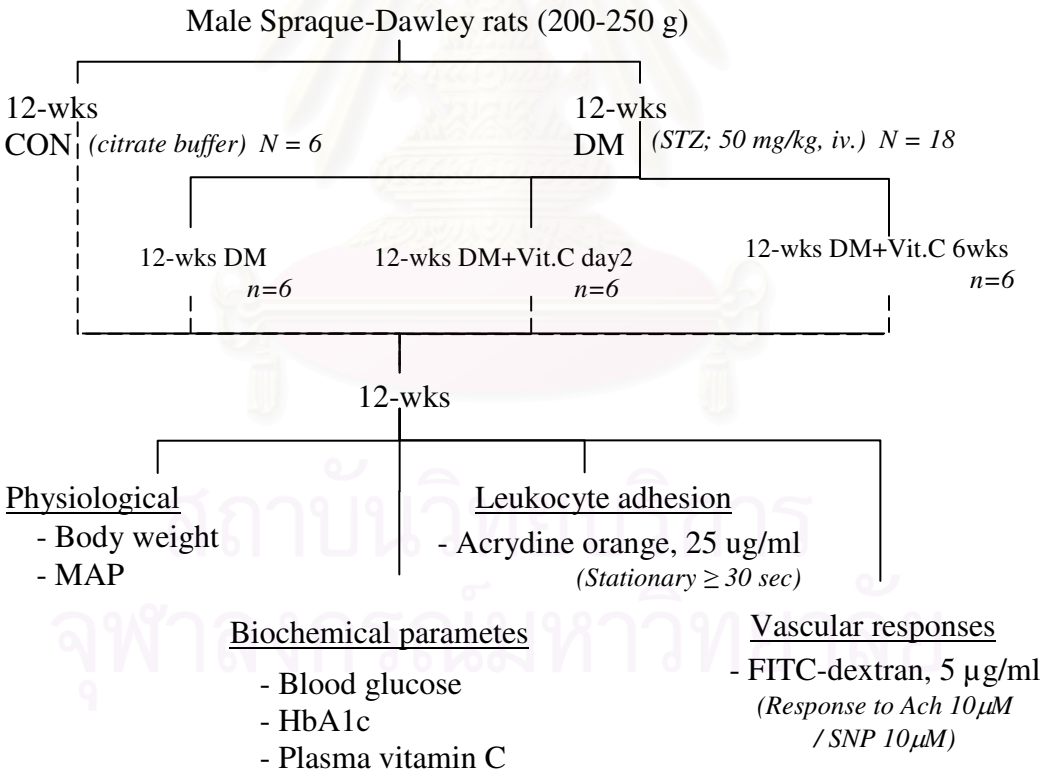


Figure 3.7 Experimental design to examine the effect of vitamin C supplementation reverses the diabetes-induced endothelial dysfunction

Protocol 3 The underlying mechanism(s) of the effect of vitamin C to reverse diabetes-induced endothelial dysfunction

Protocol 3.1 The underlying mechanism of vitamin C on ROS contents

The same as protocol 2, the animals were divided randomly into two groups of 12 weeks control (12-wks CON (N=6)), and 12 weeks diabetes (12-wks DM (N=18)). Each group was then subdivided into four subgroups of:

1) Control rats (**12-wks CON**) (n=6)
2) Diabetic rats (**12-wks DM**) (n=6)
3) Diabetic rats with vitamin C treated after 6 weeks STZ-injection (**12-wks DM+VitC$_{6wks}$**) (n=6)

   [This delayed phase of vitamin C supplementation group was designed to study the reverse effect of vitamin C.]
4) Diabetic rats with vitamin C treated since day 2th after the STZ-injection (**12-wks DM+VitC$_{day2}$**) (n=6)

   [This early phase of vitamin C supplementation group was designed to study the preventive effect of vitamin C.]

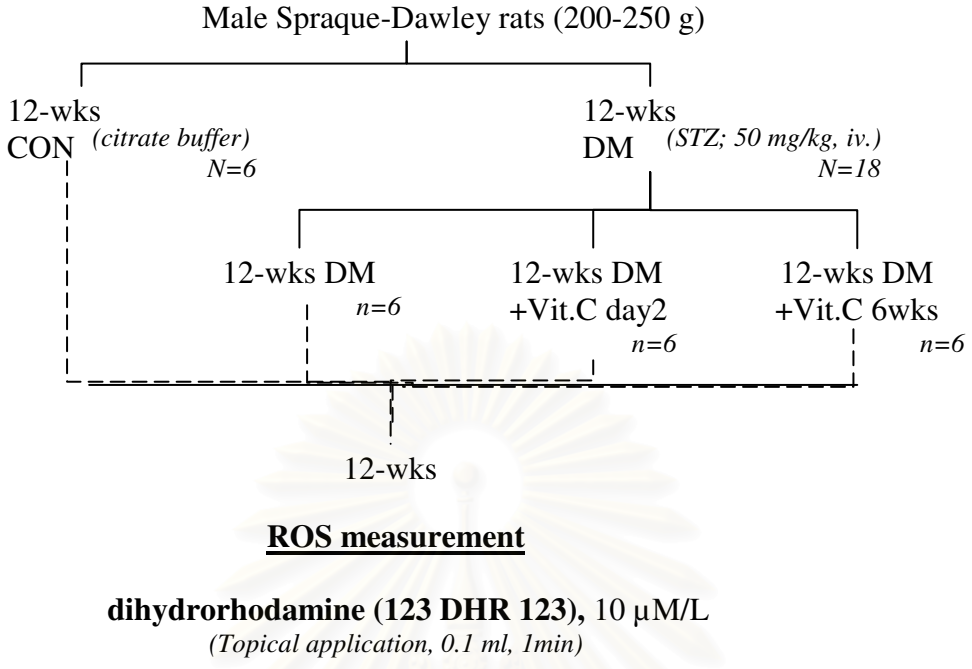


Figure 3.8 Experimental design to elucidate the underlying mechanism(s) of vitamin C on ROS contents

Direct detection of ROS contents

In this experiment, we attempted to conduct an *in vivo* direct detection of ROS contents, dihydrorhodamine 123, a hydrogen peroxide (H_2O_2)-sensitive fluoroprobe (DHR 123, Sigma-Aldrich Co., USA) was used. Using intravital fluorescence videomicroscopy, the intensity of H_2O_2 was determined by the digital image software (Image Pro Plus; Media Cybernatics, Inc, USA). DHR 123 was oxidized and changed to rhodamine 123 (green fluorescence) mostly by H_2O_2, with less sensitivity by superoxide (O_2^-), and peroxynitrite ($ONOO^-$) (Royall and Ischiropoulos., 1993, Saram *et al*., 1990; Henderson and Chappell., 1993, Crow. 1997).

After the 20-min equilibrium period, the background fluorescence images were recorded with 20x and 10x objective and eyepieces lens, respectively before the start of experiments as baseline image (0min, I_{base}). The bolus topical application of 0.1 ml 10 μM DHR 123 was used (Han *et al*., 2001). The unbranched segment of the second-order mesenteric arterioles (15 to 30 µm) was selected for observation. The mesentery was epi-illuminated by an excitation and emission wavelength at 488

nm and 525 nm, respectively. The microfluorographs of the H_2O_2-associated fluorescence within the selected area were captured at 1 min [*] after topical application the mesentery with DHR 123 as shown in following diagram (Figure 3.9) (Henderson and Chappel., 1993, Royall and Ischiropoulos.,1993 and Saram *et al*., 1990).

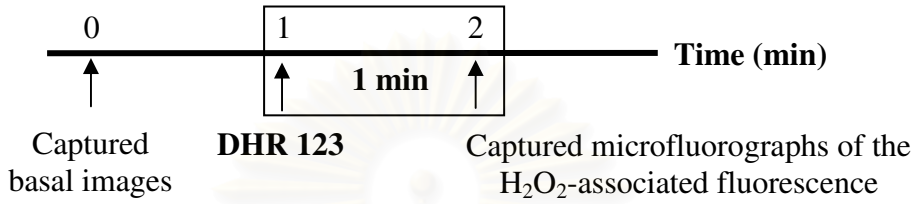


Figure 3.9 Experimental design for visualization of intravital ROS contents (Modified from Han et al., 2001)

Note [*] By bolus topical application of 0.1 ml 10 µM DHR 123, we, therefore, chose 1 min of the H_2O_2-associated fluorescence microfluorographs to visualize the ROS contents regrading this time point showed a strong signal of rodamine 123 after observing for 20 min (Figure 3.10).

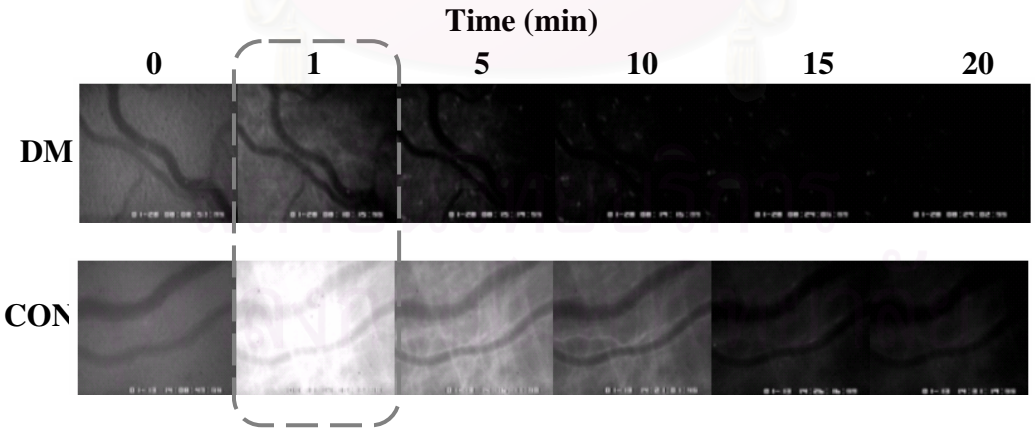
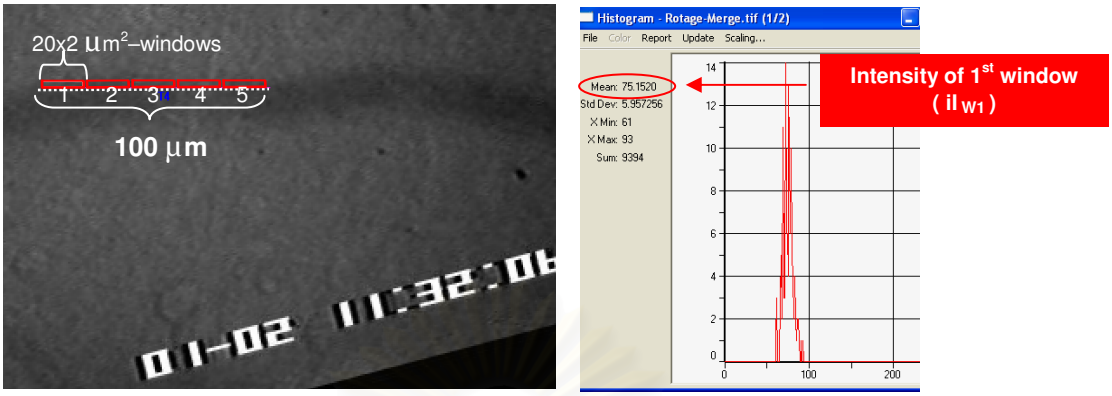


Figure 3.10 Microfluorographs of the H_2O_2-associated fluorescence taken from CON and DM at 0, 1, 5, 10, 15 and 20 min

By using digital image software (Image Pro Plus; Media Cybernatics, Inc, USA), the average fluorescence intensity of five frames of 20x2 μm^2–windows along

100 µm length of studied arterioles was evaluated as shown in (Figure 3.11).



$$\text{Average fluorescence intensity (I)} = \frac{i_{W1} + i_{W2} + i_{W3} + i_{W4} + i_{W5}}{5}$$

Figure 3.11 Method for measurement of fluorescence intensity (Modified from Han *et al*., 2001; Wood *et al*., 1999)

The average of intensity value was defined for baseline (0min, I_{base}) and at 1min (I_{1min}) after DHR application as presented by the percentage changes of H_2O_2-associated fluorescent intensity and the calculation method was shown in the following equation:

$$\%\ \text{changes of } H_2O_2\text{-associated fluorescent intensity} = \frac{(I_{1min} - I_{base})\ \text{x}\ 100}{I_{base}}$$

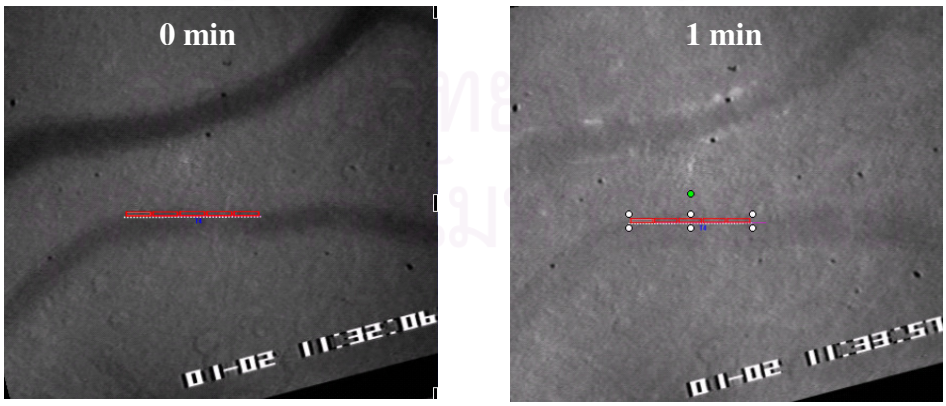


Figure 3.12 The video images of 0-min and 1-min after DHR-123 application where I_{base} and I_{1min} were determined, respectively

<u>Protocol 3.2</u> The underlying mechanism of vitamin C on NO bioavailability

<u>For this experiment we used topical application of vitamin C as its acute effect to test our hypothesis that vitamin C reverses diabetes-induced endothelial dysfunction by preserving NO bioavailability. To be noted that this experimental design differed from the previous one that we supplemented vitamin C to the animal for its long-term effect observation.</u>

Before using vitamin C to apply directly to the mesentery we aimed *<u>to examine whether the acute effect of topical application vitamin C enhances Ach-induced vasodilation. If so, what is an optimal dose for applying viamin C.</u>*

The 6 weeks control rats (6-wks CON; N=6) were used to find out dose-dependent manner of topical application vitamin C at various concentrations: 0.26, 2.6, and 26 mM (Beckman *et al*, 2001; Heitzer *et al*., 1996; Huang *et al*., 2000; Prabhakar, 2001; Taddei *et al*, 1998; Ting *et al*, 1996; Toth *et al*., 2002) in response to Ach-induced vasodilaition.

Male Spraque-Dawley rats (200-250 g)

|

6-wks CON

| *(citrate buffer)*
N=6

<u>Vascular responses</u>

- **FITC-dextran** *(response to Ach 10μM)*

- Topical application vitamin C at 0.26, 2.6, and 26 mM

Measurement of the acute effect of topical application vitamin C on endothelium-dependent vasodilation

On the day of experiment, the rat's mesentery was prepared as similar to 1st protocol. After that the second-order mesenteric arterioles (15 to 30 μm diameter) were chosen to visualize the vascular response to endothelium-dependent vasodilator, Ach. First step we mesured a baseline arteriolar diameters after 1-min preconstrict with NE (10μM; 0.1 ml). And then 5-min Ach-induced vasodilation diameters (10μM; 1 ml) were measured. After that the mesenteric arterioles were washed 5-min with

Krebs–Ringer solution (pH 7.4) to allow vessels to return to baseline. Next step, to test the acute effect of of vitamin C and its dose-dependent manner, L-ascorbic acid (2ml/ 1min [*]) at different concentration: 0.26, 2.6, and 26 mM was applied topically on the NE (10μM; 0.1 ml/1 min) preconstricted arterioles and then the vascular response to Ach-induced vasodilation (10μM; 10 ml/ 5 min) was measured as shown in following diagram (Figure 3.13). The percentage changes in diameter of mesenteric arterioles in response to Ach alone or Ach with various doses of vitamin C application was examined with the same as 1st protocol.

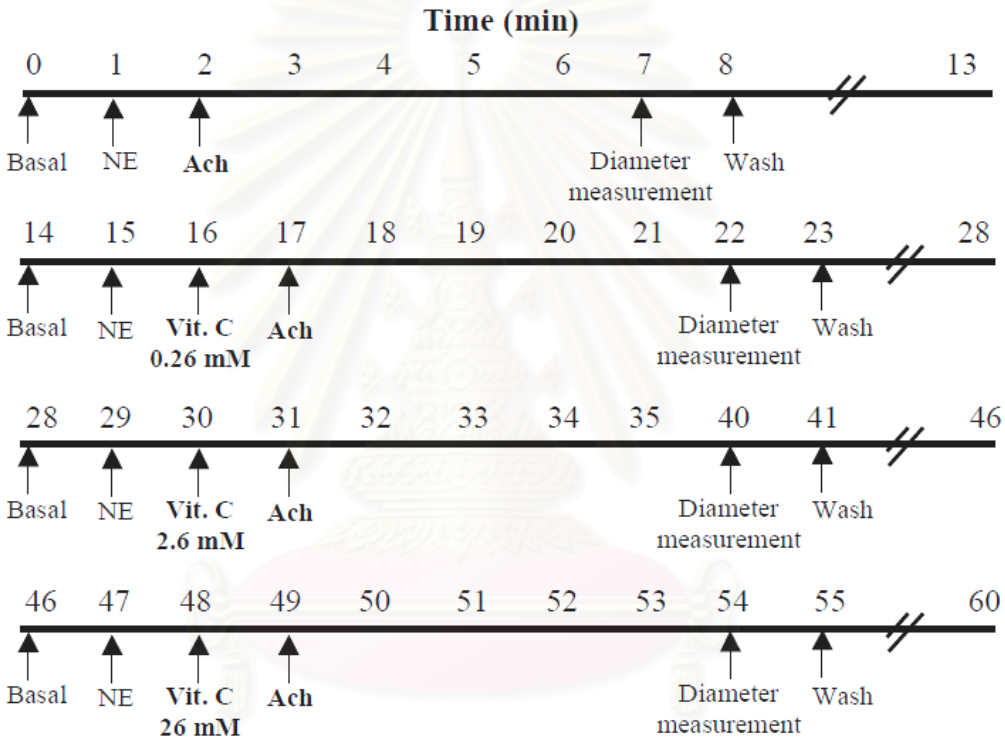


Figure 3.13 Experimental design **[#]** for visualization of vascular response to the acute effect of vitamin C application (Modified from Heitzer *et al*., 1996; Taddei *et al*, 1998.)

Note [*] The time point for 1 min application of vitamin C was chosen from our pilot study (Figure 3.14 and Table 3.1). The time-response of topically applicated vitamin C at doses 2.6 mM was observed for fifteen minutes after preconstruction with NE and recored one minute each. The optimal duration of actue effect of vitamin C application was five minutes. After passing the five minutes, the vasodilation response was decrease.

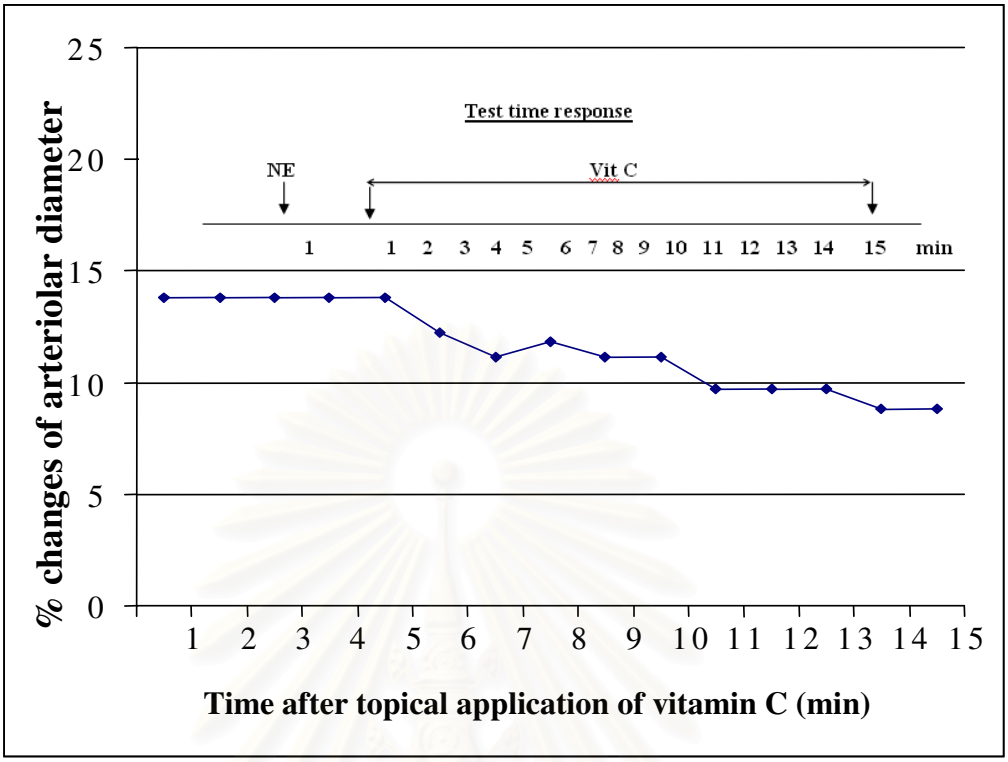


Figure 3.14 Time-response curve of the effect of topically applicated vitamin C Percentage changes of arteriolar diameters of mesenteric arterioles to the topically applicated vitamin C at dose 2.6 mM Vit.C (n=3)
Values are mean, n is number of samples

Table 3.1 Percentage changes of arteriolar diameters (% changes) of mesenteric arterioles to the topically applicated vitamin C at doses 2.6 Vit.C (n=3)
(Values = mean and n= number of samples)

Time (mins)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
% changes	13.79	13.79	13.79	13.79	13.79	12.22	11.14	11.8
Time (mins)	**9**	**10**	**11**	**12**	**13**	**14**	**15**	
% changes	11.14	11.14	9.68	9.68	9.68	8.79	8.79	

Note [#] Only this experimental design acute effect of vitamin C showed maximum arteriolar relaxtion as compare to other three designs from our pilot studies (Figure 3.15). In addition, we used Ach and NE at dose 10μM and vitamin C at dose 2.6 mM.

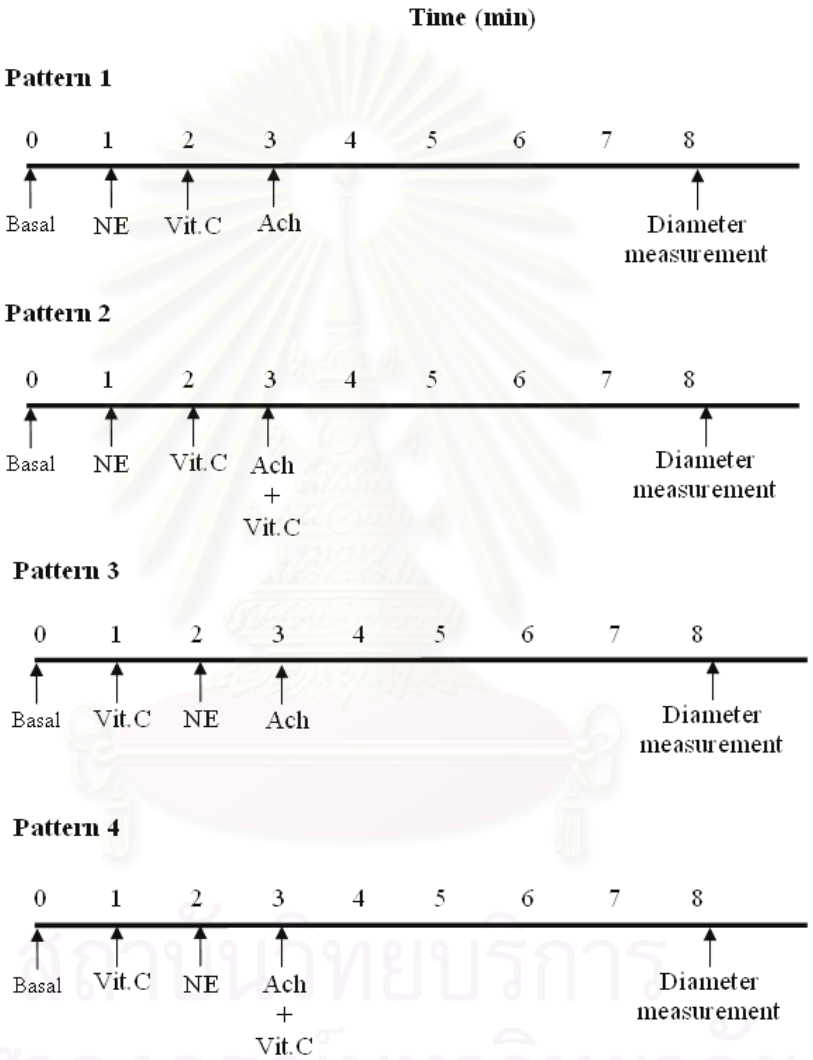


Figure 3.15 Four patterns were designed for visualization of vascular response to the acute effect of vitamin C application

Table 3.2 Percentage changes of arterial diameter of the four patterns designed for visualization of vascular reactivity to the acute effect of vitamin C application

Pattern	%Changes of arterial diameter
1	25.27 (n=2)
2	21.85 (n=2)
3	14.11 (n=2)
4	15.82 (n=2)

Note: *we found that the optimal dose of actue effect of vitamin C application was 2.6 mM; therefore, this dose was used to elucidate the underlying mechanism of vitamin C on NO bioavailability.*

The rats were divided randomly into two groups of 6 weeks control (6-wks CON (N=12)), 6 weeks diabetes (6-wks DM (N=12)). After week 6th, each group was subdivided into two subgroups:

1) Vehicle (**6-wks CON**) (n=6), (**6-wks DM**) (n=6)

2) Vehicle with 2.6 mM vitamin C-perfusion (**6-wks CON$_{Vit.C}$**) (n=6),

(**6-wks DM$_{Vit.C}$**) (n=6)

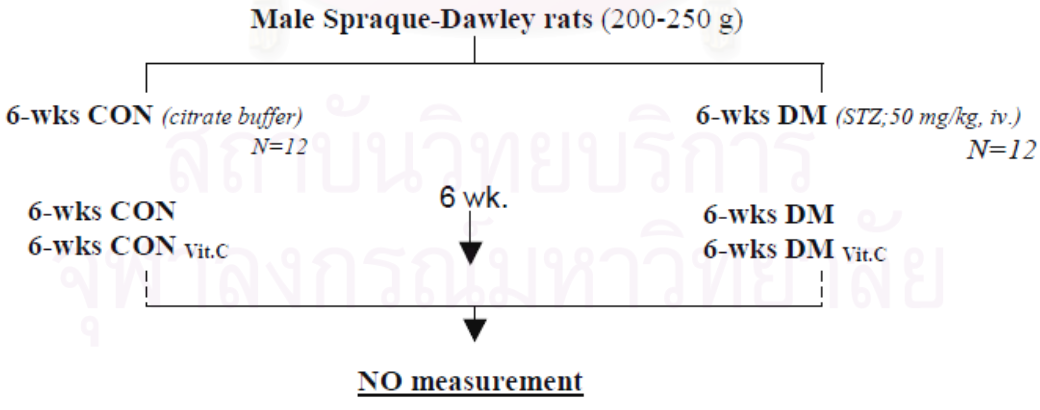


Figure 3.16 Experimental design to elucidate the underlying mechanism(s) of vitamin C on NO bioavailability

Direct detection of NO production

In this experiment, we designed to study the effect of vitamin C on NO bioavailability. *In vivo direct NO detection was visulalized using a method described by Kashiwagi S, et al., 2002 with some modification.* we used , Daiichi Pure Chemicals Co. Ltd.), a NO-sensitve fluorescent indicator (Ishikawa *et al*, 2005; Kashiwagi, *et al*., 2002; Kojima *et al*., 1998; Nakatsubo *et al*., 1998) This compound is a cell-permeable derive of DAF-2. Upon entry into the cell, DAF-2DA is transformed into the less cell-permeable DAF-2 by cellular esterases, thus, preventing loss of signal due to diffusion of the molecule from the cell. And then, this DAF-2 can react specifically with nitrosonium ion (NO^+), which derives from endogenously generated NO to yield the highly fluorescent triazolofluorescein (DAF-2T), the stable fluorogenic complex. The detection limit for NO is two to five nM at neutral pH. This compond can be utilized in cells, which produce small amounts of NO, such as endothelial cells, as well as in cells, which generate large amount of NO, such as macrophages (Ishikawa *et al*, 2005; Kashiwagi *et al*., 2002; Kojima *et al*., 1998; Nakatsubo *et al*., 1998)

As described previously, on the day of experiment, the mesentery was performed. The mesenteric microcirculation was observed under intravital fluorescence video-microscope with a 20x objective lens and a 10x eyepieces lens (Figure 1). To visualize mirocrovascular distribution of NO *in vivo*, 3 μM DAF-2DA (Daiichi Pure Chemicals Co. Ltd.) was used. In vehicle (control and diabetic) groups, the buffer containing 3 μM DAF-2DA was dissolved in Krebs-Ringer solution, 10μM Ach, and saturated with 95%N2 / 5% CO_2 to avoid autoxidation of the precursor. The buffer was perfused on the mesentery at 0.5 mL/ min at 37°C for 20 minutes (Kashiwagi *et al*., 2002). *We slightly modified the DAF-2DA solution by adding 10μM Ach for decreasing a period of the buffer perfusion* [*]. In vehicle with 2.6 mM vitamin C-perfusion (control and diabetic) groups, the buffer contained the same solutions as vehicle groups but we included 2.6 mM vitamin C and mixed it into the buffer.

To capture microfluorographs of the NO-associated fluorescence, the mesentery will be epi-illuminated by an excitation and emission wavelength of 488 and 538 nm, respectively (Ishikawa *et al*, 2005; Kashiwagi *et al*., 2002; Nakatsubo *et al*., 1998). The microscopic field containing and unbranched segment of the arteriole

(15 to 40 µm diameter) will be selected for observation. In these experiments, only those that shared the same focusing plane will be examined.

After an equilibration period for 20 min, the background fluorescence images were captured before the start of experiments as baseline image (0min, I_{base}). Next, the fluorescence images were captured at 20 min after buffer perfusion or coperfused with 2.6 mM vitamin C (20min, $I_{20\ min}$) [#] as shown in following diagram, (Figure 3.17).

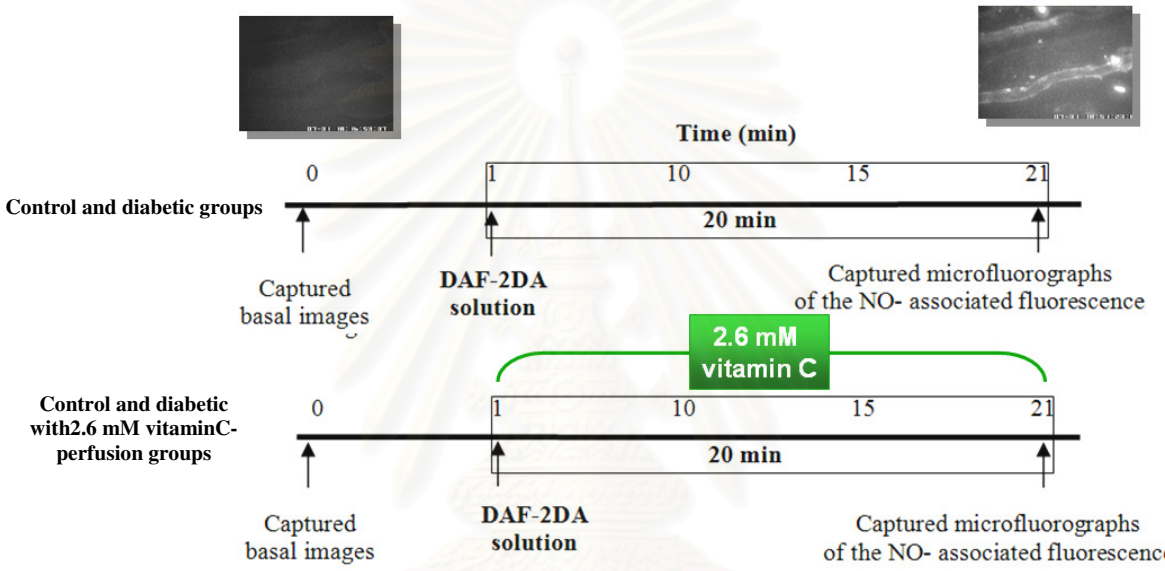
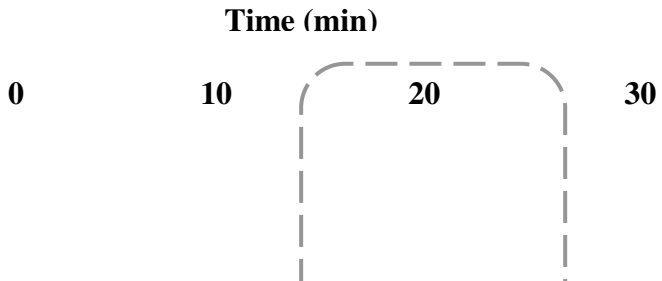


Figure 3.17 Experimental design for visualization of intravital NO imaging (Modified from Kashiwagi *et al*., 2002)

Note **[*]** By adding stimulation agonist Ach, 10µM, the expression of eNOS and the release of NO can be enhanced above basal levels after receptor stimulation.

Note **[#]** With our procedure, the first fluorescence image appeared as early as 10 min after buffer perfusion; this time point just as the vessel wall began to appear fluorescent (DAF-2T). For a measurement period, we chose 20 min of the NO-associated fluorescence microfluorographs regrading this time point showed a strong signal of DAF-2T after observing for 30 min (Figure 3.17).



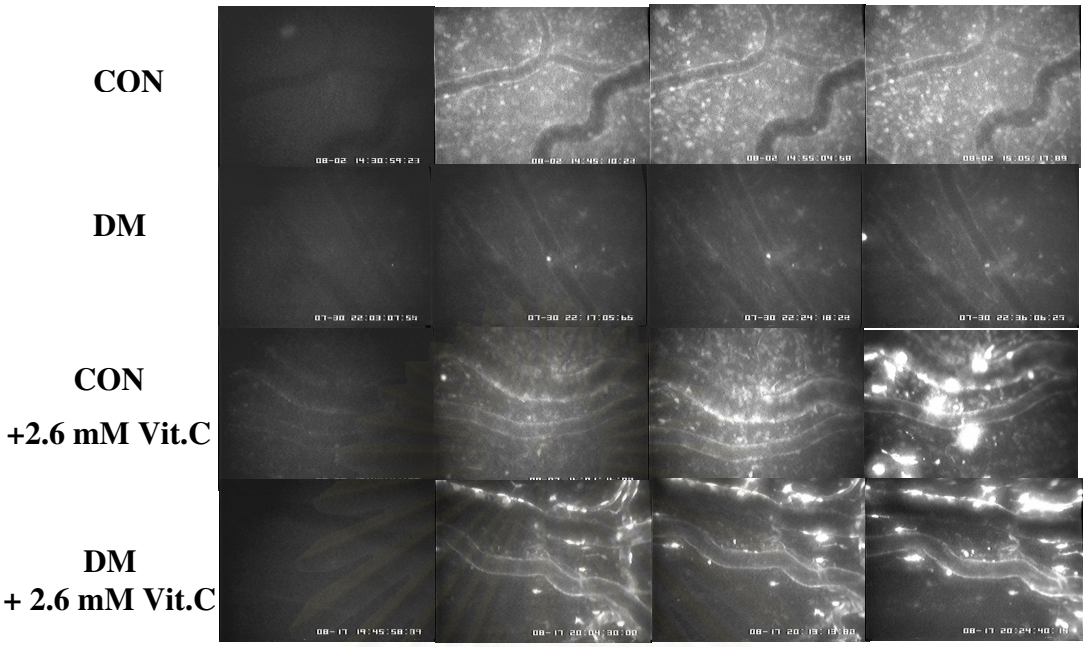


Figure 3.18 Microfluorographs of the NO-associated fluorescence taken from CON+2.6 mM Vit.C and DM+2.6 mM Vit.C at 0, 10, 20 and 30 min

The intensity levels in the areas of interests in captured image (512x512 pixels) were determined using a five-standard window frame (20x2 μm^2 each window) along 100 µm arteriolar wall (15 to 30 µM in diameter) by the digital image software (Image Pro-Plus, Media Cybernatics, Inc, USA) similar to the previous described shown in Figure 3.11.

The average intensities of baseline (0min, I_{base}) and 20min video images (I_{20min}) were determined and expressed by percentage changes of NO-associated fluorescent intensity as shown by the following equation:

$$\% \text{ changes of NO-associated fluorescent intensity} = \frac{(I_{20min} - I_{base}) \times 100}{I_{base}}$$

<u>Protocol 3.3</u>: To elucidate the underlying mechanism of vitamin C on regenerating BH_4 bioavailability

The diagram shown in Figure 3.19, indicated the conceptual framework of this experimental study targetting only on endothelial cell

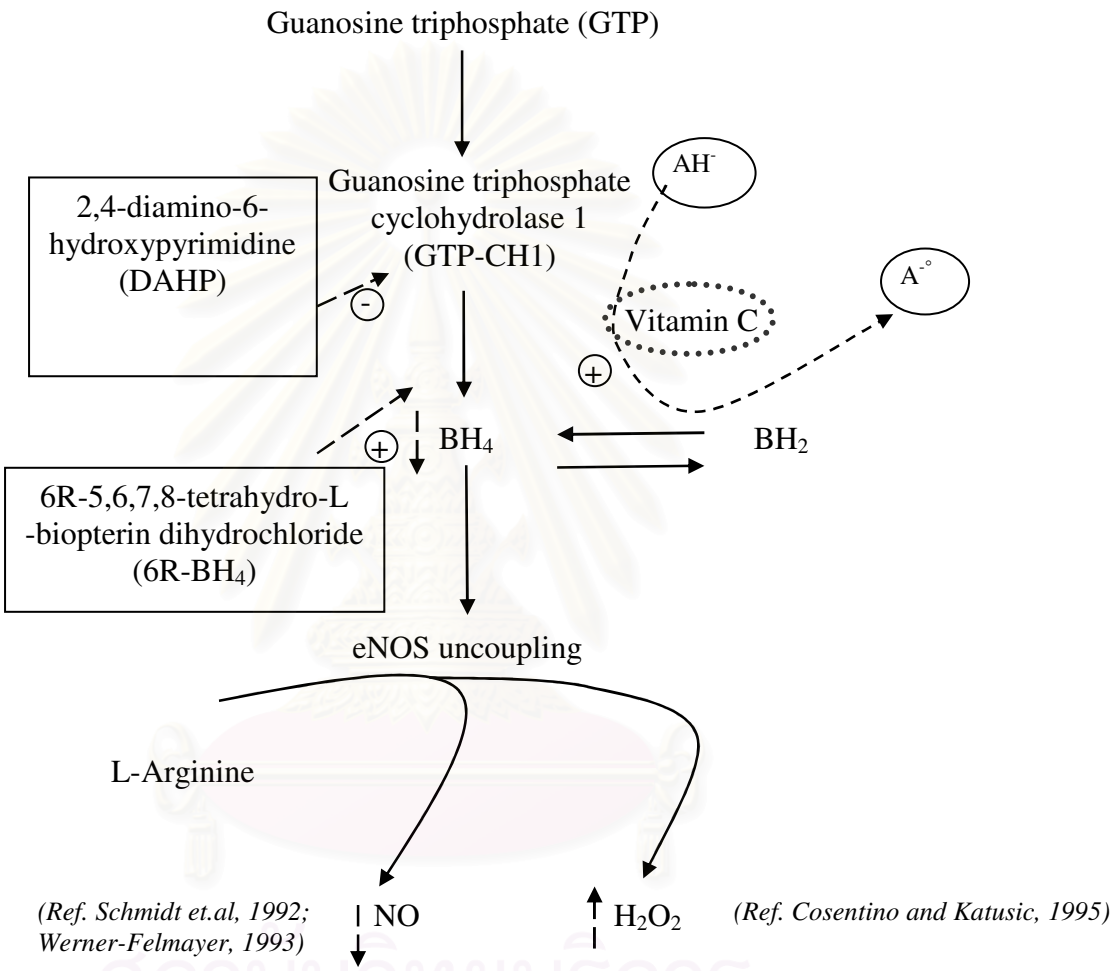


Figure 3.19 The conceptual framework desiged to find out the underlying mechanism of vitamin C on regenerating BH_4 bioavailability

This study was performed by using 2,4-diamino-6-hydroxypyrimidine (DAHP, Sigma-Aldrich Co., USA), 6R-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (6R-BH_4, Sigma-Aldrich Co., USA) and vitamin C (L-ascorbic acid, Sigma-Aldrich Co., USA)

DAHP is a compound that reduces intracellular BH4 levels. It is a selective, specific inhibitor of GTP-CH 1, a rate limiting enzyme for de novo BH_4 synthesis

(Schmidt et al., 1992, Bagi Z and Koller A, 2003). In our experiment, we used 6R-BH_4 as a positive control, which is a compound for increased intracellular BH_4 (Prabhakar 2001). The 6R-BH_4 is a cofactor for NOS and binds to the enzyme at a ratio of 1:1 BH_4:NOS (Schoedon *et al.,* 1993).

The rats were divided randomly into two groups of 6 weeks control (**6-wks CON** (N=30)), 6 weeks diabetes (**6-wks DM** (N=30)). After 6 week, each group was subdivided into six subgroups:

1) Vehicle; 30-min Krebs-Ringer perfusion
   (**6-wks CON**) (n=6)
   (**6-wks DM**) (n=6)
2) Veh+Vit.C; 1-min 2.6 mM vitamin C administration after 30-min Krebs-Ringer perfusion
   (**6-wks $CON_{Vit.C}$**) (n=6)
   (**6-wks $DM_{Vit.C}$**) (n=6)
3) DAHP; 30-min BH_4 antagonist (20mM, 2,4-diamino-6-hydroxypyrimidine (DAHP))
   (**6-wks CON_{DAHP}**) (n=6)
   (**6- wks DM_{DAHP}**) (n=6)
4) DAHP+Vit.C; 1-min 2.6 mM vitamin C administration after 30 min DAHP
   (**6-wks $CON_{DAHP+Vit.C}$**) (n=6)
   (**6-wks $DM_{DAHP+Vit.C}$**) (n=6)
5) DAHP+BH_4; 20-min BH_4 donor after 10-min DAHP
   (**6-wks $CON_{DAHP+BH4}$** ***0.1, 0.01 and 0.001 mM***) (n=6)
   (**6-wks $DM_{DAHP+BH4}$** ***0.1, 0.01 and 0.001 mM***) (n=6)
6) DAHP+BH_4+Vit.C ; 10-min DAHP followed by 20-min BH_4 donor and 1-min vitamin C
   (**6-wks $CON_{DAHP+ BH4}$** ***0.1and 0. 01 mM*** **+ Vit.C**) (n=6)
   (**6-wks $DM_{DAHP+ BH4}$** ***0.1and 0. 01 mM*** **+ Vit.C**) (n=6)

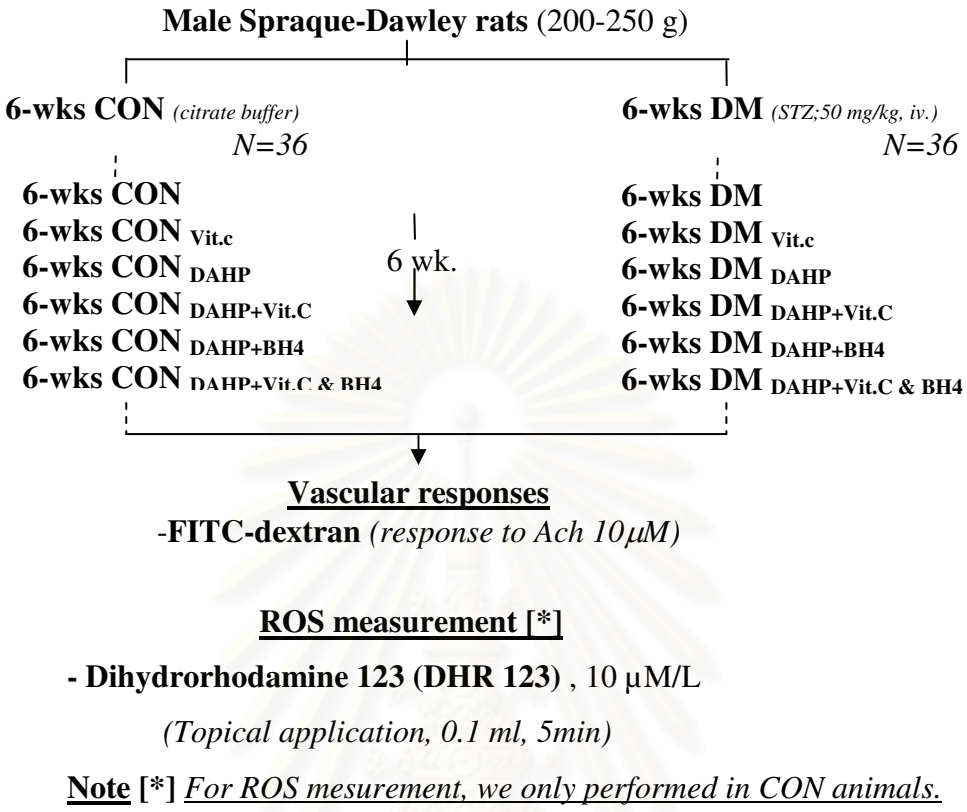


Figure 3.20 The experimental design to elucidate the underlying mechanism of vitamin C on regenerating BH4 bioavailability

Measurement of mesenteric arterioles in response to Ach-induced vasodilation as judged for NO bioavailability

On the day of experiment, the rat's mesentery was prepared as similar to 1st protocol. Changes in diameter of **6-wks CON** and **6-wks DM** arterioles (15 to 30 μm diameters) were observed in response to endothelium-dependent vasodialtor, Ach.

First group, we measured the baseline arteriolar diameter in response to Ach-induced vasodilation (10μM; 1 ml/5 min) after 30-min perfusion of Krebs-Riger solution (pH 7.4). Second group, to determine the effect of vitamin C we topically applied vitamin C (2.6 mM; 2 ml/1min) in response to Ach-induced vasodilation. Third

group, to inhibit intracellular BH_4 levels we applied DAHP (20 mM; 1 ml/5 min for 30 min [*]), in order to diminish NO production as judged by Ach-induced vasodilation. The other three groups, in the presence of DAHP vascular response to Ach (10μM; 1 ml/ 5min) was obtained after application of 6R-BH_4 with dose-dependent manner: 0.1, 0.01 and 0.001 mM (1 ml/5 min for 10 min [#]), vitamin C (2.6 mM; 2 ml/1min), and vitamin C (2.6 mM; 10 ml/1min) plus 6R-BH_4 (0.1 or 0.01 mM; 1 ml/5 min for 10 min).

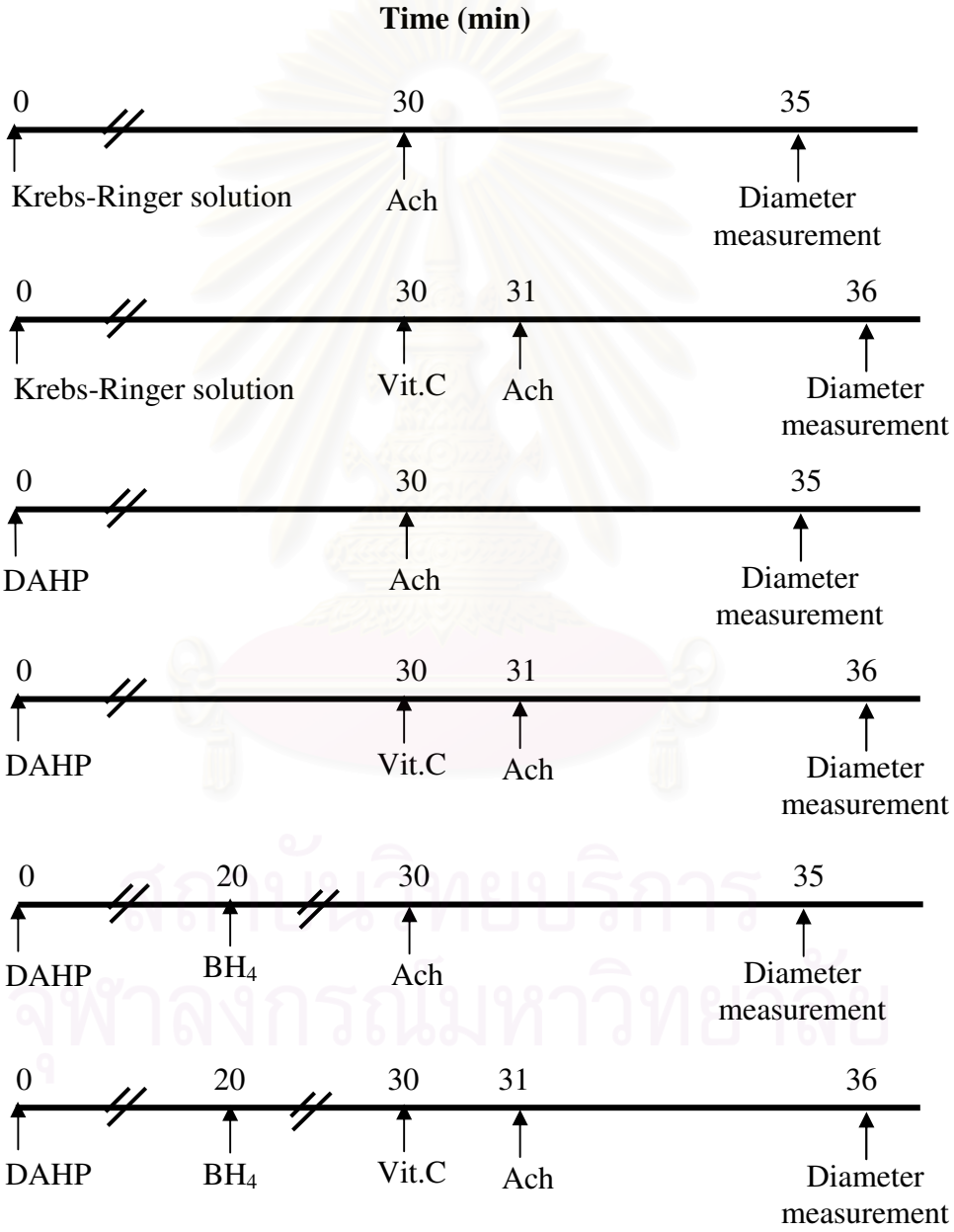


Figure 3.21 Experimental design for measuring vascular response to Ach as the effect of vitamin C and/or BH_4 application after inhibiting BH_4 biosynthetic pathway.

Note [*] As our pirot study, we knew that the inhibitory effect of DAHP to inhibit BH4 synthesis by topical accumulation with DAHP (20 mM; 6ml/30min as rate 5-min/1ml) significantly reduced Ach-induced vasodilation in the 6-wks CON rat. The result showed a maximum vasoconstriction of the mesenteric arterioles as after 20 minutes topical accumulation with DAHP (n=4) (Figure 3.22)

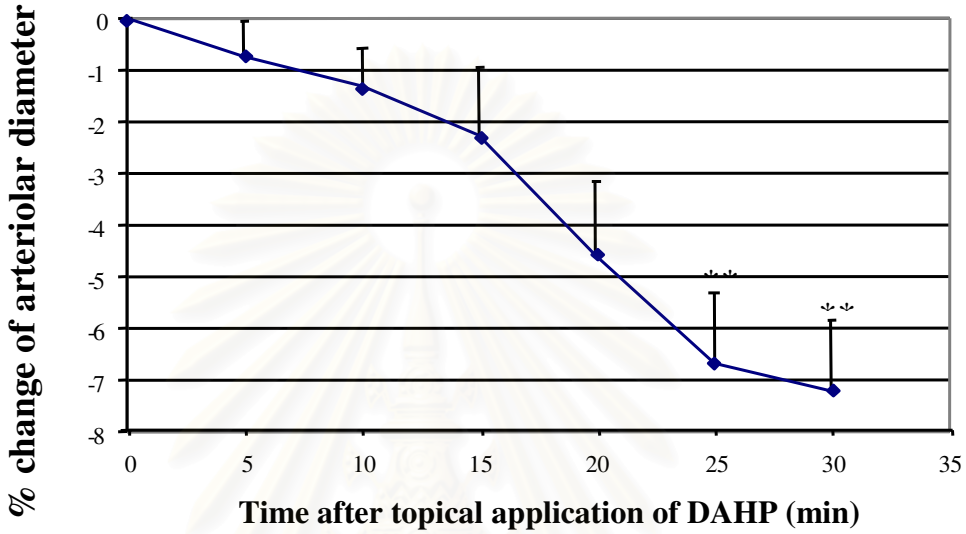


Figure 3.22 Time response curve of the effect of DAHP on percentage changes in mesenteric arteriolar diameter

Values are mean ± SEM
**$p<0.01$, significantly difference compared to 0 minute, which was the equiliblium period before applying DAHP

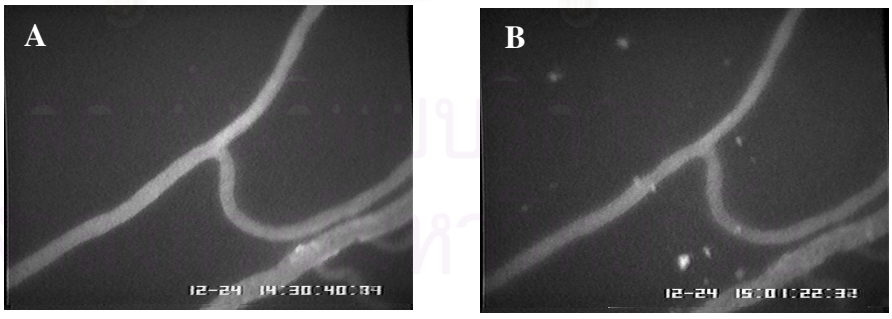


Figure 3.23 Intravital-video microscope images of mesenteric microvessels before and after topical accumulation of DAHP (20 mM) (A: Before topical accumulation, B: After topical accumulation 30 minutes) From these video-images, the result showed that Ach-induced vasodilation was reduced after topical accumulation of DAHP (B) as compared to before topical accumulation (A).

Note **[#]** 6R-BH_4 acommulation was starting 10 min before the end of DAHP perfusion with rate of toplical application 1ml each 5 min for 10 min (Heitzer et al, 2000).

To calculate the responses of mesenteric arterioles to Ach alone and/or Ach after application of the substrates, the digital image software (Image Pro-Plus, Media Cybernatics, Inc, USA) was used to examined as the previously describe (Figure 3.5 and 3.6).

Direct detection of ROS contents

To direct detection of ROS contents, DHR 123 was used as described previously with slightly different from protocol 3. In addition, the mesentery was topical application of DHR 123 (10 µM; 0.1 ml) together with Ach (10 µM; 1 ml/ 5min) (Figure 3.24). After 5 min substrates application the microfluorographs of the H_2O_2-associated fluorescence were visualized by the digital image software (Image Pro-Plus, Media Cybernatics, Inc, USA) similar to the previous described shown in Figure 3.11 and 3.12.

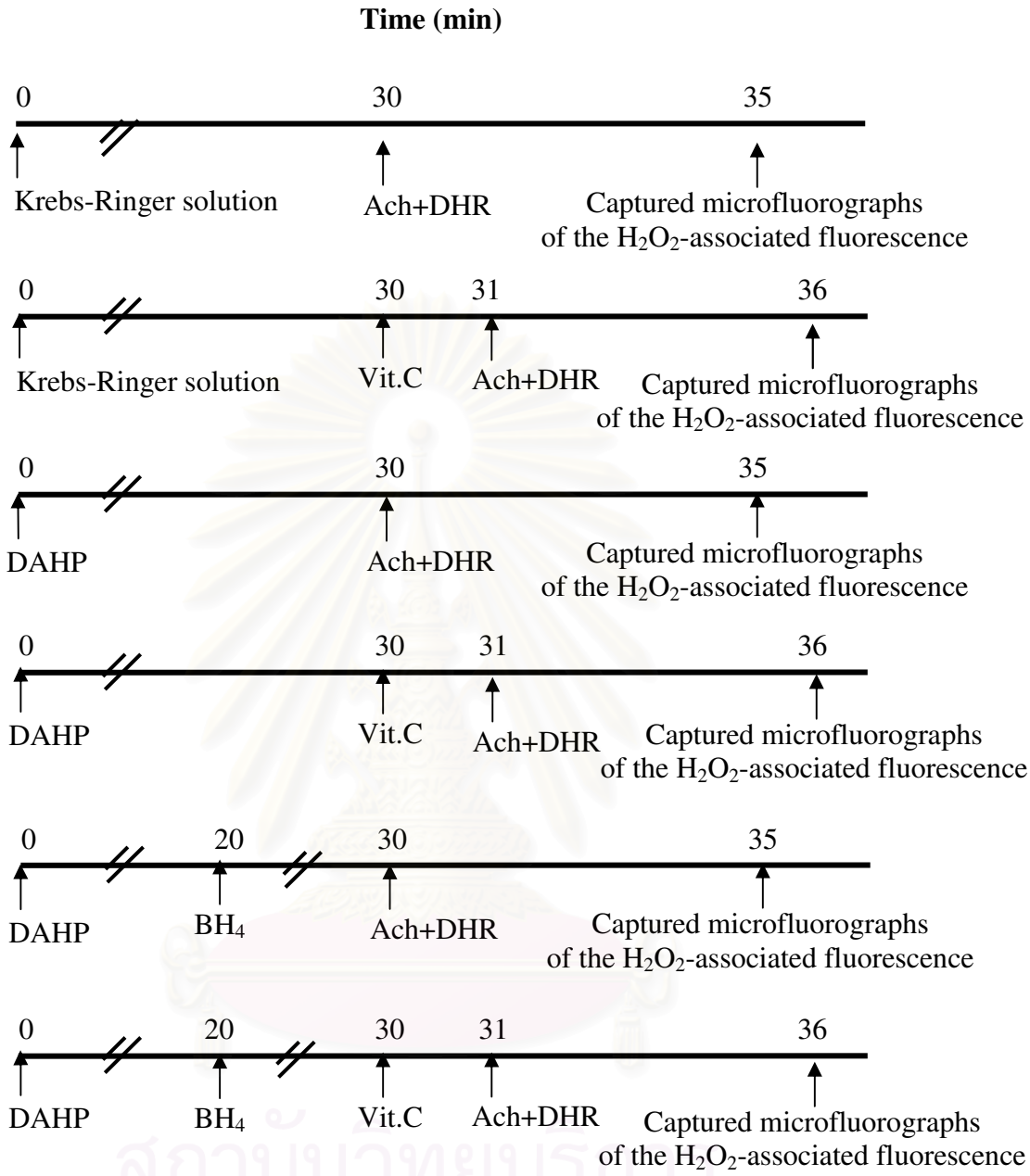


Figure 3.24 Experimental design for visualization of H_2O_2 production as the effect of vitamin C and/or BH_4 application after pretreated with BH_4-inhibitor, DAHP

Data analysis

All data were presented as means and standard errors of mean (SEM). For comparison among groups of animals, one-way analysis of variance (one-way ANOVA) was used and the differences in pairs of means among groups were made by Least significant difference (LSD) and Tukey's test as the post hoct tests. If the statistical probability (p-value) was less than or equal to 0.05, the differences were considered to be statistically significant.

CHAPTER IV

RESULTS

In the section below, we described three major results obtained from our experimental design as following:

4.1 Firstly, the occurrence of endothelial dysfunction at 6th week of diabetic induction was confirmed by the following results of:

- Measured biochemical parameters and physiological characteristics
- Number of leukocytes adhesion
- Vascular responses to endothelium-dependent and endothelium–independent vasodilators.

4.2 After the 6th week diabetes-induced endothelial dysfunction had been demonstrated, then, we aimed to evaluate whether vitamin C supplementation will be able to reverse the diabetes-induced endothelial dysfunction or not.

The following experiments were performed:

- Measurement of biochemical parameters and physiological characteristics
- Evaluation of leukocytes adhesion
- Measurement of vascular responses after the applications of endothelium-dependent and endothelium–independent vasodilators.

4.3 When the reversal effect of vitamin C supplementation was demonstrated, then, we aimed to examine as such its possible mechanism(s).

4.3.1. At first, we believed that the anti-oxidant property of vitamin C should be the major underlying mechanisms of this reversal effect. Therefore, our experimental protocol was performed in order to make the direct detection of ROS contents in diabetes and diabetes treated with vitamin C.

- Direct detection of ROS contents in long-termed vitamin C treatment

Next, we made a direct observation for NO content in in diabetes and diabetes treated with vitamin C. Our idea was that as an ROS scavenger, vitamin C should protect NO molecules from reacting with ROS, or it meant that vitamin C could increase NO bioavailability.

- Direct detection of NO production in acuted vitamin C application

4.3.2. Secondly, based on the anti-oxidant property of vitamin C, therefore, it is possible that vitamin C may preserve not only NO but also the important eNOS cofactor, BH_4, as well. In other word, it meant that BH_4 bioavailability could be enhanced through the ROS-scavenging by vitamin C.

- In order to determine the ROS scavenging effect of vitamin C on diabetes-induced ED involving BH4 bioavailibility, the acute changes in Ach-response after pretreated with BH4-inhibitor, DAHP, were investigated on both control and diabetic groups. Besides, the exogenous BH4 was also used in order to re-confirm that diabetes-induced ROS caused the decreased BH4 bioavailibity.

4.1 The occurrence of endothelial dysfunction at week 6th of diabetic induction

- **Measurement biochemical parameters and physiological characteristics**

The intravenous injection of STZ (50 mg/kg.bw) into 200-250 g male Spraque-Dawley rats caused pancreatic islet cells to damage, resulting in hyperglycemia within 48 hours. They also characterized by polydipsia, polyuria, and polyphagia throughout the experimental period, 6-wks DM group.

In the present study, the criteria used for DM was the blood glucose concentration (BG) had to be higher than 200 mg/dl. At 6th week after STZ injection, DM had significantly higher in blood glucose concentration and glycosylated hemoglobin (HbA1c) level (391 ± 32 mg/dl and 10.03 ± 0.17 %) as compared to CON group (140 ± 26 mg/dl and 5.58 ± 0.52 %) as shown in Table 4.1 and Figure 4.1. The plasma vitamin C level (Plasma Vit.C) was reduced significantly in 6-wks DM compared with their CON group (0.60 ± 0.03 and 1.33 ± 0.20 mg/dl, respectively) (Table 4.1 and Figure 4.1).

Beside the values of biochemical parameters, results of physiological characteristics were measured. The body weight (BW) was 253 ± 22 g in 6-wks DM significantly lower than 381 ± 26 g in 6wks-CON group (Table 4.1 and Figure 4.1). Mean arterial pressure (MAP) was shown in Table 4.1 and Figure 4.2. The results indicated that MAP value of 6-wks DM was significantly higher than the CON group (108 ± 5 and 82 ± 4 mmHg, respectively).

Table 4.1. Blood glucose (BG), glycosylated hemoglobin (HbA1c), plasma vitamin C (Plasma Vit.C), body weight (BW), and mean arterial blood pressure (MAP) were demonstrated for 6th week of experimental periods between control (CON) and diabetes (DM).

Group	BG (mg/dl)	HbA1c (%)	Plasma Vit.C (mg/dl)	BW (g)	MAP (mmHg)
6-wks CON	140 ± 26 (n = 6)	5.58 ± 0.52 (n = 5)	1.33 ± 0.20 (n = 6)	381 ± 26 (n = 6)	82 ± 4 (n = 8)
6-wks DM	391 ± 32 *** (n = 10)	10.03 ± 0.17 *** (n = 8)	0.60 ± 0.03 *** (n =7)	253 ± 22 *** (n=8)	108 ± 5 * (n = 8)

Values are mean + SEM

*p<0.05, ***p<0.001, significantly difference compared to 6-wks CON

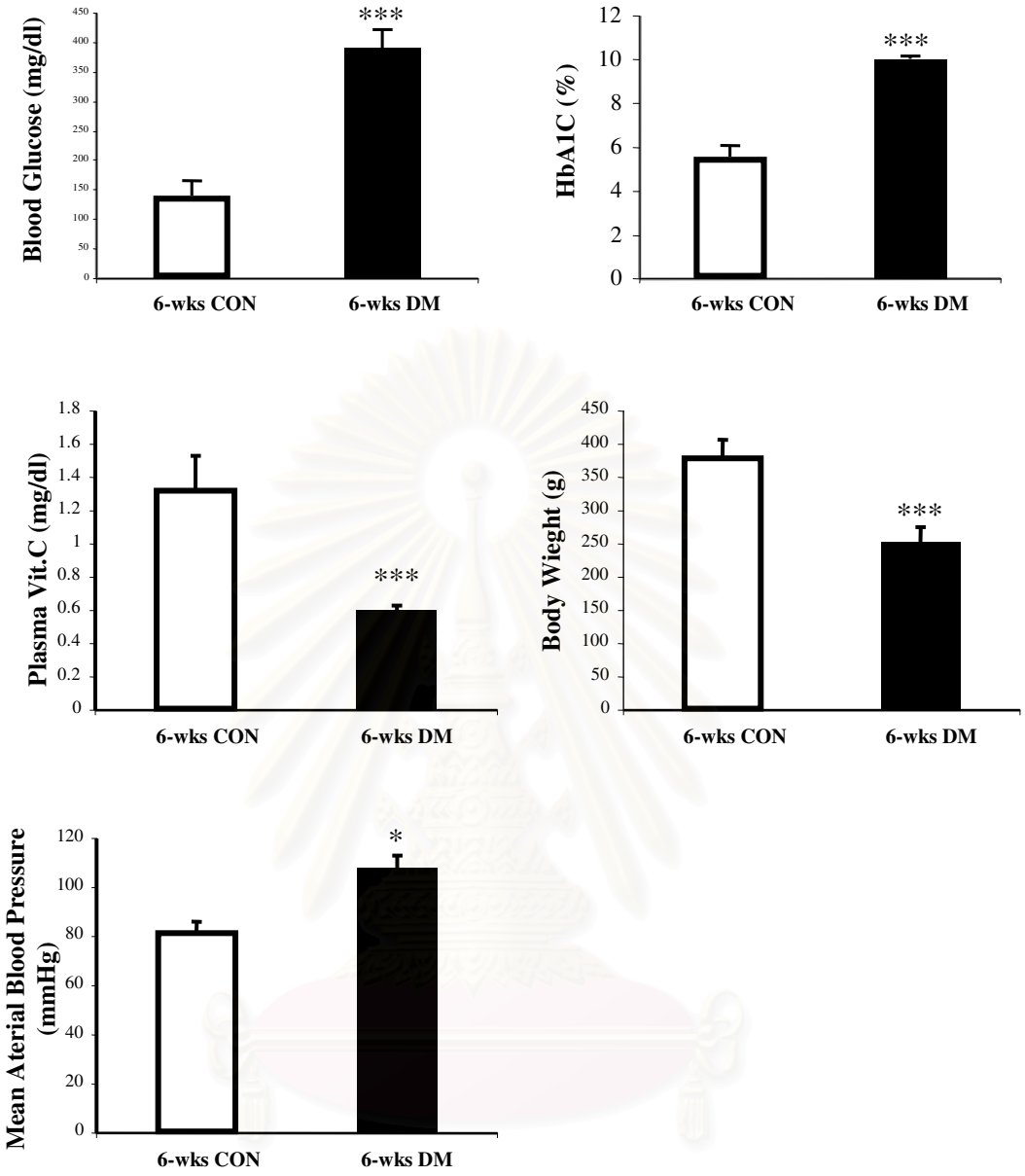


Figure 4.1. Alteration of biochemical parameters and physiological characteristics: blood glucose (BG), glycosylated hemoglobin (HbA1c), plasma vitamin C (Plasma Vit.C), body weight (BW), and mean arterial blood pressure (MAP) were demonstrated for 6th week of experimental periods between control (CON) and diabetes (DM)

Values are mean ± SEM

*p<0.05, significantly difference compared to 6-wks CON

***p<0.001, significantly difference compared to 6-wks CON

- **Evaluation of leukocytes adhesion**

In order to confirm that at 6th week after diabetic induction, the endothelium became dysfunction, the leukocyte adhesion to the endothelium of postcapillary venules was counted if it remaind stationary for ≥ 30 seconds. Numbers of leukocytes adherence (cells/100 μm-venular length) were calculated using the equation 1 and summarized in Table 4.2, Figure 4.2 and 4.3. In 6-wks DM, the adherent cells increased as 6.97 ± 0.84 cells/100 μm-venular length, which were significantly higher than 6-wks CON (0.92 ± 0.29 cells/100 μm-venular length).

- **Measurement of endothelium-dependent and endothelium–independent vasodilation**

Besides measuring the numbers of leukocytes adherence, we also verified the exhibiton of endothelial dysfunction after week 6th of diabetic induction, endothelium-dependent vasodilator, Ach, 10 μM was used to examine the endothelium function on mesenteric arterioles responses. Base on the FITC-recorded vedioimages, the percentage changes of arteriolar diameters were evaluated by using the digital image software as described previously Chapter III (Materials and Methods). Ach-induced vasodilation was significantly decreased in 6-wks DM as compared to their CON group (6.40 ± 0.79 % and 14.66 ± 0.85 %, respectively) (Table 4.3 and Figure 4.4).

To ensure that this abnormality didn't involve with smooth muscle cells, the vasodilatory response to endothelium-independent vasodilation SNP, 10 μM was examined. The results showed that there was no significant difference between 6-wks CON and 6-wks DM groups (11.89 ± 1.16 % and 10.74 ± 0.41 %, respectively) (Table 4.3 and Figure 4.4).

Table 4.2. Numbers of leukocytes adherence from postcapillary mesenteric venules between control (CON) and diabetes (DM) at 6th week of experimental periods.

Group	**Numbers of leukocytes adherence** (cells/100-μm venular length)
6-wks CON	0.92 ± 0.29 (n = 6)
6-wks DM	6.97 ± 0.84 *** (n = 10)

Values are mean ± SEM

***p<0.001, significantly difference compared to 6-wks CON

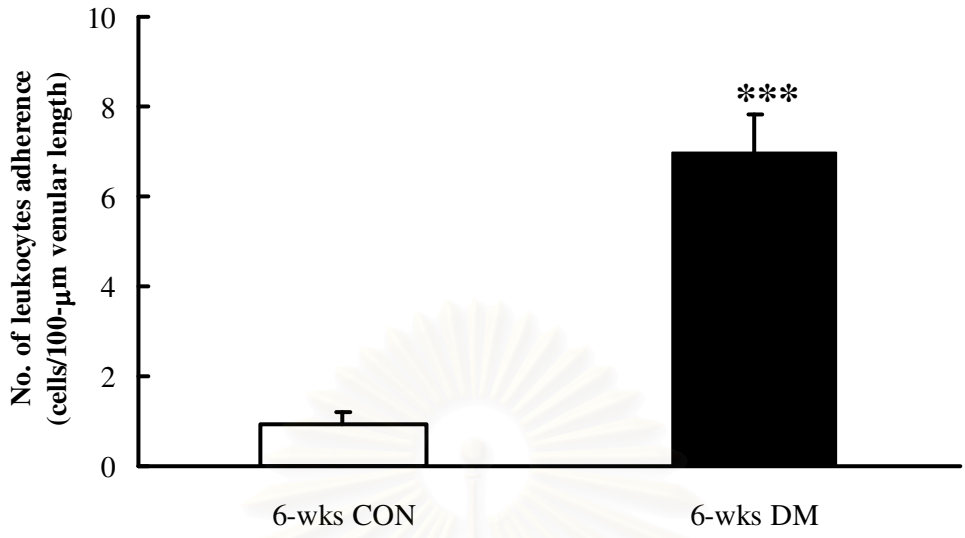
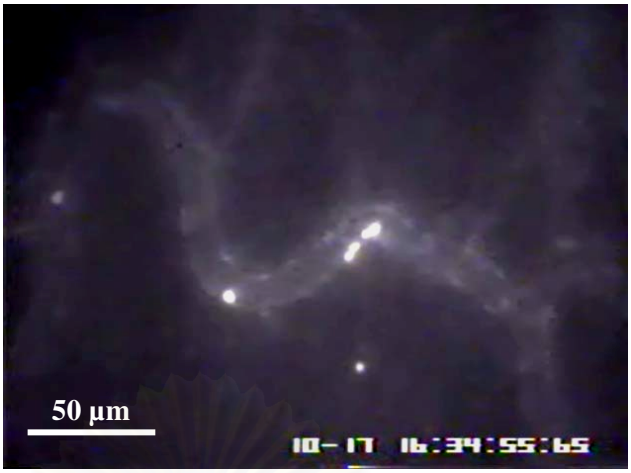


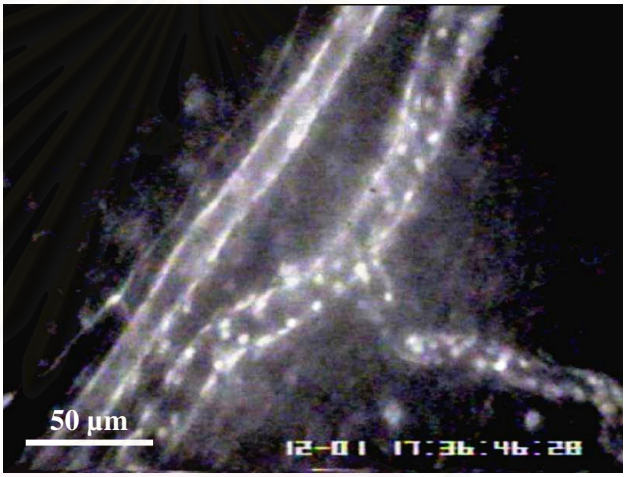
Figure 4.2. Diabetic-induced endothelial dysfunction within 6th week: numbers of leukocytes adherence from postcapillary mesenteric venules at 6th week of experimental periods between control (CON) and diabetes (DM)

Values are mean ± SEM

***p<0.001, significantly difference compared to 6-wks CON.



6-wks CON



6-wks DM

Figure 4.3. Videoimages shown a pattern of leukocyte adhere to endothelial cells from postcapillary mesenteric venules after intravenous injection with fluorescent indicator, acrydine orange at 6th week of experimental periods between control (CON) and diabetes (DM).

Table 4.3. Percentage changes of arteriolar diameters from mesenteric arterioles in responses to Ach and SNP between control (CON) and diabetes (DM) at 6th week of experimental periods

Group	% changes of arteriolar diameters	
	Ach testing	**SNP testing**
6-wks CON	14.66 ± 0.85 (n = 8)	11.89 ± 1.16 (n = 5)
6-wks DM	6.40 ± 0.79 ** (n = 7)	10.74 ± 0.41^{NS} (n = 5)

Values are mean ± SEM

**p<0.01, significantly difference compared to 6-wks CON

^{NS}, not significantly difference compared to 6-wks CON

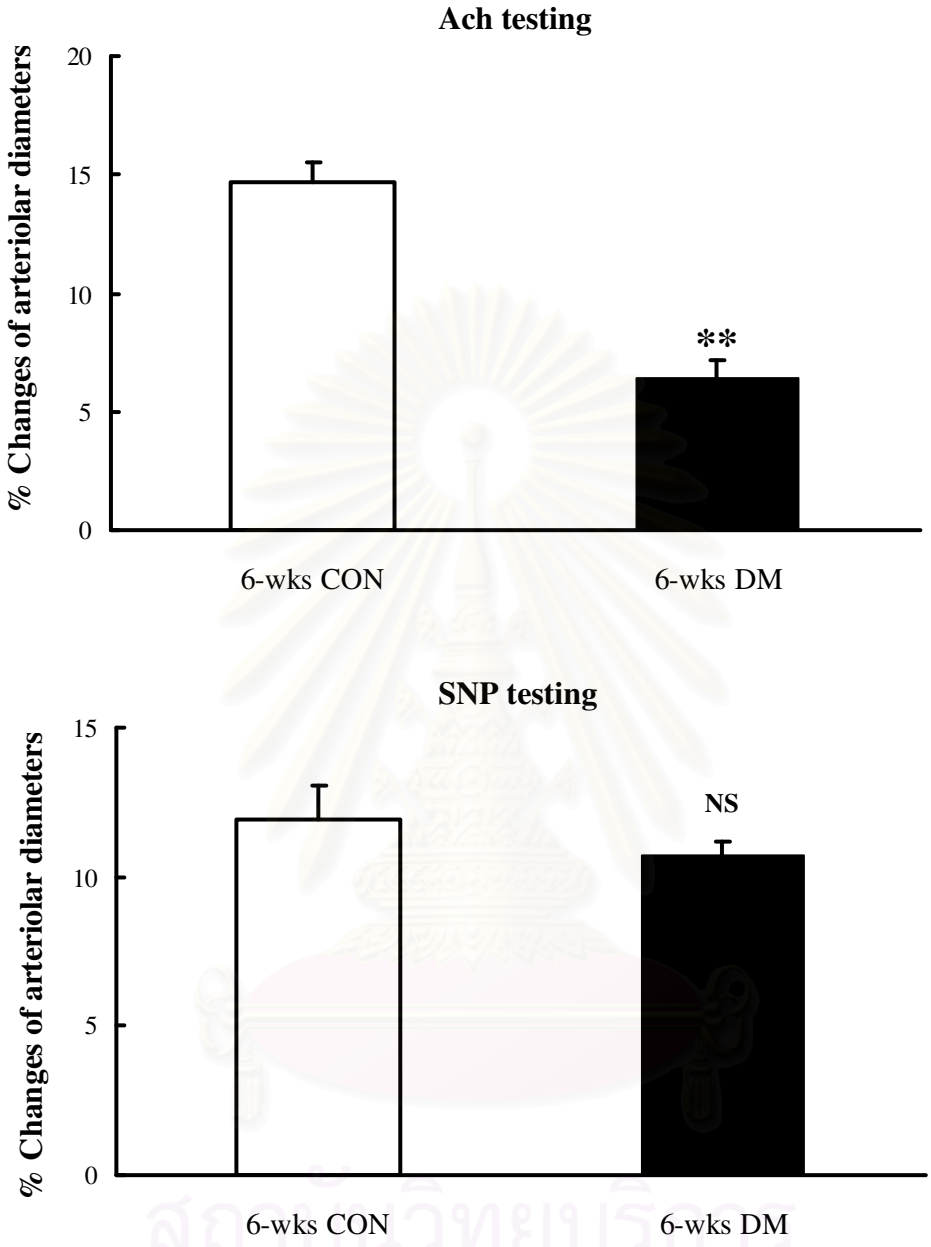


Figure 4.4. Diabetic-induced endothelial dysfunction within 6th week: percentage changes of arteriolar diameters from mesenteric arterioles in responses to Ach and SNP at 6th week of experimental periods between control (CON) and diabetes (DM)

Values are mean ± SEM

**p<0.01, significantly difference compared to 6-wks CON

NS not significantly difference compared to 6-wks CON

4.2 The effect of vitamin C supplementation to reverse the diabetes-induced endothelial dysfunction.

After confirming the endothelial dysfunction, the treatment protocol was started (DM +VitC_{6wks}) to examine whether the delayed vitamin C supplementation can reverse endothelial dysfunction. Moreover, we also desighed for testing the preventtive effect of vitamin C by early supplementation for 12 weeks before endothelium become dysfunction (12-wks DM+VitC_{day2}).

- **Measurement biochemical parameters and physiological characteristics**

By 50 mg/kg.bw of STZ injection, the hyperglycemia maintained throughout the 12 weeks period of experiment among three DM groups (12-wks DM = 418 ± 17 /dl BG; HbA1c = 10.86 ± 0.24 %, 12-wks DM +VitC_{6wks} = 408 ± 29 mg/dl BG; HbA1c = 10.34 ± 0.35, 12-wks DM+VitC_{day2} = 380 ± 19 mg/dl BG; HbA1c = 9.81 ± 0.30 %) as compared to their CON group (BG = 93 ± 7.70 mg/dl; HbA1c = 3.68 ± 0.54 %) (Table 4.4 and Figure 4.5). The plasma vitamin C level was reduced significantly in 12-wks DM in relation with their CON group (0.62 ± 0.02, and 1.30 ± 0.15 mg/dl, respectively). Contrary, delayed treatment (12-wks DM +VitC_{6wks} = 1.27 ± 0.04 mg/dl) and early supplement of vitamin C (12-wks DM +VitC_{day2} = 0.99 ± 0.03mg/dl) didn't show any statistic significant difference in the level of plasma vit C as compare to 12-wk CON (Table 4.4 and Figure 4.5).

The body weight (BW) was lost in 12-wks DM as compared to 12-wks CON group(183 ± 8 and 426 ± 5 g, respectively), while in both viltamin C supplementation, the BW was increased significantly (12-wks DM +VitC_{6wks} = 284 ± 23 g, and 12-wks DM +VitC_{day2} = 228 ± 15 g) (Table 4.4 and Figure 4.5). In comparison with their CON group, the mean arterial blood pressure (MAP) was increased in 12-wks DM (97 ± 7 mmHg and 114 ± 3, respectively). The increase in MAP was attenuated significantly with vitamin C supplementation in both delayed phase (12-wks DM +VitC_{6wks} = 95 ± 7 mmHg) and early phase (12-wks DM +VitC_{day2} = 93 ± 7 mmHg) (Table 4.4 and Figure 4.5).

Table 4.4. Blood glucose (BG), glycosylated hemoglobin (HbA1c), plasma vitamin C (Plasma VitC), body weight (BW), and mean arterial blood pressure (MAP) were demonstrated at 12th week of experimental periods in control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2rd after STZ-injection (DM +Vit.C_{day2}).

Group	BG (mg/dl)	HbA1c (%)	Plasma Vit.C (mg/dl)	BW (g)	MAP (mmHg)
12-wks CON	93± 8 (n=8)	3.68±0.54 (n=6)	1.30±0.15 (n=6)	426±4 (n=9)	97±7 (n=8)
12-wks DM	418± 17*** (n=9)	10.86±0.24*** (n=7)	0.62±0.02*** (n=7)	183±8*** (n=10)	114±3*** (n=9)
12-wks DM+Vit.C_{6wks}	408± 29*** (n=5)	10.34±0.35*** (n=5)	1.27±0.04$^{###}$ (n=5)	284±23$^{#}$ (n=6)	95±7$^{###}$ (n=7)
12-wks DM+Vit.C_{day2}	380 ± 19*** (n=8)	9.81±0.30*** (n=8)	0.99±0.03$^{###}$ (n=5)	228±15$^{#}$ (n=9)	93±7$^{###}$ (n=8)

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

$^{#}p$<0.05, $^{###}p$<0.001, significantly difference compared to 12-wks DM

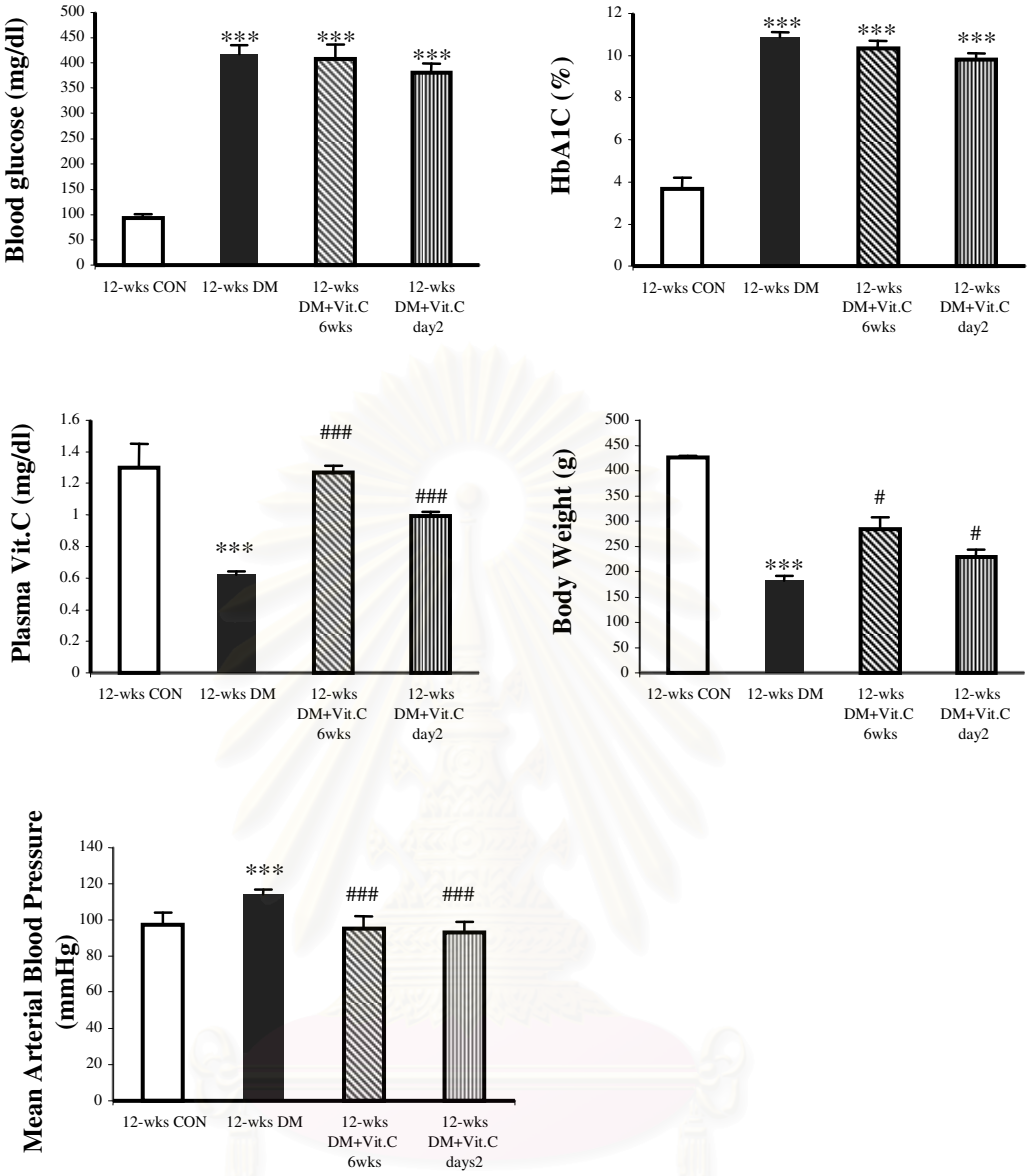


Figure 4.5. Alteration of biochemical parameters and physiological characteristics: blood glucose (BG), glycosylated hemoglobin (HbA1c), plasma vitamin C (Plasma VitC), body weight (BW), and mean arterial blood pressure (MAP) were demonstrated at 12th week of experimental periods in control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2rd after STZ-injection (DM +Vit.C_{day2})

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

$^{\#}p$<0.05, $^{\#\#\#}p$<0.001, significantly difference compared to 12-wks DM

- **Evaluation of leukocytes adhesion**

Table 4.5, Figure 4.6 and 4.7 shown the numbers of leukocyte adherence (cells/100 μm-venular length) in 12-wks CON, 12-wks DM, 12-wks DM+VitC_{6wks} and DM+ VitC_{day2} groups. Their adherent cells were changed to 2.15 ± 0. cells/100 μm-venular length in 12-wks DM +VitC_{6wks} and 4.71 ± 0.43 cells/100 μm-venular length in 12-wks DM +VitC_{day2} from 9.17 ± 1.45 cells/100 μm-venular length in 12-wks DM.

It is to be noted that this diabetes-enhanced leukocyte adhesion was significantly attenuated by delayed treatment and early supplement of vitamin C.

- **Measurement of endothelium-dependent and endothelium–independent vasodilation**

At 12^{th} week, the endothelium-dependent vasodilation in response to Ach was significantly decreased in 12-wks DM as compared to 12-wks CON (4.72 ± 1.51% and 13.86 ± 1.68 %, respectively) (Table 4.6 and Figure 4.8). Interestingly, this abnormality was restored by the delayed treatment (12-wks DM+Vit.C_{6wks} = 12.42 ± 1.11 %) and the early supplement of the vitamin C (12-wks DM+Vit.C_{day2} = 14.14 ± 1.62 %) (Table 4.6 and Figure 4.8).

In contrast, when the endothelium-independent vasodilator, SNP was used; there was no significant difference in the percentage changes of arteriolar diameters among all group of rats (12-wks CON =15.19 ± 0.90 %, 12-wks DM = 9.42 ± 1.61 %, 12-wks DM+Vit.C_{6wks} =11.86 ± 0.81 %, and 12-wks DM+Vit.C_{day2} = 12.66 ± 2.02 %) (Table 4.6 and Figure 4.8).

It is to be noted that vitamin C supplementation reversed and improved diabetes-induced endothelial dysfunction; these effect of vitamin C is mediated by the release of NO from endothelial cells but not from smooth muscle cells.

Table 4.5. Numbers of leukocytes adherence from postcapillary mesenteric venules among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2}) at 12th week of experimental periods.

Group	**Numbers of leukocytes adherence** (cells/100-μm venular length)
12-wks CON	2.00 ± 0.50 (n = 8)
12-wks DM	9.17 ± 1.45 *** (n = 6)
12-wks DM+Vit.C_{6wks}	2.15 ± 0.31 ### (n = 7)
12-wks DM+Vit.C_{day2}	4.71 ± 0.47 ## (n = 7)

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

##p<0.01, ###p<0.001, significantly difference compared to 12-wks DM

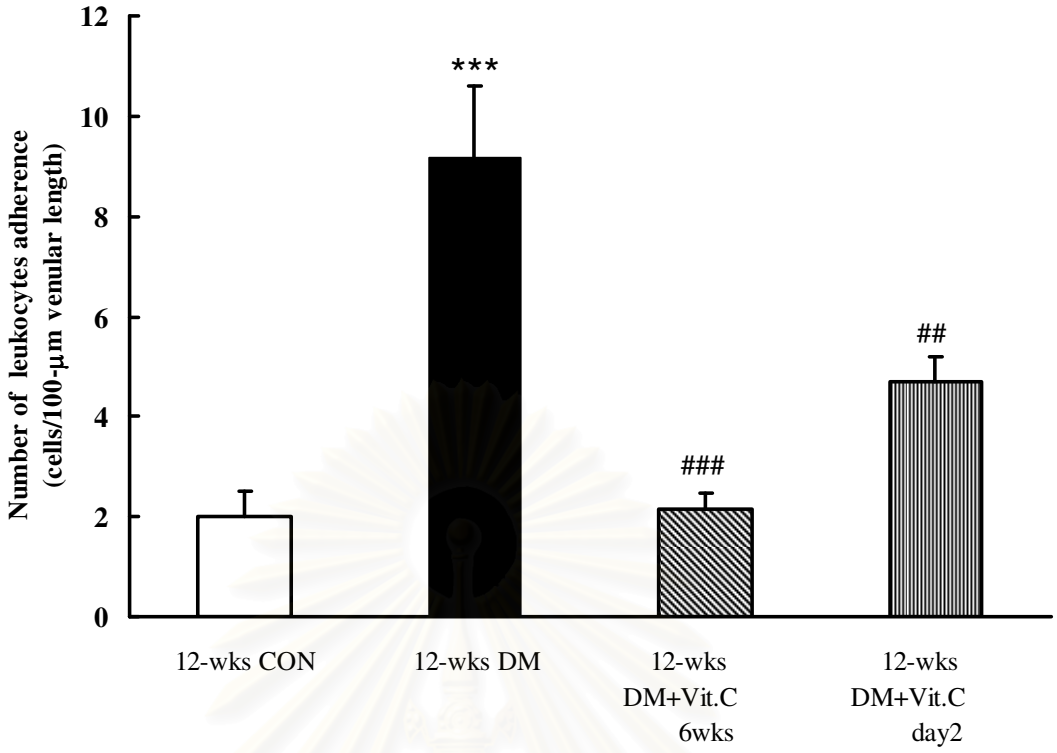


Figure 4.6. Effect of vitamin C supplementation on diabetic-induced endothelial dysfunction: numbers of leukocytes adherence from postcapillary mesenteric venules at 12 th week of experimental periods among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2})

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

$^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001, significantly difference compared to 12-wks DM

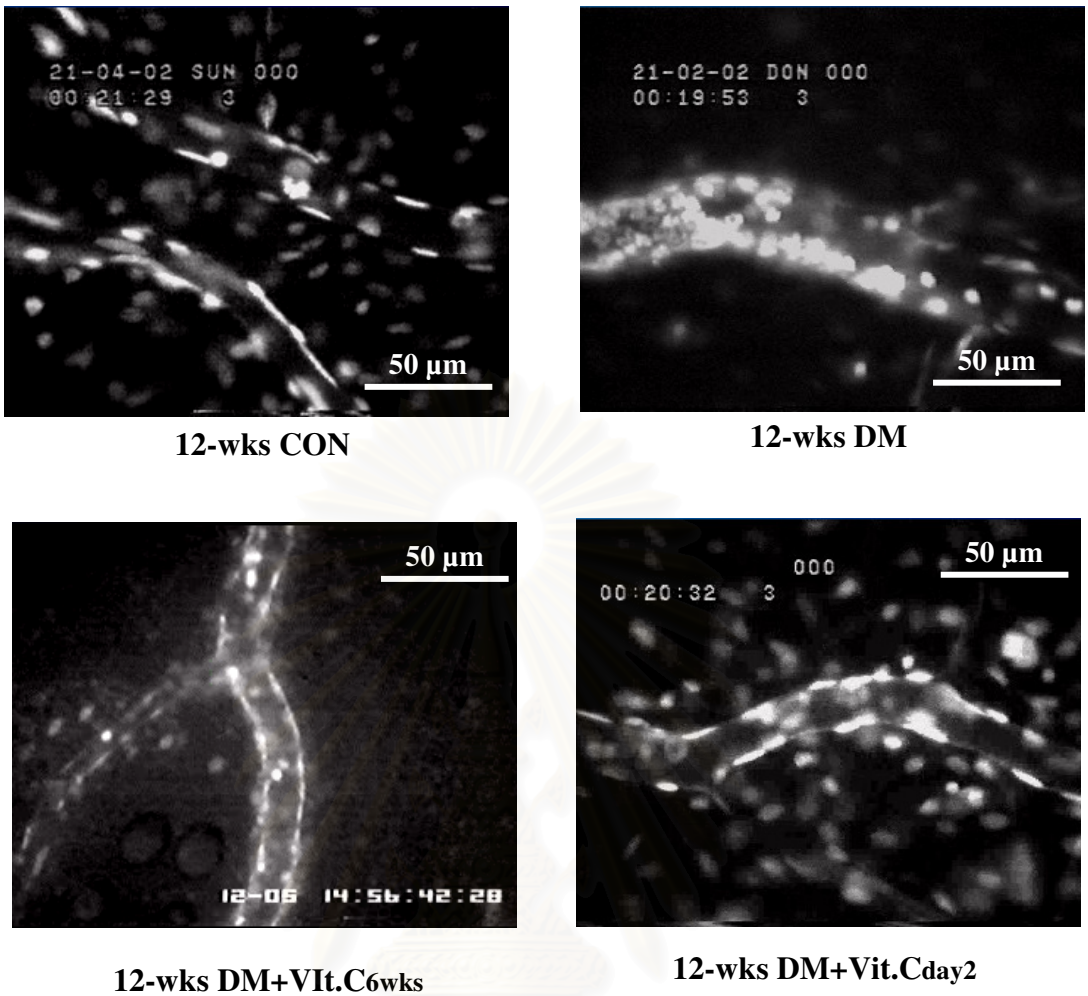


Figure 4.7. Videoimages shown a pattern of leukocyte adhere to endothelial cells from postcapillary mesenteric venules after intravenous injection with fluorescent indicator, acrydine orange at 12th week of experimental periods among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2}).

Table 4.6. Percentage change of arteriolar diameters from mesenteric arterioles in responses to Ach and SNP among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2}) at 12th week of experimental periods.

Group	% changes of arteriolar diameters	
	Ach testing	**SNP testing**
12-wks CON	13.86 ± 1.68 (n = 9)	15.19 ± 0.90 (n = 6)
12-wks DM	4.72 ± 1.51 *** (n = 7)	9.42 ± 1.61 ^{NS} (n = 6)
12-wks DM+Vit.C_{6wks}	12.42 ± 1.11$^{\#\#}$ (n = 6)	11.86 ± 0.81 ^{NS} (n = 5)
12-wks DM+Vit.C_{day2}	14.14 ± 1.62$^{\#\#}$ (n = 7)	12.66 ± 2.02 ^{NS} (n = 7)

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

^{NS}, not significantly difference compared to 12-wks CON

$^{\#\#}p$<0.01, significantly difference compared to 12-wks DM

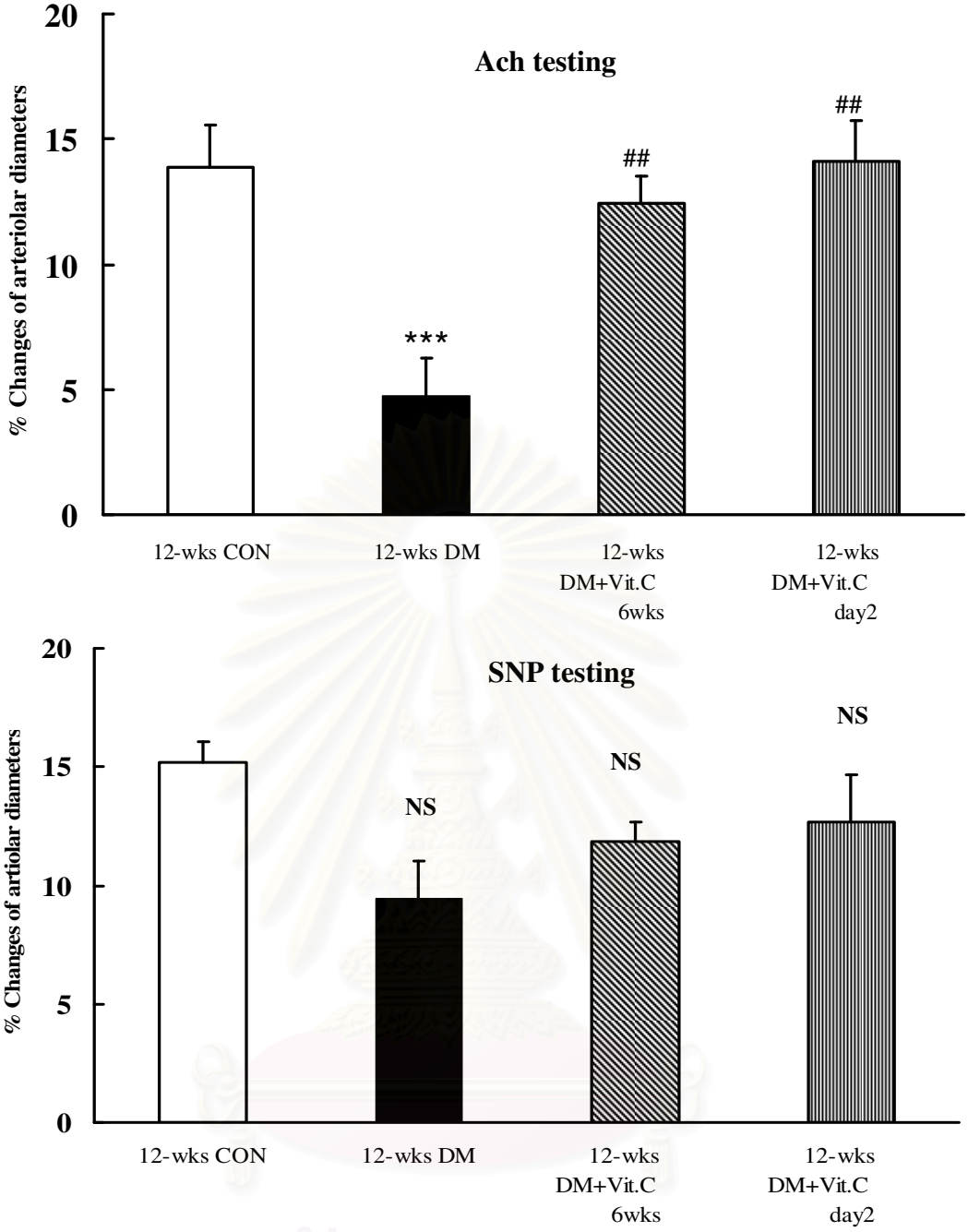


Figure 4.8. Effect of vitamin C supplementation on diabetic-induced endothelial dysfunction: percentage changes of arteriolar diameters from mesenteric arterioles in responses to Ach and SNP at 12th week of experimental periods among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2})

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

^{NS}, not significantly difference compared to 12-wks CON

$^{\#\#}p$<0.01, significantly difference compared to 12-wks DM

4.3 The underlying mechanism(s) of the effect of vitamin C to reverse diabetes-induced endothelial dysfunction

1) The underlying mechanism of vitamin C on sacvenging ROS

- **Direct detection of ROS contents in long-termed vitamin C treatment**

Table 4.7, Figure 4.9 and 4.10 indicated that the 1-min H_2O_2-associated fluorescent intensity was significantly increased in 12-wks DM compared with 12-wks CON (165.89 ± 24.59 % and 34.08 ± 15.6 %, respectively). However, the decrease in the percentage change of H_2O_2-associated fluorescent intensity was observed in both vitamin C supplementation as delayed phase (DM +Vit.C_{6wks} = 26.02 ± 14.56 %) and early phase (DM +Vit.C_{day2} = 32.86 ± 8.03 %).

It is to be noted that the delayed treatment and early supplement of vitamin C could similarly attenuate the increase in ROS contents induced in diabetes.

Table 4.7. Percentage changes of H_2O_2-associated fluorescent intensity from mesenteric arterioles among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2}) at 12th week of experimental periods

Group	% changes of H_2O_2-associated fluorescent intensity
12-wks CON	34.08± 15.6 (n = 5)
12-wks DM	165.89± 24.59 *** (n = 5)
12-wks DM+Vit.C_{6wks}	26.02± 14.56 ### (n = 5)
12-wks DM+Vit.C_{day2}	32.86± 8.03 ### (n = 6)

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

###p<0.001, significantly difference compared to 12-wks DM

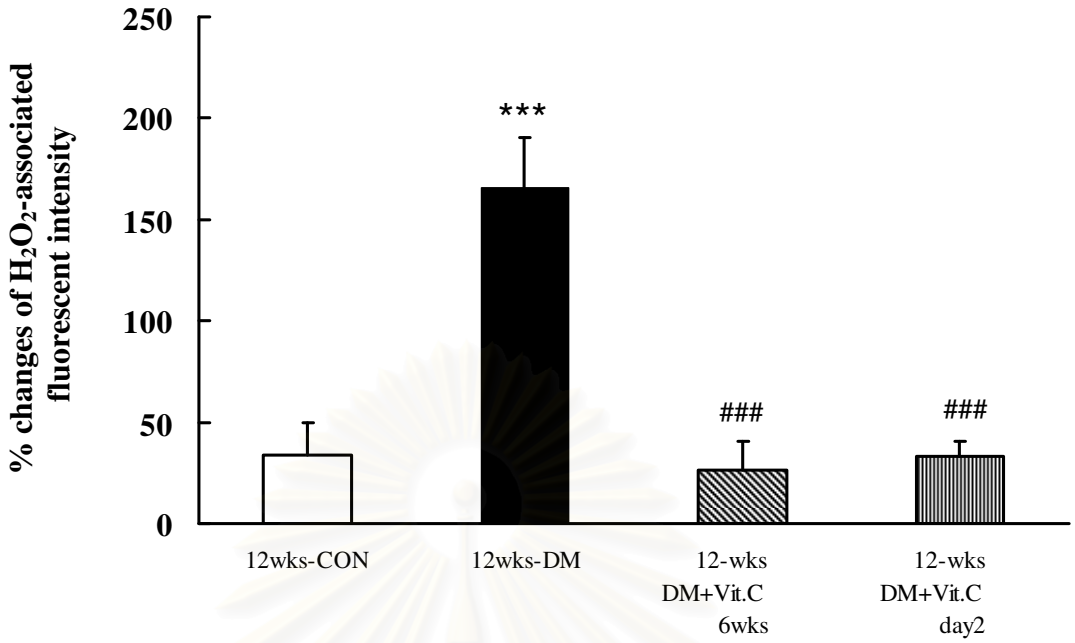


Figure 4.9. Effect of vitamin C supplementation on ROS contents: percentage changes of H_2O_2-associated fluorescent intensity from mesenteric arterioles among control (CON), diabetes (DM), diabetes treated with vitamin C (1 g/l) starting on week 6th after STZ-injection (DM +Vit.C_{6wks}) and diabetes treated with vitamin C (1 g/l) starting on day 2nd after STZ-injection (DM +Vit.C_{day2}) at 12th week of experimental periods

Values are mean ± SEM

***p<0.001, significantly difference compared to 12-wks CON

###p<0.001, significantly difference compared to 12-wks DM

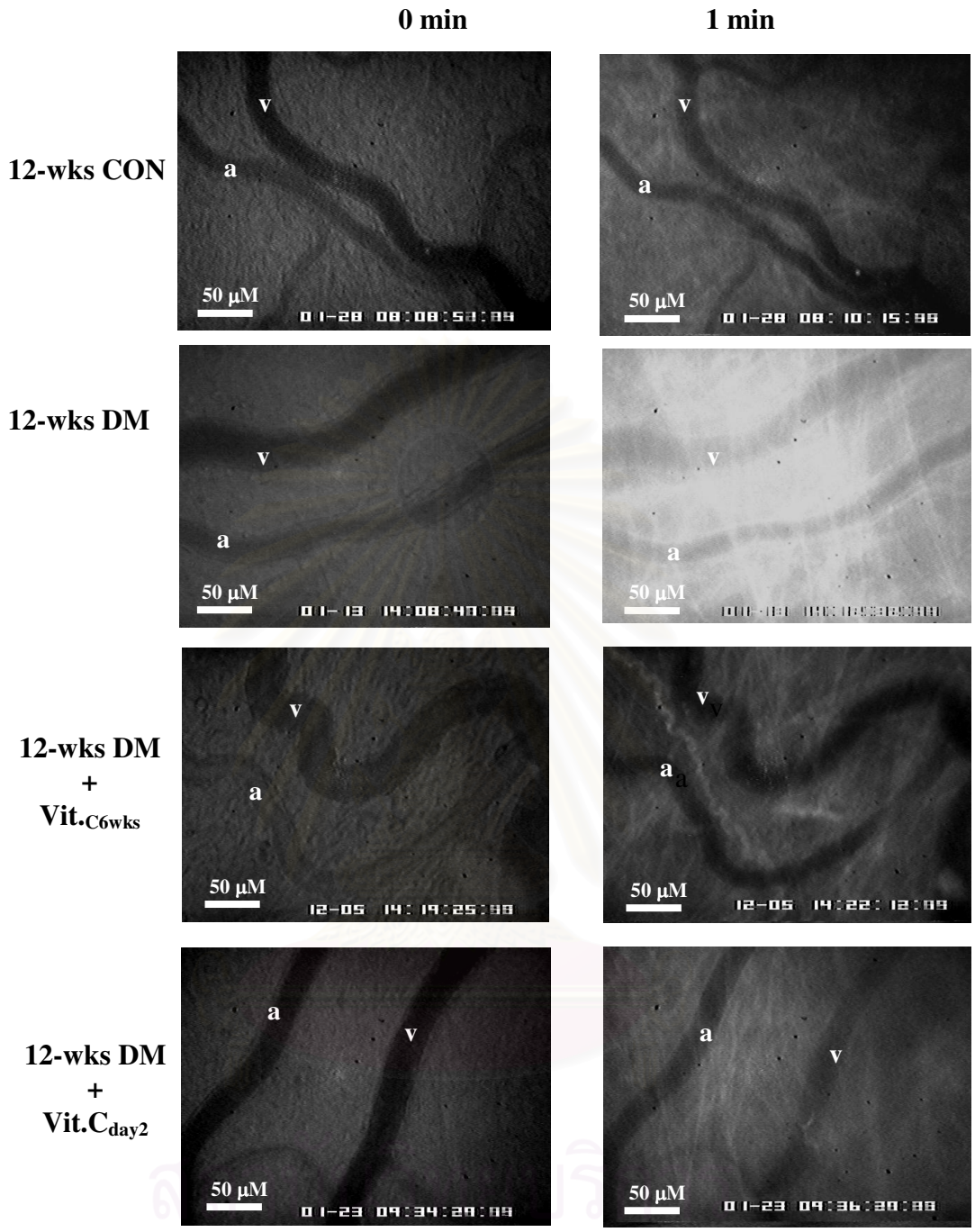


Figure 4.10. The intravital video-microscopic images showing spatial alteration in H_2O_2-associated fluorescence in mesenteric microcirculation before (as baseline, Right) and after 1 min topical application of DHR 123, a H_2O_2 sensitive fluorochrome (Left). The ROS generations were observed along arteriolar wall.
(a, arteriole; v, venule)

2) The underlying mechanism of vitamin C on NO bioavailability

- **Measurement of the acute effect of topical application vitamin C on endothelium-depentdent vasodilation**

In order to examine the mechanism(s) underlying the effect of vitamin C on endothelial cells, the acute effect of topical application vitamin C was set up to determine its action. The topically applicated vitamin C at verious doses (0.26, 2.6, and 26 mM) were observed on mesenteric arterioles in response to endothelium-depentdent vasodilator, Ach (10^{-5} M).

Table 4.8, Figure 4.11 demonstrated an optimal dose of vitamin C. After topical application vitamin C at dose 2.6 mM for one minute, the vitamin C-induced vasodilation to Ach was significantly higher than the absence of vitamin C applying, baseline (23.49 ± 3.03 % and 14.53 ± 1.43 %, respectively). This 2.6 mM vitamin C-induced vasodilation to Ach was also singnificantly higher than the other two doses--0.26 and 26 mM (12.21± 1.54 % and 13.47 ± 2.50, respectively). In contrast, there were no significant differences among the baseline, vitamin C at dose 0.26 or 26 mM.

Table 4.8. Percentage changes of arteriolar diameters from mesenteric arterioles to Ach in absence, or presence of topical application of vitamin C at veriouse doses (0.26, 2.6, and 26 mM)

Group	% Changes of arteriolar diameters
Ach	14.53 ± 1.43 (n = 13)
0.26 mM Vit.C -Ach	12.21± 1.54 ^{NS} (n = 9)
2.6 mM Vit.C-Ach	23.49 ± 3.03 $^{**, \#\#, ++}$ (n = 9)
26 mM Vit.C-Ach	13.47 ± 2.50 ^{NS} (n = 11)

Values are mean ± SEM

$^{**}p<0.01$, significantly difference compared to Ach

^{NS}, not significantly difference compared to Ach

$^{\#\#}p<0.01$, significantly difference compared to 0.26 mM Vit.C-Ach

$^{++}p<0.01$, significantly difference compared to 26 mM Vit.C-Ach

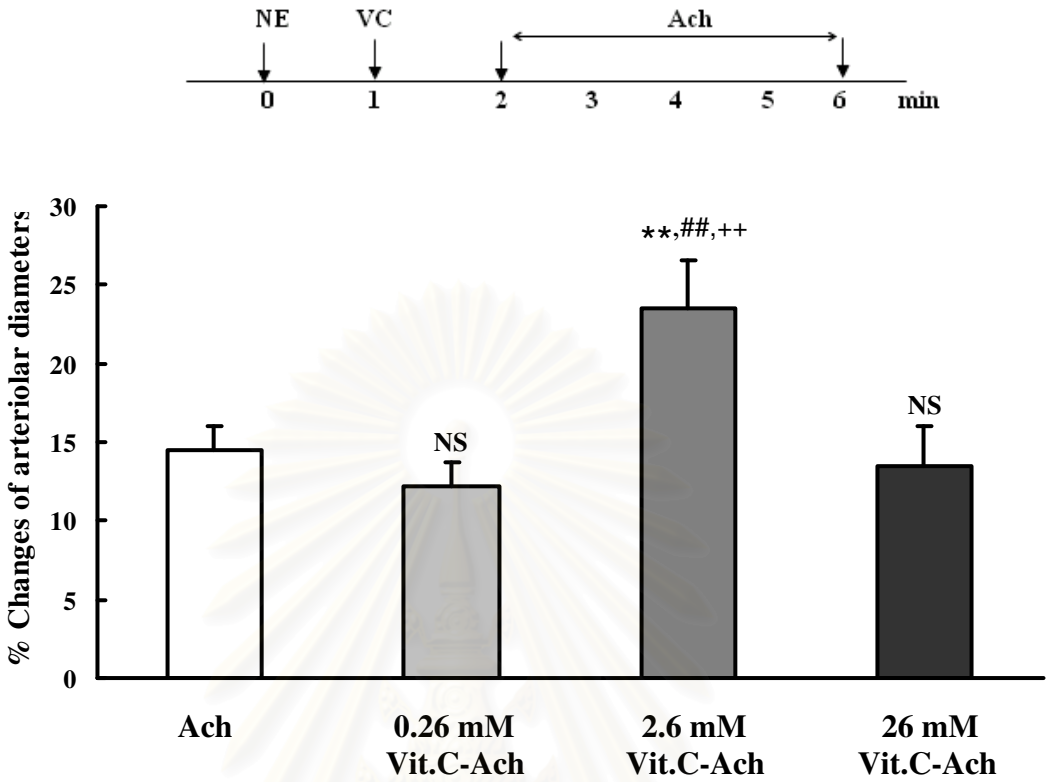


Figure 4.11. Dose-responses of the acute effects of topical application of vitamin C: on percentage changes of arteriolar diameters in mesenteric arterioles as compared to baseline values (Ach)

Values are mean ± SEM

$^{**}p<0.01$, significantly difference compared to Ach

^{NS}, not significantly difference compared to Ach

$^{\#\#}p<0.01$, significantly difference compared to 0.26 mM Vit.C-Ach

$^{++}p<0.01$, significantly difference compared to 26 mM Vit.C-Ach

- **Direct detection of NO production in acute vitamin C application**

In 6-wks DM the NO-associated fluorescent intensity in arteriolar wall was significantly reduced at 20 min in relation to 6-wks CON group (55.00 ± 10.39 % and 157.01 ± 30.82 %, respectively) (Table 4.9, Figure 4.12 and 4.13). Interestingly, 2.6 mM vitamin C-perfusion for 20 min in both DM and CON rats significantly enhanced the NO level compared to their groups. (CON+2.6 mM Vit.C= 235.43 ± 28.00 % and DM+2.6 mM Vit.C = 147.21 ± 21.98 %, respectively) (Table 4.9, Figure 4.12 and 4.13).

It is to be noted that vitamin C could enhance NO bioavailability even in the DM group—the condition of decreased NO bioavailability.

Table 4.9. Percentage changes of NO-associated fluorescent intensity from mesenteric arterioles among control (CON), diabetes (DM), control with 2.6 mM vitamin C-perfusion (CON + 2.6 mM Vit.C), and diabetes with 2.6 mM vitamin C-perfusion (DM + 2.6 mM Vit.C) at 6th week of experimental periods.

Group	**% Changes of NO-associated fluorescence intensity** (Ach perfusion)
6-wks CON	157.01 ± 30.82 (n = 5)
6-wks DM	55.00 ± 10.39 * (n = 5)
6-wks CON + 2.6mM Vit.C	235.43 ± 28.00 $^{\#\#\#, *, +}$ (n = 6)
6-wks DM+2.6mM Vit.C	147.21 ± 21.98 $^{\#\#\#, NS}$ (n = 5)

Values are mean ± SEM

$^{*}p<0.05$, significantly difference compared to 6-wks CON

^{NS}, not significantly difference compared to 6-wks CON

$^{\#\#\#}p<0.001$, significantly difference compared to 6-wks DM

$^{+}p<0.05$, significantly difference compared to 6-wks DM + 2.6 mM Vit.C

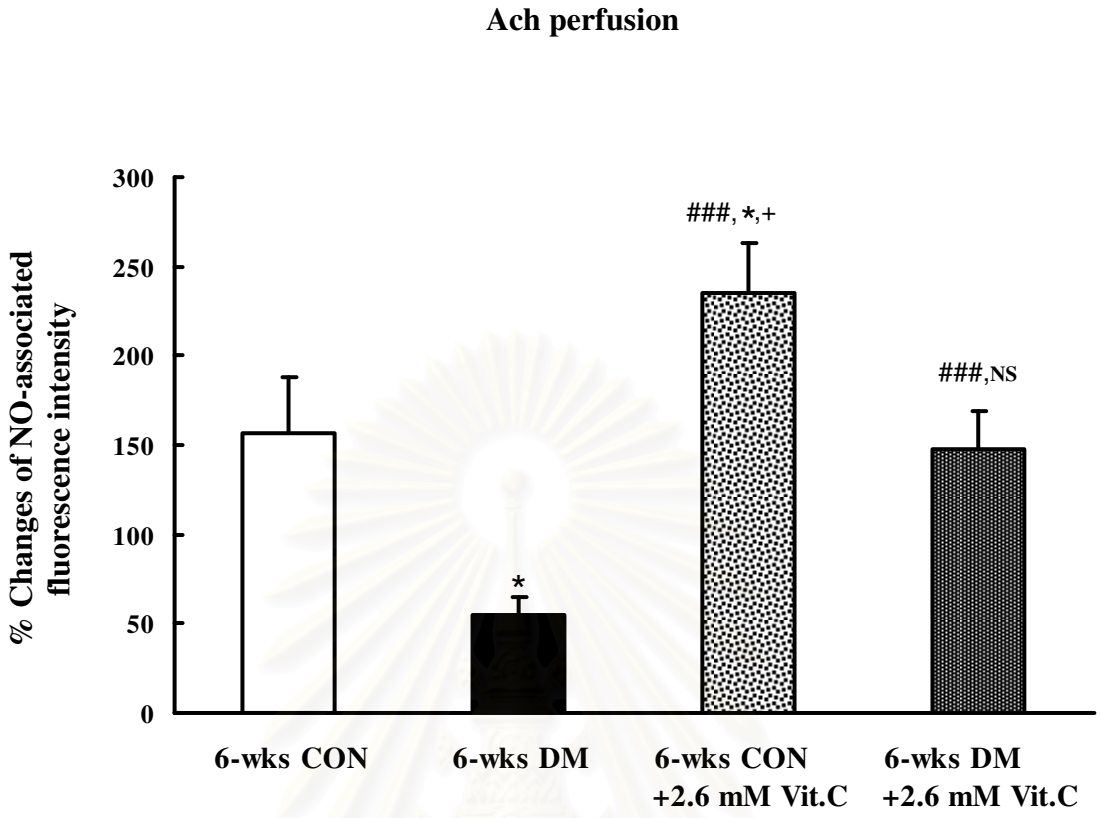


Figure 4.12. Effect of vitamin C administration on NO bioavailability: percentage changes of NO-associated fluorescent intensity from mesenteric arterioles among control (CON), diabetes (DM), control with 2.6 mM vitamin C-perfusion (CON + 2.6 mM Vit.C), and diabetes with 2.6 mM vitamin C-perfusion (DM + 2.6 mM Vit.C) at 6th week of experimental periods

Values are mean ± SEM

*p<0.05, significantly difference compared to 6-wks CON

^{NS}, not significantly difference compared to 6-wks CON

$^{###}p$<0.001, significantly difference compared to 6-wks DM

^{+}p<0.05, significantly difference compared to 6-wks DM + 2.6 mM Vit.C

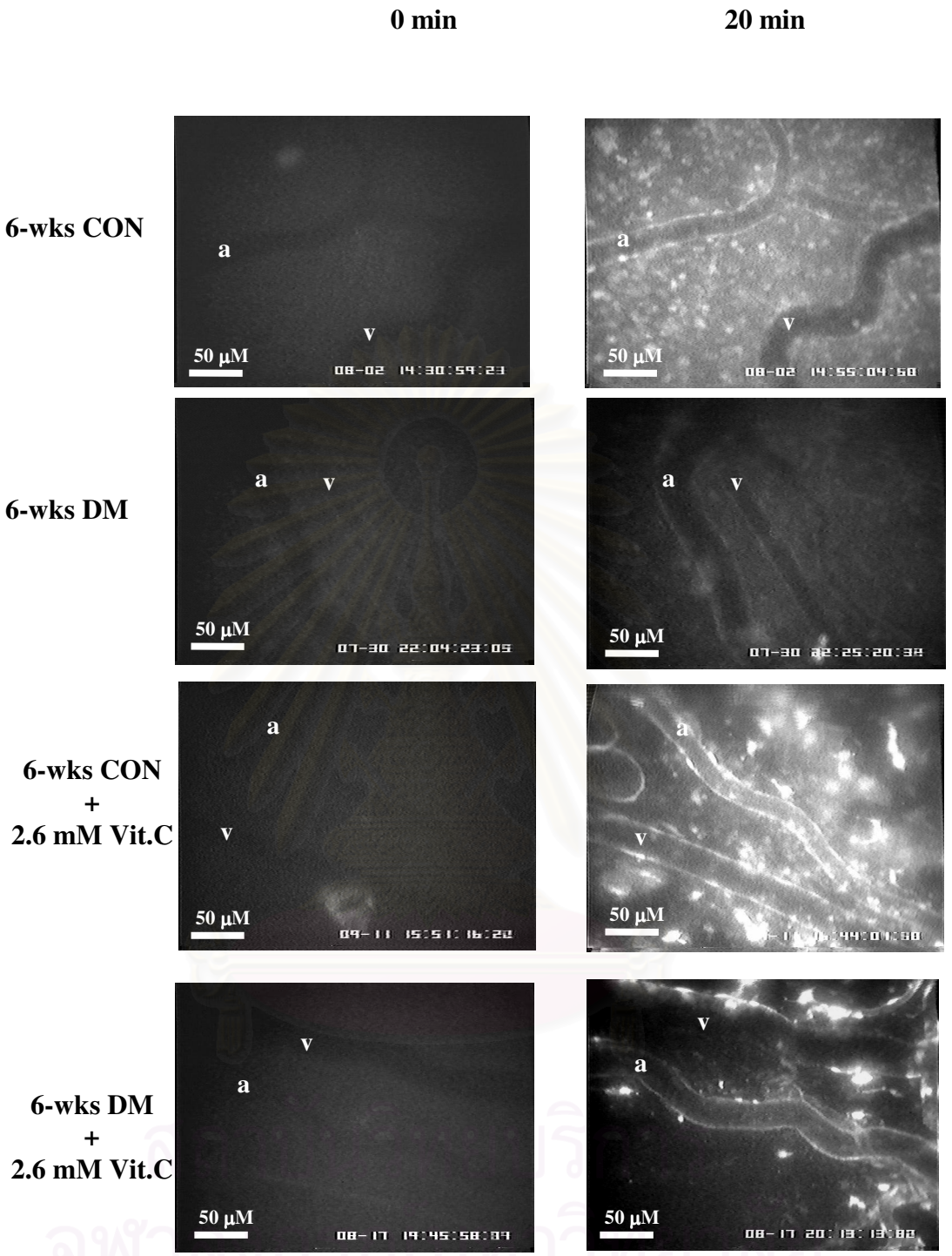


Figure 4.13. The intravital video-microscopic images showing spatial alteration in NO-associated fluorescence in mesenteric microcirculation before (as baseline, Right) and after 20 min perfusion of DAF-2DA, a NO sensitive fluorochrome (Left). The NO bioavailability was observed along arteriolar wall.
(a, arteriole; v, venule)

3) The underlying mechanism of vitamin C on preserving BH_4 bioavailability

: Role of BH_4 deficiency-mediated eNOS uncoupling

- **Measurement of fuctional NO bioavailability via Ach-induced vasodilation in acuted vitamin C application**

To examine the underlining mechanism of the effect of vitamin C on preserving BH_4 bioavailability, firstly, we attempted to conduct our experimental protocol *in vivo* to exhibit a BH_4 deficiency-mediated eNOS uncoupling model in 6-wks CON rats which assessed by measuring a functional NO bioavailability via the vascular diameter change in response to Ach-induced vasodilation in the mesenteric arteriole. Our result showed that topical accumulation with DAHP (20 mM; 6ml/30min as rate 5-min/1ml) significantly reduced Ach-induced vasodilation in CON in relation to their Vehicle group (DAHP-treated CON = 4.34 ± 0.78 % and Vehicle-treated CON = 13.22 ± 1.24 %, respectively) (Table 4.10, Figure 4.14).

As expected for positive control, treatment with BH_4 donor (6R-5,6,7,8-tetrahydro-L-biopterin dihydrochloride;6R-BH_4) should able to restore the uncoupled eNOS.

Table 4.10, Figure 4.14 showed that after 20 min treated DAHP, topical accumulation with BH_4 donor (5 min/1ml for 10 min) caused a dose-dependent vasodilatation in response to Ach in 6-wks CON as compare to their DAHP group (DAHP+ BH_4 0.1 mM-treated CON = 17.40 ± 1.12 %, DAHP+ BH_4 0.01 mM-treated CON = 10.38 ± 0.96 %, and DAHP+ BH_4 0.001 mM-treated CON = 7.72 ± 0.78 % and DAHP-treated CON = 4.34 ± 0.78 %, respectively).

To be noted that, NO-mediated endothelium-dependent vasodilation was abolished when BH_4 cofactor was not available for NO synthesis, as a result eNOS become dysfunction or call uncoupling.

Table 4.10. Percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4-inhibitor (DAHP, 20 mM) and DAHP plus BH_4 donor at different concentrations in control (CON) at 6th week of experimental periods

Group	% Changes of arteriolar diameters
	Ach testing
	6-wks CON
Vehicle	13.22 ± 1.24 (n = 8)
DAHP	4.34 ± 0.78 *** (n = 7)
DAHP + BH_4 0.1 mM	17.40 ± 1.12 $^{@@@}$ (n = 6)
DAHP + BH_4 0.01 mM	10.38 ± 0.96 $^{@@@}$ (n = 7)
DAHP + BH_4 0.001 mM	7.72 ± 0.78 $^{@}$ (n = 7)

Values are mean ± SEM

$^{***}p<0.001$, significantly difference compared to 6-wks vehicle

$^{@}p<0.05$, $^{@@@}p<0.001$, significantly difference compared to 6-wks DAHP

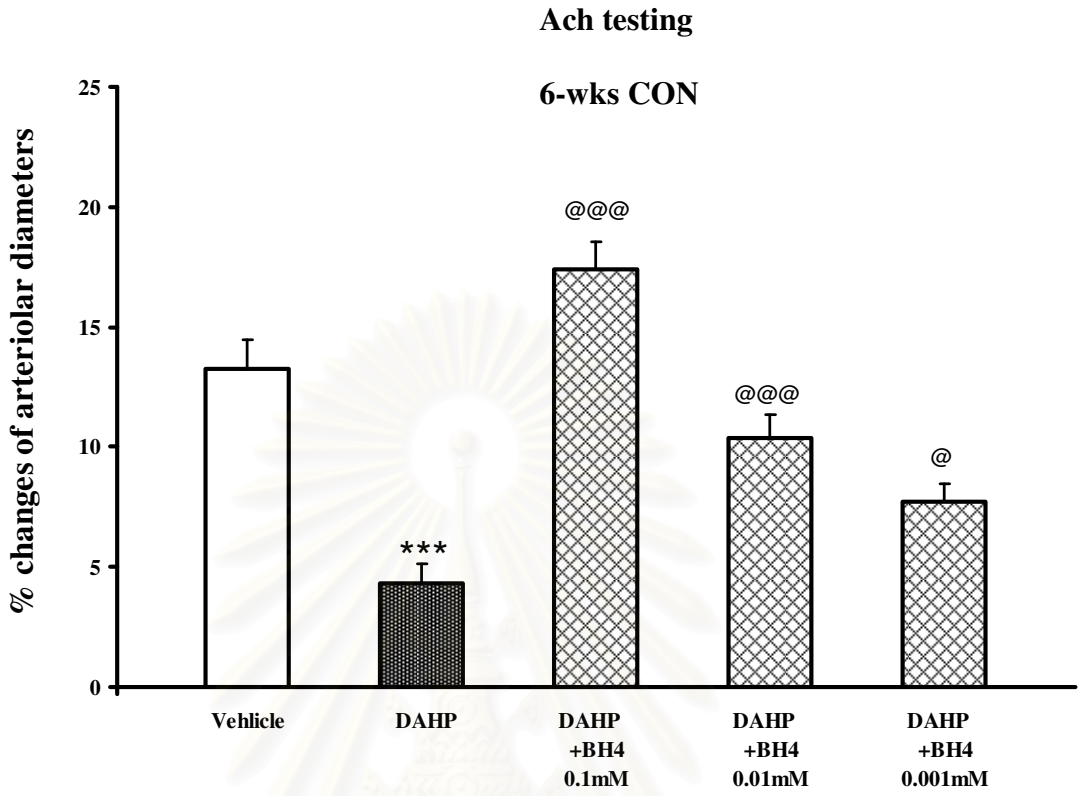


Figure 4.14. Role of BH_4 deficiency-mediated eNOS uncoupling: percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4-inhibitor (DAHP, 20 mM) and DAHP plus BH_4 dornor at different concentrations in control (CON) at 6^{th} week of experimental periods

Values are mean ± SEM

***$p<0.001$, significantly difference compared to 6-wks vehicle

$^{@}p<0.05$, $^{@@@}p<0.001$, significantly difference compared to 6-wks DAHP

: Effect of vitamin C to preserve BH_4 bioavailability-mediated eNOS coupling

- **Measurement of fuctional NO bioavailability via Ach-induced vasodilation in acuted vitamin C application**

Recent literature reported that vitamin C has been shown to increase intracellular BH_4 levels (Huang A, 2000) by mechanisms that are unclear at present. Vitamin C is believed to interfere with BH_4 oxidation and thereby maintain it in a reduced state (Toth M, et al., 2002). Base on these recent observations, we examined the effects of vitamin C on DAHP-mediated BH_4 depletion in 6-wks rats.

An off-line analysis of the vascular diameter changes in the mesenteric arteriole showed that a decreased BH_4 production by DAHP, a GTP-CH 1 inhibitor, significantly reduced Ach-induced vasodilation as compare to their Vehicle group (DAHP-treated CON = 4.34 ± 0.78 % , and Vehicle-treated CON = 13.22 ± 1.24 %, respectively) (Table 4.11, Figure 4.15). Interestingly, an abnormality in NO-mediated endothelium-dependent vasodilation in DAHP group was improved by treating with vitamin C, BH_4 and vitamin C in combination with BH_4 (DAHP+Vit.C-treated CON = 17.98 ± 1.42 %, DAHP+BH_4 0.01 mM-treated CON = 10.38 ± 0.96 %, DAHP+BH_4 0.1 mM-treated CON = 17.40 ± 1.12 %, DAHP+ BH_4 0.01 mM+Vit.C-treated CON = 17.52 ± 1.36 % and DAHP+BH_4 0.1 mM+Vit.C-treated CON = 22.10 ± 1.41 %, respectively).

Moreover, the vitamin C in combination with BH_4-treated group showed synergistic decreased reduced Ach-induced vasodilation as dose dependent maner of BH_4 compare with DAHP+BH_4 0.01 mM group (DAHP+ BH_4 0.01 mM+Vit.C-treated CON = 17.52 ± 1.36 %, DAHP+BH_4 0.1 mM+Vit.C-treated CON = 22.10 ± 1.41 % and DAHP+BH_4 0.01 mM-treated CON = 10.38 ± 0.96 %, respectively).

Beside, vitamin C alone could significantly enhance NO-mediated endothelium-dependent vasodilation from Vehicle group's baseline (Vit.C-treated CON = 22.44 ± 3.22 % and Veh-treated CON = 13.22 ± 1.24 %, respectively).

It is to be noted that vitamin C could preserve BH_4 bioavailability to mediate eNOS coupling as a result of the enhanced NO-mediated vascular reactivity.

Table 4.11. Percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4-inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in control (CON) at 6th week of experimental periods

Group	% Changes of arteriolar diameters
	Ach testing
	6-wks CON
Vehicle	13.22 ± 1.24 (n = 8)
Vit.C	22.44 ± 3.22 *** (n = 8)
DAHP	4.34 ± 0.78 *** (n = 7)
DAHP + Vit.C	17.98 ± 1.42 @@@ (n = 6)
DAHP + BH_4 0.01 mM	10.38 ± 0.96@ (n = 6)
DAHP + BH_4 0.1 mM	17.40 ± 1.12 @@@,++ (n = 6)
DAHP + BH_4 0.01 mM + Vit.C	17.52 ± 1.36 @@@, ++ (n = 6)
DAHP + BH_4 0.1 mM + Vit.C	22.10 ± 1.41@@@, +++ (n = 6)

Values are mean ± SEM

***p<0.001, significantly difference compared to 6-wks vehicle

@p<0.05, @@@p<0.001, significantly difference compared to 6-wks DAHP

++p<0.01, +++p<0.001, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

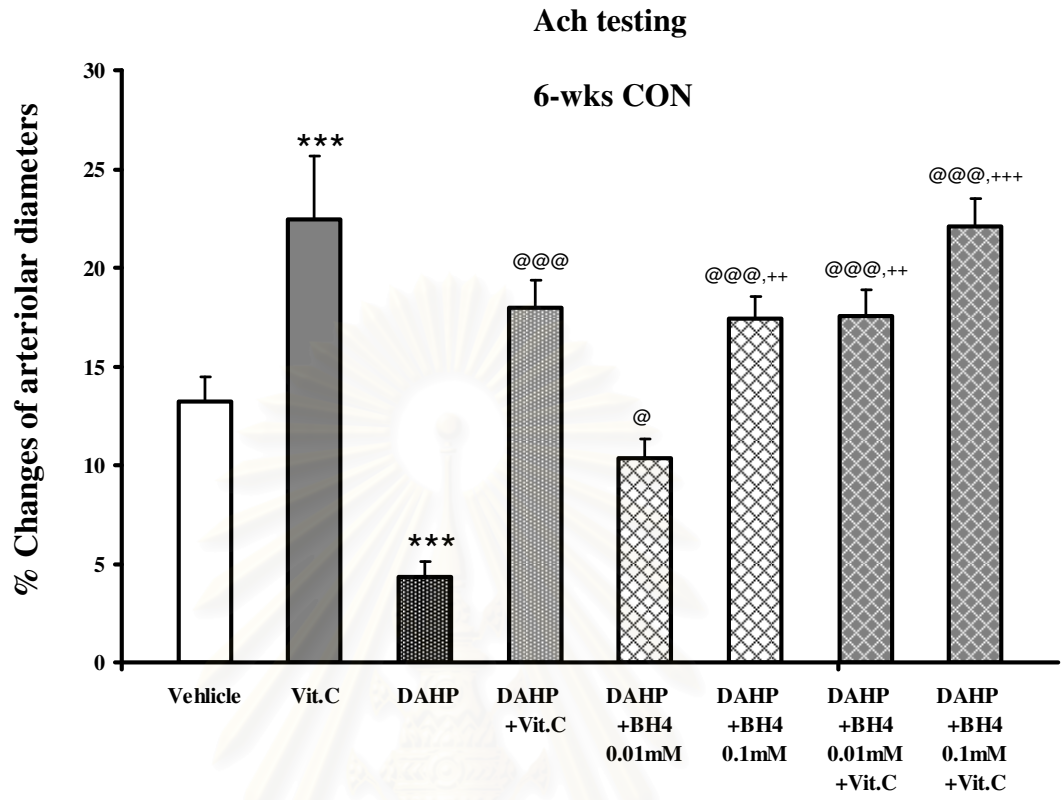


Figure 4.15. Effect of vitamin C to preserve BH_4 bioavailability-mediated eNOS coupling: percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4-inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in control (CON) at 6^{th} week of experimental periods

Values are mean ± SEM

***$p<0.001$, significantly difference compared to 6-wks vehicle

$^{@}p<0.05$, $^{@@@}p<0.001$, significantly difference compared to 6-wks DAHP

$^{++}p<0.01$, $^{+++}p<0.001$, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

- **Direct detection of ROS contents in acuted vitamin C application**

Table 4.12, Figure 4.16 and 4.17 indicated that the 5-min H_2O_2-associated fluorescence was significantly increased when BH_4 synthesis was blocked by DAHP in relation to vehicle-treated group (DAHP-treated CON = 169.61 ± 6.46 % and Vehicle-treated CON = 54.48 ± 14.90 %, respectively). Whereas the enhance of H_2O_2 contents could be altered by treating with vitamin C, BH_4 and vitamin C in combination with BH_4 groups (DAHP+Vit.C-treated CON = 115.66 ± 10.96 %, DAHP+BH_4 0.1 mM-treated CON = 63.79 ± 2.85 % and DAHP+BH_4 0.1 mM+Vit.C-treated CON = 33.89 ± 7.62 %, respectively).

Especially, the vitamin C in combination with BH_4-treated group showed synergistic decreased H_2O_2 contents as compare to both CON groups treated with vitamin C or BH_4 alone (DAHP+BH_4 0.1 mM+Vit.C-treated CON = 33.89 ± 7.62 %, DAHP+Vit.C-treated CON = 115.66 ± 10.96 %, and DAHP+BH_4 0.1 mM-treated CON = 63.79 ± 2.85 %, respectively).

Beside, vitamin C alone could significantly decrease a slight increase of H_2O_2 contents in Vehicle group (Vit.C-treated CON = 7.33 ± 1.97 % and Veh-treated CON = 54.48 ± 14.90 %, respectively).

It is to be noted that vitamin C could preserve BH_4 bioavailability to mediate eNOS coupling as a result of the depletion of the ROS contents.

Table 4.12. Percentage changes of H_2O_2-associated fluorescent intensity from mesenteric arterioles in the presence or absence of BH_4-inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in control (CON) at 6th week of experimental periods.

Group	% Changes of H_2O_2-associated fluorescent intensity Ach testing
	6-wks CON
Vehicle	54.48 ± 14.90 (n = 4)
Vit.C	7.33 ± 1.97 *** (n = 6)
DAHP	169.61 ± 6.46 *** (n = 4)
DAHP + Vit.C	115.66 ± 10.96 @@@ (n = 3)
DAHP + BH_4 0.1 mM	63.79 ± 2.85 @@@ (n = 3)
DAHP + BH_4 0.1 mM + Vit.C	33.89 ± 7.62 @@@, &&&, + (n = 3)

Values are mean ± SEM

***p<0.001, significantly difference compared to 6-wks vehicle

@p<0.05, @@@p<0.001, significantly difference compared to 6-wks DAHP

&&& p<0.001, significantly difference compared to 6-wks DAHP+Vit.C

+p<0.05, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

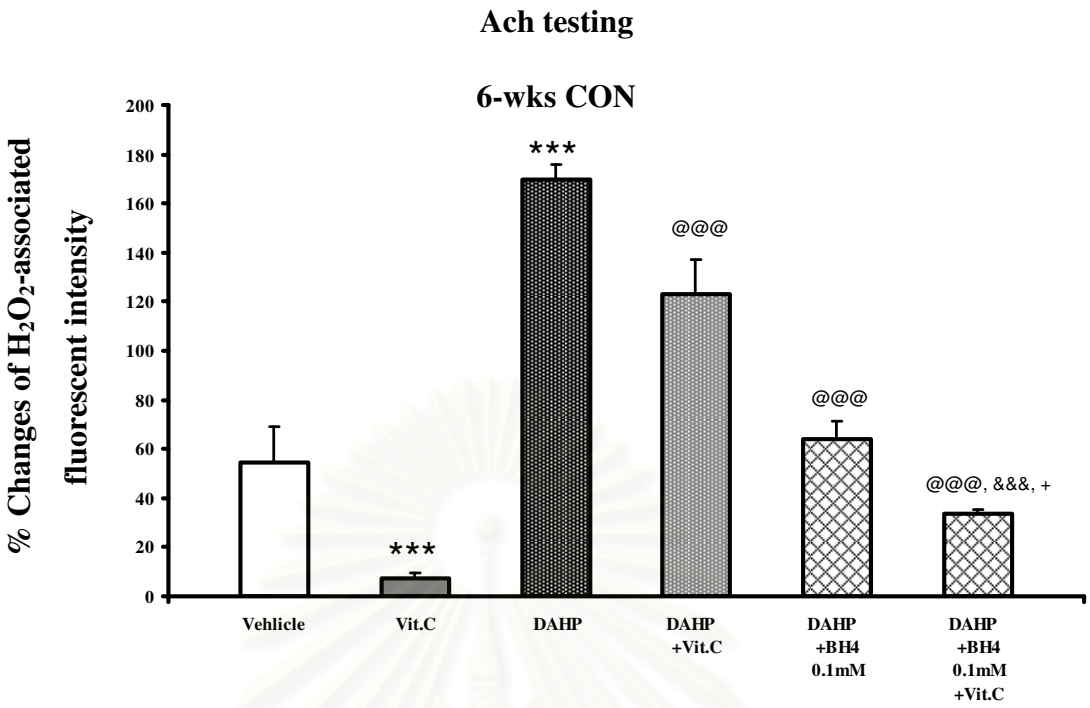


Figure 4.16. Effect of vitamin C to preserve BH_4 bioavailability-mediated eNOS coupling: percentage changes of H_2O_2-associated fluorescent intensity from mesenteric arterioles in the presence or absence of BH_4-inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in control (CON) at 6th week of experimental periods.

Values are mean ± SEM

***p<0.001, significantly difference compared to 6-wks vehicle

$^{@}p$<0.05, $^{@@@}p$<0.001, significantly difference compared to 6-wks DAHP

$^{\&\&\&}$ p<0.001, significantly difference compared to 6-wks DAHP+Vit.C

^{+}p<0.05, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

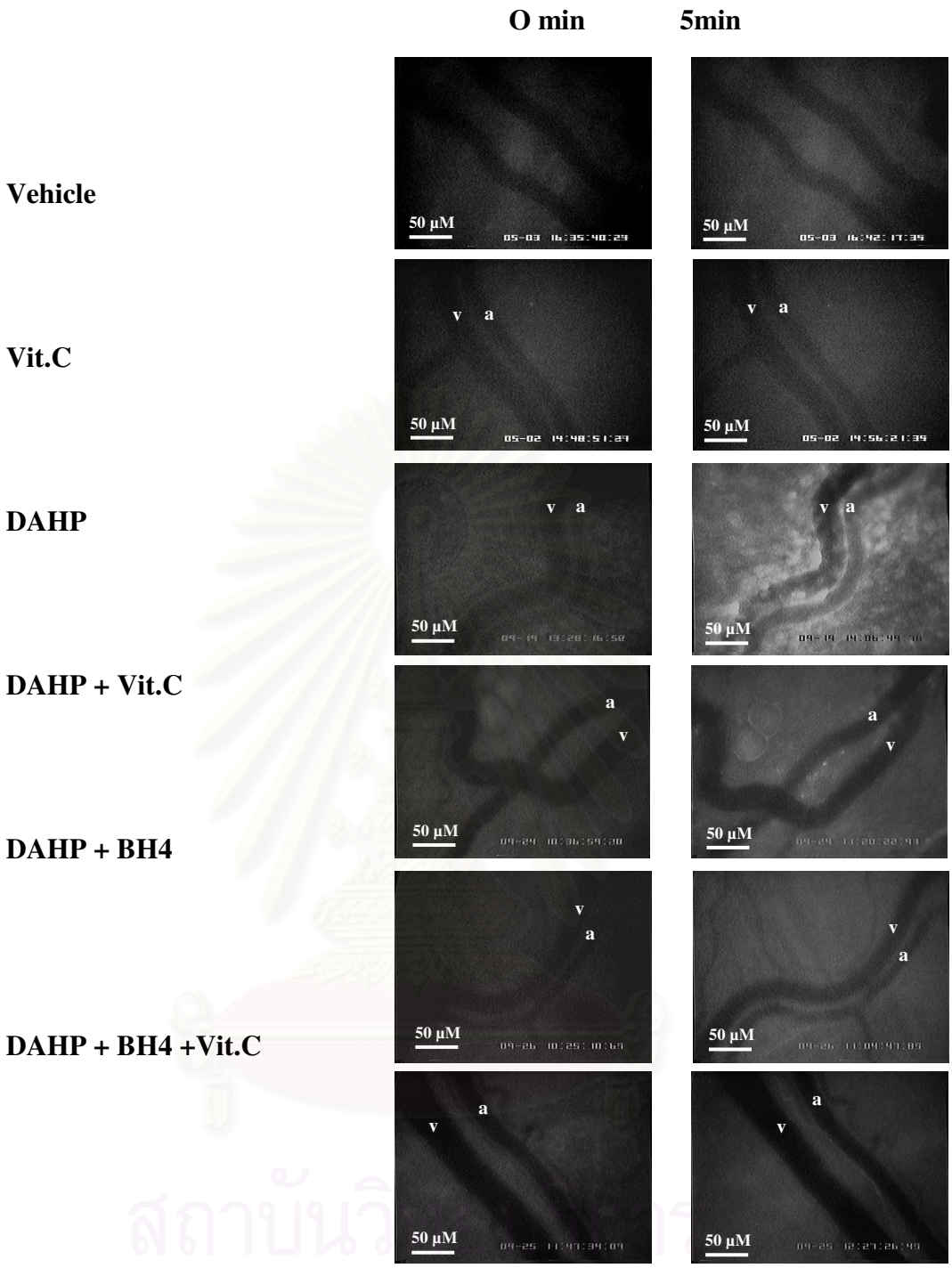


Figure 4.17. The intravital video-microscopic images showing spatial alteration in H_2O_2-associated fluorescence in mesenteric microcirculation before (as baseline, Right) and after 1 min topical application of DHR 123, a H_2O_2 sensitive fluorochrome (Left). The ROS generations were observed along arteriolar wall.
(a, arteriole; v, venule)

: Role of hyperglycemia-mediated eNOS uncoupling through BH_4 deficiency

- **Measurement of fuctional NO bioavailability via Ach-induced vasodilation in acuted vitamin C application**

As our previous results suggested that altered NO-mediated endothelium-dependent vasodilation seen in STZ-induced type 1diabetic rats can be reversed by vitamin C. Therefore, we further intended to conduct a hyperglycemia-mediated eNOS uncoupling through BH_4 deficiency model in 6 week DM rats before observing the underlying mechanism of the effect of vitamin C on BH_4 bioavialability.

We found that a decreased BH_4 production by topical accumulation with DAHP (20 mM; 6ml/30min as rate 5-min/1ml) significantly reduced Ach-induced vasodilation in DM compared with their vehicle group (DAHP-treated DM = 3.81 ± 0.60 % and Vehicle-treated DM = 7.69 ± 0.87 %, respectively) (Table 4-12, Figure 4-16).

Table 4.12, Figure 4.16 showed that after 20 min treated DAHP, topical accumulation with BH_4 donor (5 min/1ml for 10 min) caused a dose-dependent vasodilatation in response to Ach in DM as compare to their DAHP group (DAHP+ BH_4 0.1 mM-treated DM = 12.48 ± 1.19 %, and DAHP+BH_4 0.01 mM-treated DM = 7.22 ± 0.73 %, and DAHP-treated DM = 3.81 ± 0.60 %, respectively). However, except the dose of 0.001 mM BH_4 in DM showed unchanged Ach-induced vasodilaition (DAHP+ BH_4 0.001 mM-treated DM = 4.64 ± 0.42 %) compared with their DAHP group.

To be noted that especially in diabetic condition when BH_4 cofactor was not available for NO synthesis, NO-mediated endothelium-dependent vasodilation was even worse as a result of hyperglycemia-mediated eNOS uncoupling.

Table 4.13. Percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4-inhibitor (DAHP, 20 mM) and DAHP plus BH_4 donor at different concentrations in diabetes (DM) at 6th week of experimental periods.

Group	% Changes of arteriolar diameters
	Ach testing
	6-wks DM
Vehicle	7.69 ± 0.87 (n = 7)
DAHP	3.81 ± 0.60 $^{\#\#}$ (n = 6)
DAHP + BH_4 0.1 mM	12.48 ± 1.19 $^{+++}$ (n = 6)
DAHP + BH_4 0.01 mM	7.22 ± 0.73 $^{+}$ (n = 7)
DAHP + BH_4 0.001 mM	4.64 ± 0.42 (n = 7)

Values are mean ± SEM

$^{\#\#}p<0.01$, significantly difference compared to 6-wks vehicle

$^{+}$ $p<0.05$, $^{+++}$ $p<0.001$, significantly difference compared 6-wks DAHP

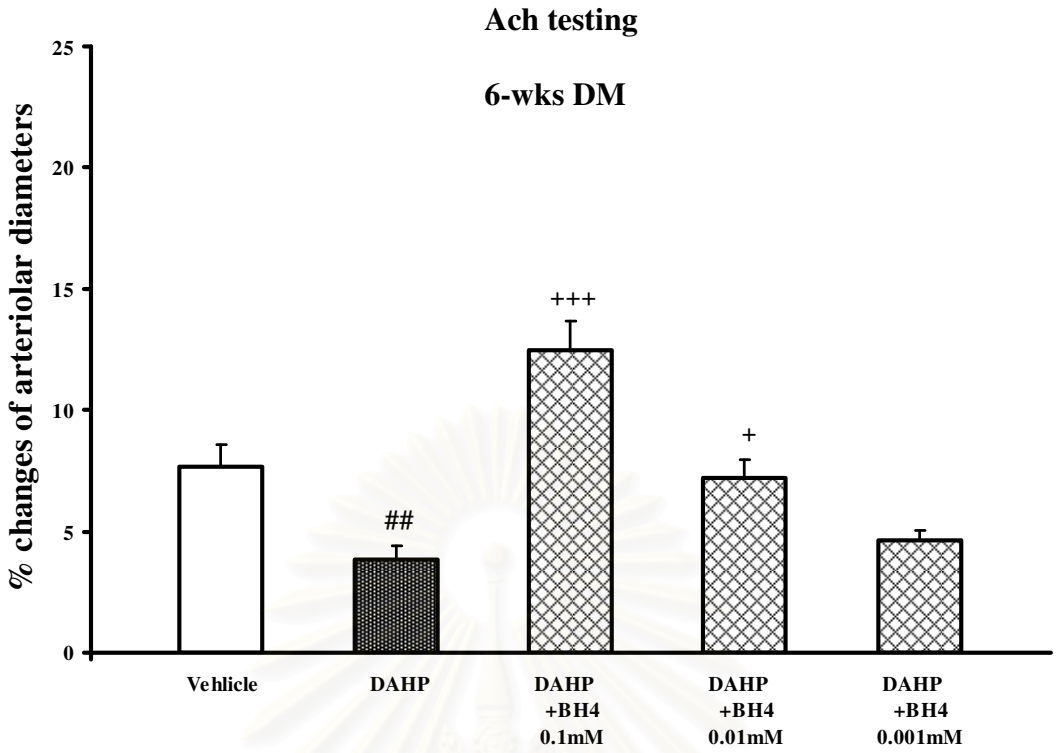


Figure 4.18. Role of hyperglycemia-mediated eNOS uncoupling through BH_4 deficiency: percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4 synthetic inhibitor (DAHP, 20 mM) and DAHP plus BH_4 dornor at different concentrations in diabetes (DM) at 6^{th} week of experimental periods

Values are mean ± SEM

$^{##}p<0.01$, significantly difference compared to 6-wks vehicle

$^{+}p<0.05$, $^{+++}p<0.001$, significantly difference compared to 6-wks DAHP

: Effect of vitamin C to preserve BH_4 bioavailability restores hyperglycemia-mediated eNOS uncoupling

- **Measurement of fuctional NO bioavailability via Ach-induced vasodilation in acuted vitamin C application**

As our previous results (Table 4.6 and Figure 4.8), it is demonstrated that abnormalities in NO-mediated endothelium-dependent vasodilation in STZ-induced diabetic rats are reversed with vitamin C. After we could conduct the hyperglycemia mediated-eNOS uncoupling, we, therefore, intented further to examine the underlying mechanism of the effect of vitamin C on BH_4 bioavialability in 6 week DM rats.

We found that a decreased BH_4 production by DAHP significantly reduced Ach-induced vasodilation as compare to their Vehicle group (DAHP-treated DM = 3.81 ± 0.60 % , and Vehicle-treated DM = 7.69 ± 0.88 %, respectively) (Table 4-14, Figure 4-19). Vitamin C, BH_4, and vitamin C in combination with BH_4 groups could significantly alter an abnormality in NO-mediated endothelium-dependent vasodilation in DAHP group (DAHP+Vit.C-treated DM = 11.30 ± 1.34 %, DAHP+BH_4 0.01 mM-treated DM = 7.22 ± 0.73 %, DAHP+BH_4 0.1 mM-treated DM = 12.48 ± 1.19 % , DAHP+ BH_4 0.01 mM+Vit.C-treated DM = 13.61 ± 0.95 % and DAHP+BH_4 0.1 mM+Vit.C-treated DM = 19.07 ± 2.34 %, respectively).

The group of vitamin C in combination with low dose BH_4, 0.01 mM, showed two-time increase in Ach-induced vasodilation compare with DAHP+BH_4 0.01 mM group (DAHP+ BH_4 0.01 mM+Vit.C-treated DM = 13.61 ± 0.95 % and DAHP+BH_4 0.01 mM-treated DM = 7.22 ± 0.73 %, respectively). Moreover, vitamin C in combination with high dose BH_4, 0.1 mM, group significantly enhanced Ach-induced vasodilation in relation to DAHP groups treated- vitamin C or BH_4 alone (DAHP+BH_4 0.1 mM+Vit.C-treated DM = 19.07 ± 2.34 %, DAHP+Vit.C-treated DM = 11.30 ± 1.34 %, DAHP+BH_4 0.01 mM-treated DM = 7.22 ± 0.73 % and DAHP+BH_4 0.1 mM-treated DM = 12.48 ± 1.19 %, respectively).

Beside, vitamin C alone could significantly enhance NO-mediated endothelium-dependent vasodilation from Vehicle group's baseline (Vit.C-treated DM = 15.05 ± 3.22 % and Veh-treated DM = 7.69 ± 0.88 %, respectively).

It is to be noted that vitamin C could preserve BH_4 bioavailability in the condition of hyperglycemia-mediated eNOS uncoupling as a result of the enhanced enhance NO-mediated endothelium-dependent vasodilation.

Table 4.14. Percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4 synthetic inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in diabetes (DM) at 6th week of experimental periods.

Group	% Changes of arteriolar diameters Ach testing
	6-wks DM
Vehicle	7.69 ± 0.88 (n = 7)
Vit.C	15.05 ± 3.22 # (n = 5)
DAHP	3.81 ± 0.60 ## (n = 6)
DAHP + Vit.C	11.30 ± 1.34 +++ (n = 6)
DAHP + BH_4 0.01 mM	7.22 ± 0.73 + (n = 7)
DAHP + BH_4 0.1 mM	12.48 ± 1.19 +++ (n = 6)
DAHP + BH_4 0.01 mM + Vit.C	13.61 ± 0.95 +++, bbb (n = 6)
DAHP + BH_4 0.1 mM + Vit.C	19.07 ± 2.34 +++, aaa, bbb, ccc,dd (n = 6)

Values are mean ± SEM

#$p<0.05$,##$p<0.01$, significantly difference compared to 6-wks vehicle

+$p<0.05$, +++$p<0.001$, significantly difference compared to 6-wks DAHP

aaa$p<0.001$, significantly difference compared to 6-wks DAHP+Vit.C

bbb$p<0.001$, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

ccc$p<0.001$, significantly difference compared to 6-wks DAHP+BH_4 0.1 mM

dd$p<0.01$, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM+Vit.C

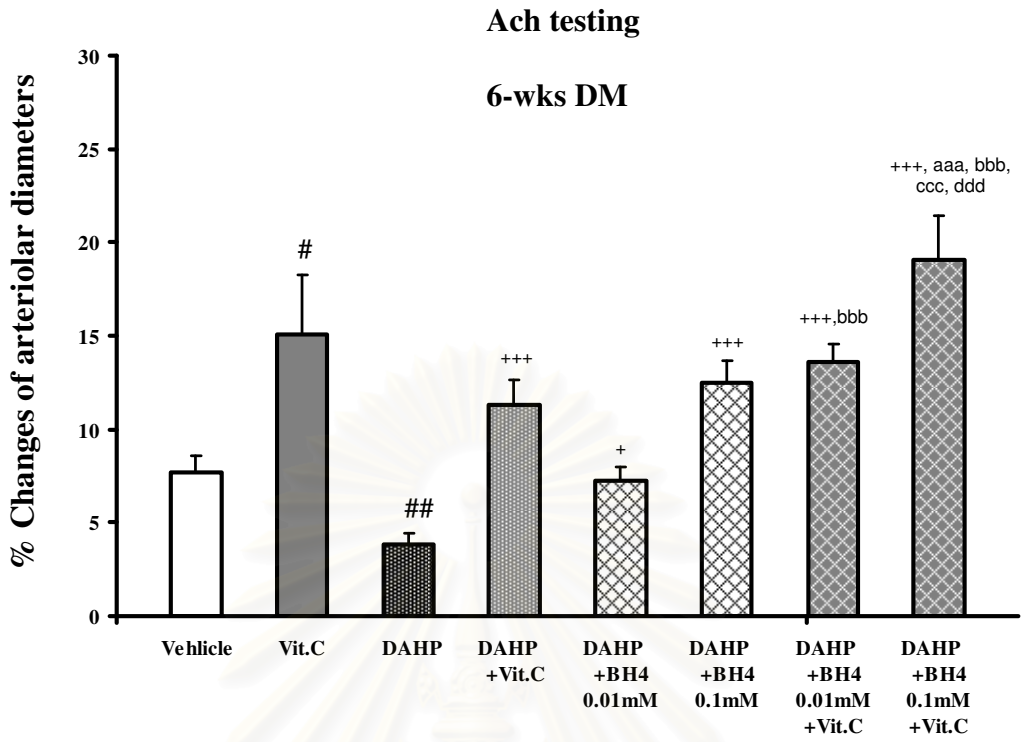


Figure 4.19. Effect of vitamin C to preserve BH_4 bioavailability restores hyperglycemia-mediated eNOS uncoupling: percentage changes of arteriolar diameters from mesenteric arterioles in response to Ach in the presence or absence of BH_4 synthetic inhibitor (DAHP, 20 mM), vitamin C (2.6 mM) application, DAHP plus vitamin C, and DAHP plus BH_4 donor (0.1 or 0.01 mM) in combination of vitamin C in diabetes (DM) at 6th week of experimental periods

Values are mean ± SEM

#p<0.05, *##p*<0.01, significantly difference compared to 6-wks vehicle

^{+}p<0.05, ^{+++}p<0.001, significantly difference compared to 6-wks DAHP

^{aaa}p<0.001, significantly difference compared to 6-wks DAHP+Vit.C

^{bbb}p<0.001, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM

^{ccc}p<0.001, significantly difference compared to 6-wks DAHP+BH_4 0.1 mM

^{dd}p<0.01, significantly difference compared to 6-wks DAHP+BH_4 0.01 mM+Vit.C

CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to elucidate the effects of vitamin C supplementation whether it could reverse diabetic-induced endothelial dysfunction and its roles in reactive oxygen species (ROS), and bioavailability of nitric oxide (NO), and tetrahydrobiopterin (BH_4) in diabetic rat model. Discussion is expressed in relation to the heading topics as following:

The STZ - Rat Model: *Biochemical and Physiological Parameters*

The results indicated that the intravenous injection of streptozotocin (STZ; 50 mg/kg.bw, *iv.*) caused pancreatic islet cells to damage, resulting in hypoinsulinemia and hyperglycemia within 48 hours. The hyperglycemia maintained throughout the experimental periods, 6^{th} week and 12^{th} week. Our data indicated that blood glucose levels are increased to $\geq$3-fold and $\geq$4-fold in 6-wks and 12-wks DM rats, respectively, as compared to those in CON rats. Beside, the values of glycosylated hemoglobin (HbA_{1C}) in 6-wks and 12-wks DM (10.03±0.17 and 10.86±0.24 %, respectively) were significantly above their age-match controls (5.58±0.52 and 3.68±0.54%, respectively) ($p<0.001$).

Similar to human hemoglobin, the value of HbA_{1C} reflects the hyperglycemia better than blood glucose. The normal range of human total %hemoglobin is 5.80-7.20% (Raj *et al.,* 2000). Glycosylation is a nonenzymatic reaction that occurs with a rate directly proportionate to the concentration of glucose in the blood. In the normal range of blood glucose concentrations (approximately 80-140 mg/dl after an overnight fast), 6 to 7% of the human hemoglobin is glycosylated to form HbA_{1C} (Raj *et al.,* 2000; Smith *et al*., 2005). Hemoglobin turnover into the blood as red blood cells are phagocytosed and their hemoglobin degraded and new red blood cells are derived from reticulocytes. The average lifespan of human red blood cell is 120 days. Thus the extent of HbA_{1C} is a direct reflection of the average blood glucose concentration to which the cell has been exposed over its 120-days lifespan. Therefore, the value of

HbA_{1C} is a better indicator for hyperglycemic control than intermediate blood glucose level.

Beside in severe hyperglycemia, the DM rats also showed the abrupt appearance of polyphagia, polydipsia, polyuria and weight loss. In diabetes, the hyperosmolarity in urine was enhanced by large amounts of secreted glucose and ketone bodies. Therefore, the increased urinary water volume (polyuria) was induced by this condition of hyperosmotic diuresis. Regard to this polyuria, dehydration and the hyperosmolarity of the body fluids are obtained and they can further activate the osmoreceptors at the thirst centers in brain causing polydipsia (Smith *et al*., 2005). With insulin deficiency, the scales swing from insulin-promoted anabolism to catabolism of proteins and fats which tends to induce a negative energy balance, and which, in turn, leads to excessive huger (polyphagia) (Cotran. 1999). These symptoms are accompanied by fatique, weight loss and muscle weakness (Champe *et al*., 2005). According to body weight loss, our data also indicated for a significant difference in body weight between DM (183±8 g) and CON rats (426±4 g) ($p<0.001$) with the range similar to the previous reports (Jariyapongskul *et al*., 2002; Chakraphan *et al*., 2002 and 2005; Sridulyakul *et al*., 2003 and 2004).

By using invasive measurement (carotid arterial cannulae), our data showed that mean arterial blood pressure (MAP) was significantly elevated in both 6-wks and 12-wks DM rats (108±5 and 114±3 mmHg, respectively) compared to their CON (82±4 and 97±7 mmHg, respectively) ($p<0.05$ and $p<0.001$). In addition, at both 6-wks and 12-wks DM rats, systolic blood pressure (SBP) (130±7 and 136±5 mmHg, respectively) and diastolic blood pressure (DBP) (99±4 and 103±6 mmHg, respectively) were significantly higher than age-match CON (SBP; 100±6 and 116±4 mmHg, DBP; 80±4 and 88±6 mmHg) ($p<0.05$ and $p<0.001$). These findings indicated that the enhanced blood pressure in DM rats should be involved through the increase in total peripheral resistance (TPR). Thereby, the increased TPR can impair vascular endothelial function as shown by our results of an altered vasoreactivity; the mesenteric arteries from 6-wks and 12-wks DM rats exhibited a significantly reduced Ach-induced endothelium-dependent vasodilation (6.40±0.79 and 4.72±1.51%, respectively) ($p<0.01$ and $p<0.001$), which will be described in the following session. Following Lindsay *et. al.,* 1997, they found that the inhibition of NO synthesis using N^{ω}-nitro-L-arginine methyl ester (L-NAME) in spontaneously diabetic BB rats

increased MAP as which indicating that abnormality of vascular endothelial function plays an important role to elevate arterial blood pressure. In agreement with our previous studies, we reported that eNOS protein levels in heart homogenate from DM rats was significantly decreased compared with those from CON rats (Sridulyakul *et al*., 2003 and 2004). As such results, it can be described that the relative role of NO bioavailability could account for the development of hypertension at the early phase of diabetic rats. Therefore, the impaired vascular endothelial function which involved NO bioavailability would be increased TPR, which, in turn, lead to increase MAP.

Vitamin C and STZ-Rats

Why plasma vitamin C is decreased in diabetes?

Interestingly, we found that significant amounts of plasma vitamin C were significantly declined in DM rats at 6-wks and 12-wks (0.60 $\pm$ 0.03 and 0.62$\pm$0.02 mg/dl, respectively) *compared to those CON rats* (1.33 $\pm$ 0.20 and 1.30$\pm$0.15 mg/dl, respectively) ($p<0.001$). In agreement with previous studies, they have been reported that vitamin C levels were significantly lower in plasma and tissues of diabetic animals and humans than normal (Will and Byer 1996; Kashiwaki *et al*, 2000 and 2002; Chakraphan. 2002; Jariyapongsakul *et al*., 2002; Sridulyakul *et al*., 2004).

Although the mechanisms for this evidence has not been fully understood, several studies found that vitamin C status depends on the interactions of dietary vitamin C intake, plasma insulin concentrations, glucose and renal reabsorbtion (Cunningham.1998; Will and Byer 1996). The cellular uptake of vitamin C (as from plasma) can occur by two mechanisms. Firstly, an active transport of vitamin C is documented (Moser U. 1986; Will and Byer. 1996).With regard to this active transport, insulin has been shown to accelerate vitamin C clearance from plasma, and presumably into cells since there will be no increase in urinary vitamin C excretion (Sherry and Ralli. 1948). The previous observation of a clear late-phase insulin release following an intravenous infusion of vitamin C could explain the variability seen in tissue vitamin C stores in IDDM (Kodama *e .al*., 1993). Secondly, hyperglycemia has been shown to inhibit vitamin C transport. The inhibition of vitamin C uptake by hyperglycemia was demonstrated *in vitro* in the absence of insulin and may not, therefore, be important in normal physiology. The glucose transport system, especially GLUT-1, also transports the minor oxidized and

uncharged species, dehydro ascorbic acid (DHA) with a subsequent intracellular reduction to vitamin C. Earlier studies showed that DHA competes for the glucose transport on an equimolar basis with the transport surrogates 2-deoxy-glucose and 3-Omethyl-glucose or further downregulates the V_{max} of the uptake mechanism by chronic exposure of cell to high glucose levels. (Dai and McNeill, 1995; Siman. 1997; Stankova *et al*., 1984).

For the decrease plasma vitamin C levels, it can be explained by other several factors, including the impaired hepatic and renal regeneration, increased urinary excretion as well as impaired hepatic biosynthesis of vitamin C in diabetic rats (Kashiba. 2002; Will and Byer. 1996).

Effects of vitamin C supplementation and biochemical and physiological parameters in STZ-rats

As the results obtained form DM+Vit.C_{6wks} and DM+Vit.C_{day2} groups, it demonstrated that supplementation with vitamin C could reduce the increased in MAP (95±7 and 93±7 mmHg, respectively) ($p<0.001$). It should be noted that vitamin C supplementation was able to prevent hypertension even after endothelial dysfunction had been occurred almost of 6 weeks. With regard to the study from Salonen *et al*.1988, they found the positive association between plasma vitamin C and serum 6-ketoprostaglandin-$F_{e1\alpha}$. Base on their report, it should be assumed that the increase in plasma vitamin C could enhance the prostacyclin synthesis. On the other hand, our obtain data from leukocyte adhesion and Ach-induced endothelium-dependent vasodilation, led us to assume that supplementation of vitamin C could enhance NO bioavailability as described in the following session. With our point of view, we believed that the ability of vitamin C could improve NO bioavailability directly, so, as a consequence, it could improve MAP that was exhibited in DM rats.

However, supplementation of vitamin C did not show any statistic significant difference in the level of blood glucose (DM+Vit.C_{6wks};408±29 mg/dl and DM+Vit.C_{day2}; 380±19 mg/dl), or HbA_{1c} (DM+Vit.C_{6wks};10.34±0.35 % and DM+Vit.C_{day2}; 9.81±0.30 %) as compared to their DM (418±17 mg/dl and 10.86±0.24 %, respectively) in consistent with the previous results taken at the same experimental periods (12-wks) from Jariyapongskul *et al*.,2002.

To study the effect of vitamin C supplementation, we previously gave supplemented vitamin C solution *ad libitum* to the rats at the concentration of 1 g/ L (Chakraphan., 2002; Dai and McNeill., 1995; Jariyapongsakul *et al*., 2002). The results provided an evidence that this amount of vitamin C used in our study was sufficient for sustained plasma vitamin C concentration in both groups of DM rats as compared to the control (DM +$VitC_{6wks}$ = 1.27 ± 0.04 mg/dl, DM +$VitC_{day2}$ = 0.99 ± 0.03mg/dl, and CON = 1.30 ± 0.15 mg/dl, respectively)(not significantly difference) (Table 4.4, Figure 4.5).

Therefore, our findings from the 1st protocol provided evidence that delayed vitamin C supplementation could reverse diabetes-induced endothelial dysfunction in the rat mesentery in addition to its therapeutic use. Moreover, we also confirmed the preventive effect of vitamin C on diabetes-induced endothelial dysfunction as well.

Such the exact mechanisms underling the effect of vitamin C to improved endothelial dysfunction in diabetic rats will be discussed in the following session.

Diabetes-Induced Endothelial Dysfunction in STZ-Rats

In this study, the diabetes-induced endothelial dysfunction was characterized by number of leukocytes adherence and endothelial-dependent vasodilation.

- *Leukocyte and endothelial cell interaction*

Our results demonstrated that in 6-wks and 12-wks DM, the numbers of leukocytes adherence increased to 6.97 ± 0.84 and 9.17 ± 1.45 cells/100 μm vessel length, respectively, which were significantly higher than their age-match CON (0.92 ± 0.29 and 2.00 ± 0.50 cells/100 μm) ($p<0.001$). From the results of the leukocytes adherence study, it implied that leukocyte adhesion increased in associated with diabetes progression. Moreover, the increased number of leukocyte rolling was also reported by the previous studies as well (Schaffler *et al*., 1998; Booth *et al*., 2001). It is well known that the sequence of leukocyte-endothelial interaction could be described into three steps as shown in Figure5.1

In the first step, leukocyte rolling is activated by the expression of selectins. The rolling state is then progressed to firming state of leukocyte adherence by the

expression of adhesion molecules i.e. intracellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) (Packer L et al., 2000). Interestingly, the enhanced expression of ICAM-1 and P-selectin has been reported in diabetic human retina and choroids (Packer L et al., 2000). This diabetes- induced adhesion molecules expression was further supported by several studies. For instance, Morigi et al., 1998, found that glucose-induced leukocyte adhesion was dependent upon the up-regulation of adhesion molecules including, E-selectin, ICAM-1 and VCAM-1. The up-regulation of ICAM-1 gene had been found in human endothelial cells exposure to high glucose concentration (25 mM) (Chettab et al., 2002).

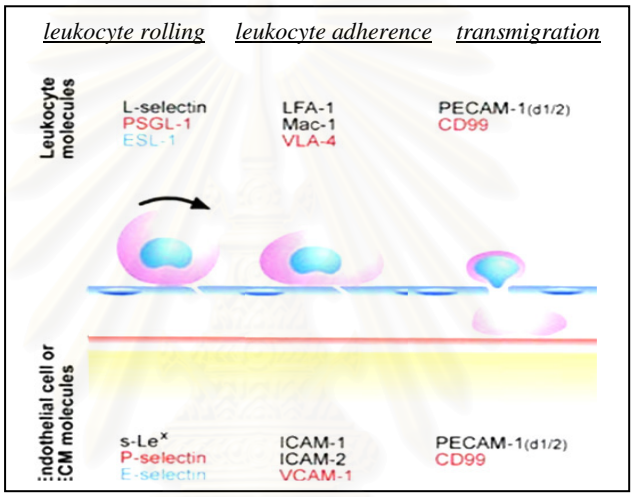


Figure 5.1. The schematic representation showed the sequential steps in leukocyte emigration are controlled by specific adhesion molecules on leukocytes and endothelial cells (modified from Muller 2002).

Even though it still not clear how diabetes cause the increased adhesive molecules expression, there are at least two pathways been reported. Firstly, it was believed that advanced glycosylation end-products (AGEs)- receptors (RAGEs) specific for **AGEs interaction generate free radicals that further activate NF-kB**. Then activated NF-kB could cause the up-regulation of those adhesive molecules. At present, it is quite known that the activity of two transcriptional factors, NF-κB and AP-1 (activator protein-1), is regulated by intracellular redox states (Sen and Packer. 1996). When these transcriptional factors bind to the specific binding sites in the regions upstream of various genes such as VCAM-1, ICAM-1 and then regulate the

expression of those genes (Sen and Packer. 1996; Dandona *et al.,* 2000; Kashiwagi. 2001).

Secondly, it is believed that diabetes-induced endothelial cell dysfunction which results to the **deficiency of NO bioavailability** is the underlined mechanism for the up-regulation of adhesive molecules expression (Murohana *et al*., 1996; Morigi *et al*., 1998; Lefer *et al*., 1999; Huvers. 1999; Kubes *et al*.1991).

As regards these two possible concepts, it implied that **hyperglycemia induced free radicals** is the important factor underlined the interaction of leukocytes and endothelial cells as shown by the following diagram (Figure 5.2)

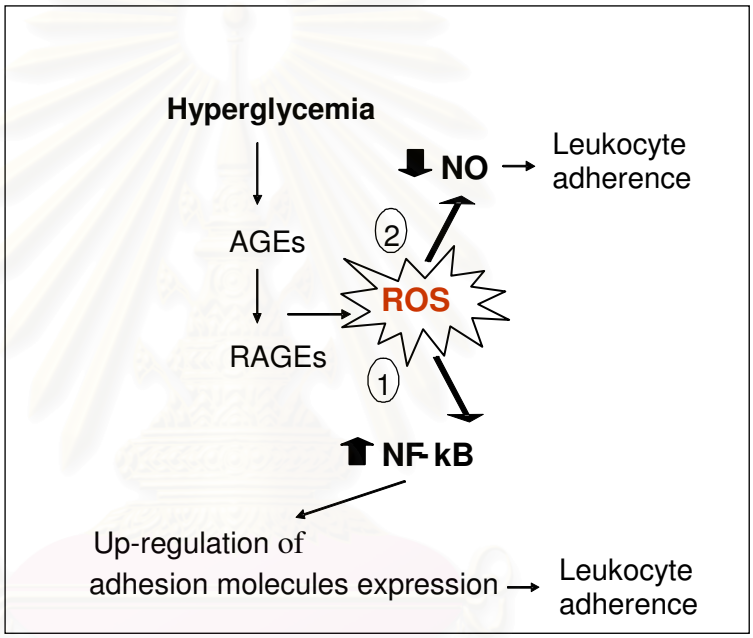


Figure 5.2. The two possible pathways of diabetes-induced leukocyte-endothelial cell interaction are: 1) an ehanced NF-kB activation 2) a deficiency of NO bioavailability

- *Endothelium-dependent vasodilation*

We also studied the endothelial dysfunction by determining an altered vascular reactivity. By using 10 μM Ach, we found a significant reduction in the endothelium-dependent vasodilation to Ach in mesenteric arterioles in both 6-wks and 12-wks DM rat, (6.40±0.79 and 4.72±1.51%, respectively) compared to their age-matched CON rats (14.66±0.85 and 13.86±1.68%, respectively) ($p<0.01$, $p<0.001$). In agreement

with our results, Taylor et al., 1992, showed the deceased Ach-induced vasodilation in mesenteric resistance arterioles in 6-wks of STZ-induced DM rats. Moreover, by using the same STZ-rat model, Diederich et al., 1994 found that the concentrations of Ach were required up to 63 and 100 nM in order to reach the 50% relaxation (ED50) after 16 and 24 wk of diabetes, respectively.

As shown in Table 4.3 and Figure 4.4, our study showed no impairment of the 10 μM -SNP vascular response in the diabetic vessels, therefore, it implied that this abnormality of vascular response observed since 6^{th} week in diabetic rats is endothelial-dependent vasodilatation.

As regards the results of leukocyte adherence and Ach-vascular reactivity, we, therefore, confirmed that our methodology using the single intravenous injection of 50 mg/kg.bw of STZ could induce the endothelial dysfunction significantly as early as 6-wks and prolong throughout the 12-wks experimental period.

Effect of Vitamin C Supplementation On the Diabetes-Induced Endothelial Dysfunction

The results of leukocyte-endothelial cells interaction and Ach-induced endothelium-dependent vasodilation confirmed that the abnormality of hyperglycemia-induced endothelial dysfunction occurred at 6-wks period after STZ injection. Therefore, we aimed to examine whether this endothelial dysfunction will be able to reverse by vitamin C supplementation or not. And if it could do what will be the possible mechanism(s).

Our data obtained form the diabetic group that was treated with vitamin C started at 6-wks after STZ injection (DM+Vit.C_{6wks} group) demonstrated that vitamin C supplementation could REVERSE diabetes-induced endothelial dysfunction. As shown in Table 4.5 and Figure 4.6, the findings indicated that number of leukocytes adherence were significantly decreased in DM+Vit.C_{6wks} group (2.15±0.31 cells/100-μm venular length ($p<0.001$)). The reversal effect of vitamin C supplementation was also demonstrated by the results of Ach-induced endothelium-dependent vasodilation as well (12.42±1.11 % ($p<0.01$)).

Our obtained data from DM+Vit.C_{day2} group, demonstrated that the early vitamin C supplementation started at 2 weeks after STZ-injection could PREVENT the abnormalities of leukocyte–endothelium interaction (4.71±0.47 cells/100-µm venular length ($p<0.01$)) and Ach-induced endothelium-dependent vasodilation (14.14±1.62 % ($p<0.01$)). These preventive roles of vitamin C were actually reported in our previous studies (Jariyapongskul *et al*., 2002 and Chakraphan *et al.,* 2002). However, as to compare the reversal effect with the preventive effect, the following analytical data can be remarked.

Our results have shown that the reversal effect of vitamin C could give the same benefit as its preventive action. It could be noticed from the results shown in Table4.5 and 4.6 that the values of leukocyte–endothelium interaction and Ach-induced endothelium-dependent vasodilation between DM+Vit.C_{6wks} and DM+Vit.C_{day2} groups have shown no significant difference. Base on these obtained data, therefore, the supplementation of vitamin C should be recommended for both therapeutic and preventive aspects for diabetic care.

- ***Vitamin C and its potential effect on leukocyte-endothelial cells interaction***

Our finding has shown that vitamin C supplementation could reduce leukocyte adhesion even after endothelial cells became dysfunction. The results of both groups of DM+Vit.C_{6wks} and DM+Vit_{day2} (2.15±0.31 and 4.71±0.47 cells/100-µm venular length, respectively)($p<0.001$ and $p<0.01$) indicated the roles of vitamin C in the reversal and prevention of the diabetes-induced leukocyte-endothelial cell interaction.

In regard to the diagram shown in Figure 5.2, our obtained data of H_2O_2-associated fluorescence using DHR123 demonstrated that long-term vitamin C supplementation significantly decreased ROS production in DM rats as well (Figure 4.12). Therefore, the effect of vitamin C may be described mainly through its anti-oxidant property. The idea is that vitamin C could scavenger hyperglycemia-induced ROS directly, therefore, it can prevent endothelial dysfunction or restore the endothelial function back to normal. Less ROS is produced, less adhesion molecules can be activated to express on the surface of endothelial cells.

At this point of view, it might be suggested that vitamin C as a potent free radical scavenger may have a capacity to suppress the pro-inflammatory cytokines-

mediated the expression of the adhesive proteins, which in turn, to prevent leukocyte-endothelial interaction found in DM rats.

- ***Vitamin C and its potential effect on Ach-induced endothelium-dependent vasodilation***

Moreover, at arteriolar site, the attenuated relaxant responses to Ach in DM rat has been restored and prevented with vitamin C supplementation (12.42±1.11 and 14.14±1.62 %, respectively) ($p<0.01$). Taken together with the result of NO-associated fluorescence using DAF-2DA, 20-min after 2.6 mM vitamin C administration was significant increase in NO production in DM rats as well (Figure 4.12). Base on our results, it is demonstrated that vitamin C supplementation could evoked NO molecule to release even in diabetic mesenteric arterioles.

In addition, the endothelium-dependent vasodilation is one of the local control factors of peripheral blood flow. If the vessel is denuded of endothelium, therefore the vessel could not be dilatation due to the NO was abolished (Kuo *et al.,* 1996). Therefore, endothelial dysfunction is accompanied by **1) decreased production of NO by ROS** and/or **2) decreased NO bioavailability via its eNOS cofactor tetrahydobiopterin (BH_4) deficiency** (Shinozaki, *et al* 1999; Thum *et al* 2007). With these point of view of the possible mechanism(s) of vitamin C to reverse diabetes-induced endothelial dysfunction will be discussed in the following sessions.

<u>The Mechanism of Vitamin C to Decline Diabetes-Induced ROS Contents</u>

Up to now, much interest has been paid to the first postulated mechanism; **1) the endothelial dysfunction is accompanied by decreased production of NO by ROS**. Especially, the role of ROS on diabetic endothelial dysfunction has brought a lot of attention focused on its mechanism. Table 4.7 showed a greatly increased intracellular H_2O_2-dependent endothelial cells in DM rats as compared to CON (165.89±24.59 and 34.08±15.6, respectively ($p<0.001$)). Similar to a recent data, Thum et. Al., 2007 provided an evidence for increased superoxide radical (O_2^-) within bone marrow of diabetic rats. And also Zang *et al.,* 2004, they reported that high glucose (44mM, 5 hr) stimulated H_2O_2 production in isolated rat aortas using the Amplex Red assay (Amplex Red reagent (200mol/l) and horseradish peroxidase (1unit/ml)) and measuring in a microplate reader 530 nm.

Moreover, by using DAF-2DA to direct detection of NO content we found a significant reduction in NO contents in arteriolar walls of 6-wks DM rats as compared to CON (55.00±10.39 and 157.01 ± 30.82%, respectively) ($p<0.05$) (Table 4.9). Moreover, we demonstrated that the endothelial dysfunction was established on 6-wks after STZ-injection as characterized with a decreased Ach-induced vasodilation as well as increased leukocyte adhesion (Table 4.2 and 4.3). Similar observation had been made in 5-days human aortic endothelial cells; they found that high glucose decreased NO using electron paramagnetic resonance (Cai *et al* 2005). It has been hypothesized that hyperglycemic condition could cause an excessive production of ROS, such as O^{-}_{2}, to oxidize the existing NO before it can reach its target tissues. The interactions between NO and O^{-}_{2} occur at an extremely rapid rate, **~3-time** faster than the reaction rate for O^{-}_{2} with SOD (Beckman and Koppenol.1996). In hyperglycemia, this O_2^{-} may quench NO, thereby reducing the efficacy of a potent endothelium-derived vasodilator system (Jariyapongskul *et al*., 2003; Benz *et al.,* 2002; Meininger *et al.,* 2000).

Our results indicate that hyperglycemia triggers the up-regulation of ROS production, which, in turn, inhibits endothelial NO bioavailability (Figure 5.3).

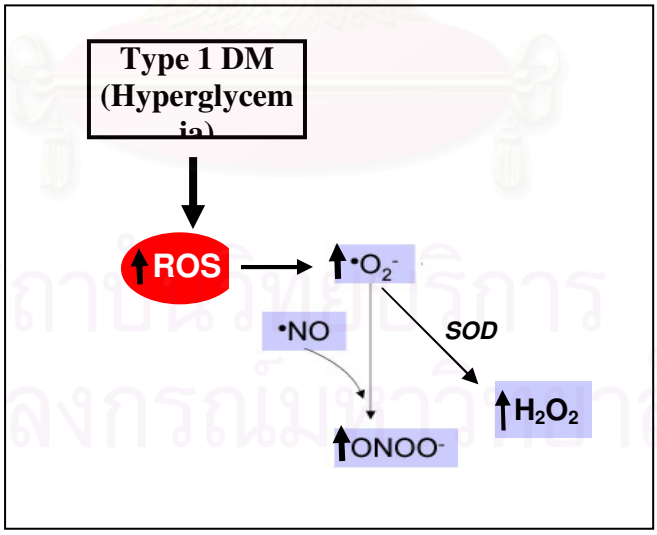


Figure 5.3. Hyperglycemia-induced overproduction ROS^{-}, which in turn, lead to deplete NO bioavailability

Base on our finding of ROS by using DHR123 (Table 4.7), the increase of H_2O_2-associate fluorescence in arteriolar endothelial cell of DM rats could be

reversed by supplementation of vitamin C at both delayed phase (at week 6th after STZ injection) and early phase (at day 2 after STZ injection) (26.02±14.56 and 32.86±8.03, respectively) ($p<0.001$). Our result provided an evidence that vitamin C supplementation appeared to scavenge both O_2^- and H_2O_2 which was localized in intracellular endothelial cells, demonstrating a potentiality of vitamin C as a ROS scavenger.

In agreement with earlier studies by Nishikimi *et al*., 1975 and Nandi and Chatterjee. 1987, they reviewed that in rats, the average tissue concentration of vitamin C was **10^{-3} M** and that of superoxide dismutase (SOD) was **10^{-6} M** which is about 1000 times less than tissue concentration of vitamin C. Although, the rate constant for the reaction between SOD and O_2^- (K_{SOD}) was **1.9 x 10^9 mol · L^{-1} · s^{-1}**, which is approximately 23 times more than the rate constant of vitamin C (K_{AH2})--**8.2 x 10^7 mol · L^{-1} · s^{-1}** (Nishikimi *et al*., 1975). Regarding the rate constant of vitamin C is lower than SOD, therefore, if we need vitamin C to have a potential efficacy for scavenging O_2^-, we have to supplement vitamin C with a high dose at least similar to the tissue concentrations of vitamin C. Moreover, Jackson *et al.,* 1998 examined the effect of vitamin C in isolated rabbit artery in condition of a steady flux of O_2^- (1.5-2.3 μM/ min). Their results shown that 10 mM vitamin C partially inhibited the effect of O_2^- by enhanced arterial relaxation. ***Therefore, our findings could demonstrate the ability of anti-oxidant property of vitamin C on reducing hyperglycemia-induced ROS.***

Moreover, in Table 4.9, we found that 20-min after 2.6 mM vitamin C administration, the NO-associated fluorescence were significant increased in both groups of CON and DM (235.43±28.00 and 147.21±21.98%, respectively) as compared to their based line values (157.01±30.82 and 55.00±10.39%) ($p<0.05$ and $p<0.001$). In addition, in DM group, acute administration of vitamin C can restore the Ach-agonist evoked NO released to normal (Table 4.9) as similar to the results of chronic vitamin C supplementation for 6-wks or 12-wks also restored Ach-induced vasodilation, which demonstrated the functional NO bioavailability (Table 4.6). So, it led us to suggest that vitamin C could enhance NO bioavailability even in the DM group—the condition of decreased NO bioavailability. In agreement with the previous reports and our current finding, they showed that vitamin C supplementation improved endothelial dysfunction in smokers, patients with hypercholesterolemia, coronary artery disease and diabetic patients and rats (Heitzer *et al,* 1996; Ting *et al,*

1996; Ting *et al,* 1997; Gokce *et al,* 1999). ***Therefore, our findings could demonstrate the ability of vitamin C-mediated NO bioavailability can be possible by inhibiting NO depletion by ROS in DM.***

Base on our findings, we propose the mechanism of vitamin C on ROS scavenging and as a consequence, it preserves NO bioavailability from ROS interaction as shown in Figure 5.4.

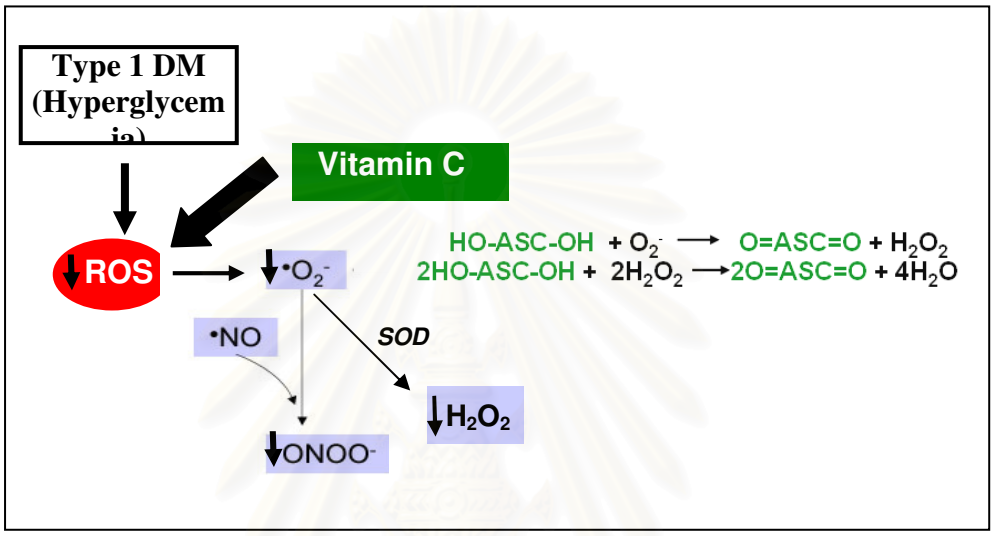


Figure 5.4. The schematic representation showed the proposed mechanism of vitamin C (HO-ASC-OH) to scavenge ROS.

The Mechanism of Vitamin C to Preserve Diabetes-Altered BH_4 Bioavailability

According to our hypothesis, the possible underlying molecular mechanism(s) by which vitamin c can reverse diabetes-increased ROS production that disturb endothelial function, are following:

1) Vitamin C could possibly decrease the production of ROS by its direct scavenging reaction as shown in the following equation,

$$\underset{\textit{(vitamin C)}}{\text{HO-ASC-OH}} + O_2^- \rightarrow \text{O=ASC=O} + H_2O_2$$

$$\underset{\textit{(vitamin C)}}{2\ \text{HO-ASC-OH}} + 2\ H_2O_2 \rightarrow \text{O=ASC=O} + 4\ H_2O$$

Thereby, it could enhanced Ach-induced vasodilatation *as we have discussed above for the results of DAF-2DA and Ach-responses.*

2) Vitamin C could possibly reverse diabetes-induced endothelial dysfunction via its ability to enhance eNOS activity indirectly by providing BH_4 bioavailability, in other word, it may protect the eNOS proteins from uncoupling state, *as we are going to discuss in the following session.*

Role of eNOS uncoupling in the absence of BH_4 bioavailability

We, therefore, are much attention on the potential involvement of BH_4 in endothelial dysfunction. Our present studies provide an evidence of uncoupled eNOS in the deficiency of BH_4. By using the perfusate contained 2,4-diamino-6-hydroxypyrimidine (DAHP), the inhibitor of key enzyme, GTP-CH 1, in the pathway BH_4 synthesis, into the study area of mesenteric arterioles, we found a significant decreased Ach-induced vasodilation in 6-wks CON rat (4.34±0.78% ($p<0.001$)) (Table 4.10). Our obtained data from DAHP-treated CON group showed the significant **~3-time (13% / 4%) decreased vascular response** as compared to their baseline response (Table 4.10). It implied that DAHP-induced BH_4 deficiency can mediate eNOS uncoupling via the decreased NO bioavailability. In agreement with Meinnger CJ et al., 2000, who revealed that a deficiency in BH_4, cofactor of eNOS, was responsible for impaired NO synthesis in the coronary endothelial cells isolated from the spontaneously diabetic BB rats.

Since endothelial dysfunction by the loss of BH_4 is sufficient to produced eNOS uncoupling which prefers to produced O_2^- and/or H_2O_2 rather than NO (Berka *et al.,*1996; Landmesser *et al.,* 2003; Heiter *et al.,*2000; Shinozaki *et al.,*1999; Sun *et* al., 2001; Werner *et al.,* 2003). Moreover, our finding in Table 4.12 indicated that there was **~3-time increase in H_2O_2-associated fluorescence** when BH_4 synthesis was blocked by DAHP (169.61% / 54.48%). Therefore, at this point of our interpretation , it is important to indicate that by using the novel fluorochrome, DHR-123, specific detecting *in vivo* H_2O_2 contents, the results could provide the strong evidence for the *direct* role of BH_4 deficiency in relation to the eNOS uncouple formation and consequently produced ROS to NO molecule proximately in **1:1** ratio as a following equation,

$$1\ O_2 + 1\ H_2O \rightarrow \mathbf{1\ H_2O_2}$$

$$1\ O_2^- + \mathbf{1\ NO} \rightarrow 1\ ONOO^-$$

In order to re-confirm that DAHP caused the BH_4 deficiency-mediated eNOS uncoupling, we used 6R-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (6R-BH_4,) as a positive control, which is a compound for increased intracellular BH_4. Interestingly, the supplementation of exogenous BH_4 into the study area of mesenteric microcirculation after the 10-min DAHP-induced BH_4 deficiency could increase Ach-induced vasodilatation in a dose-dependent manner (Table 4.10).The similar effects of BH_4 on enhancing Ach-vasorelaxation (0.02 mM incubation for 30 min and 0.05 mM infusion for 10 min) were shown prior in patients with smoking by Higman *et al.,* 1996; Heitzer *et al.,* 2000.

Effect of vitamin C to preserve BH4 bioavailability-mediated eNOS coupling

By using the same experimental setup of DAHP-induced BH_4 deficiency, our results have shown that 1-min 2.6mM vitamin C administration after 30-min DAHP could markedly increase in endothelium-dependent vasodilation to Ach (17.98±1.42%) as compared to DAHP group (4.34±0.78%) ($p<0.001$, Table 4.11). Moreover, when we used DHR 123 to detect *in vivo* H_2O_2 contents (Table 4.12), we also found that the 1-min vitamin C administration could decrease H_2O_2-contents in DAHP-induced BH_4 deficiency up to ~1.5 time. (DAHP / DAHP+Vit.C :169.61 / 115.66*).* ***Corresponding to the results of Ach-induced vasodilation, the results showed that*** the 1-min 2.6mM vitamin C administration could ***enhance vasodilatation up to*** ~4.14 times (DAHP+Vit.C / DAHP : 17.98 / 4.34). *Interestingly, this vitamin C administration was able to increase endothelial vascular relaxation via Ach proximately in the same range as exogenous 0.1mM BH_4 given* (DAHP+Vit.C / DAHP+BH4 = 17.98± 1.42 : 17.40± 1.12).

Toth *et al.,* 2002, who characterized the mechanism of the chemical interaction between L-ascorbic acid (vitamin C) and BH_4 *in vitro.* They found that 3 mM vitamin C provided an almost perfect stabilization of 25 µM BH_4. Moreover, several *in vitro* findings, vitamin C is believed to interfere with BH_4 oxidation and thereby maintain it in a reduced state, which in turn, to restore endothelial function, to coupled eNOS, by increasing intracellular BH_4 (Huang *et al,* 2000; Heller *et al,* 2001; Kuzkaya *et al,* 2003; Toth *et al,* 2002). Since vitamin C could reduce the BH_2 to regenerate BH_4 as shown in the following equation:

$$HO\text{-}ASC\text{-}OH + BH_2 \rightarrow O{=}ASC{=}O + BH_4$$

(vitamin C)

At this point, it may be suggested that in the situation of DAHP-induced BH_4 deficiency, eNOS uncoupling could produce as which they will generate O_2^- and consequently convert to H_2O_2- as demonstrated by DHR 123. And also when eNOS uncoupling, less of NO could be produced, therefore, the Ach-induced vasodilatation was significantly decreased. This experimental DAHP-induced BH_4 deficiency was set up in order to imitate the condition of diabetes-induced endothelial dysfunction. Therefore, this kind of experimental findings could be lead us to the possible mechanism of beneficial effect of vitamin C on diabetic endothelial cell as shown in the following details.

Moreover, we also examine the NO contents using a novel fluorescent dye sensitive to NO, DAF-2DA, in order to monitor the Ach-evoked NO release in relation to Ach-induced vasodilation. As shown in Table 4.9, the 20-min administration of vitamin C alone markedly enhanced ~1.5-time and ~2.7-time of DAF-2T increasing as compared to the baseline values of CON and DM, respectively. Whereas the 1-min administration of 2.6mM vitamin C could increase Ach-induced vasodilation up to ~1.6-time in CON (Table 4.8).

Moreover, the Vit.C in combination with high dose of BH_4-treated group (DAHP+$BH_4$0.1 mM+Vit.C-treated CON group) can be a very effective treatment for enhancing Ach-induced vasodilation compared with DAHP+BH_4 0.01 mM group (Table 4.11). Beside, as compare to DAHP group, the depletion of H_2O_2 content by vitamin C, BH_4, and combined vitamin C and BH_4 was ~32%, 61%, and 80%, respectively (Table 4.12), indicated that the group of combination showed a synergistic effect to decline ROS production. Our data demonstrated that the combination of vitamin C and BH_4 showed a very strong powerful to reduce ROS production and also to enhance NO bioavailability, therefore, it might be a good clinical implication for using these two agents for diabetic patients.

At this point of view, we concluded that because of an enhanced regeneration of BH_4, vitamin C can improve the uncoupled eNOS and enhance its enzymatic activity as demonstrated by increased Ach-induced vasodilation which was associated with the decreased H_2O_2 contents (Figure 5.5).

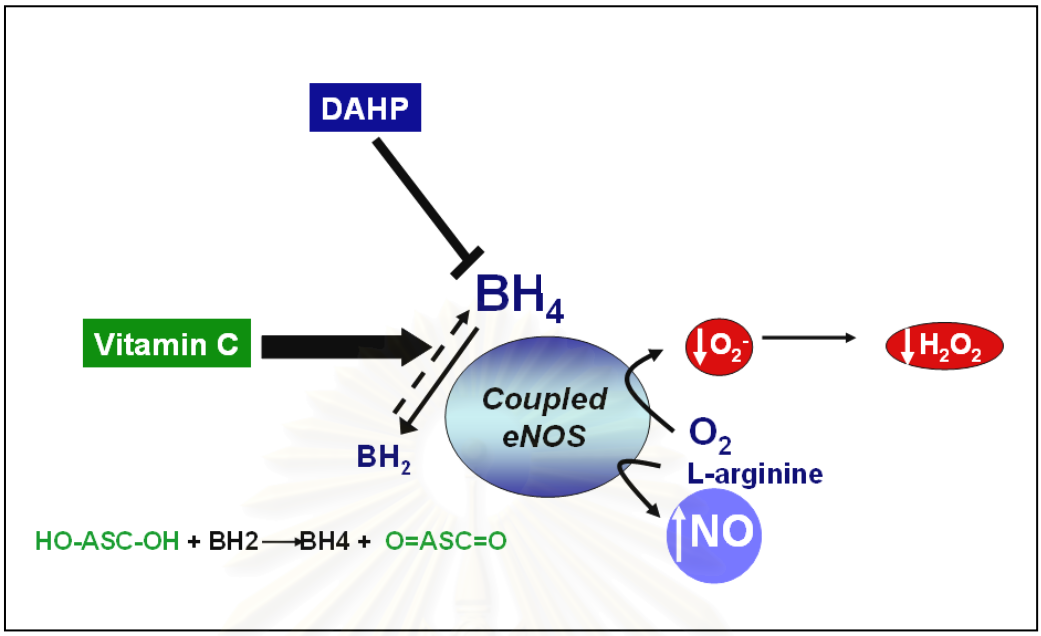


Figure 5.5. The diagram represents the proposed mechanism of vitamin C in reversing diabetes-induced endothelial cells in associated with BH_4.

Role of hyperglycemia-induced eNOS uncoupling in the absence of BH_4 bioavailability

Several studies indicate a close relationship between BH_4 bioavailability and eNOS function in diabetes, supporting the idea of reduced the bioavailability of BH_4-promoted eNOS uncoupling which contributes to further reduced NO bioavailability and generate O_2^- and/or H_2O_2 rather than NO

A recent study by Thum *et al* 2007, they found a significant reduction of intracellular BH_4 levels after challenging endothelial progenitor cells (EPCs) with high glucose concentration, whereas oxidized BH_2 levels significantly increased. Consistent to Sinozaki *et al.,* 1999, provided the same result in high fructose-fed rats due to enhance formation of O_2^-, which was caused by relative deficiency of BH_4 and elevation of BH_2 levels in aortic tissue. Base on their findings, therefore, we proposed that diabetes-induced ROS could be related to the deficiency of BH_4 by O_2^- oxidation, resulting in enhanced BH_2 ($BH_4 + O_2^- \rightarrow BH_2 + H_2O_2$).

With this point of view, we investigate in a further group of DM rats which is much more complicate than the study in normal rats as shown by their baseline values of Ach-induced vasodilation in DM is 7.69±0.87% whereas in CON is

13.22±1.24% (Table 4.31 and 4.10, respectively). As such result, the addition of BH_4 synthetic inhibitor, DAHP, showed greatly decrease in the Ach-induced vasodilation in DM rats (3.81±0.60%, Table 4.13), which was worsen than the effect of DAHP in CON rats (4.34±0.78%, Table 4.10). This indicated that the DAHP-induced BH_4 deficiency was potentially capable of exhibition uncoupled eNOS in DM rats with a higher degree rather than normal rats.

As shown in Table 4.13, when BH_4 was inhibited by DAHP, the decrease in Ach-induced vasodilation was indicated in DM rats and it could be corrected by the moderate (0.01 mM) and high doses (0.1 mM) of BH_4 donor applications. However, the low dose of BH_4 donor (0.001 mM) caused no changed in diabetic Ach-induced vasodilation, whereas, in CON rats all doses of BH_4 donor showed significantly increase in Ach-induced vasodilaition (Table 4.10). ***Therefore ,it indicated that higher BH_4 concentrations was necessary for improving eNOS coupling to increase NO bioavailability in DAHP-treated DM rats than those of CON rats.***

Effect of vitamin C to preserve BH4 bioavailability restores hyperglycemia-mediated eNOS uncoupling

Table 4.14, we found that abnormality in Ach-induced vasodilation in DM rats was improved by vitamin C compared to DAHP group (11.30±1.34 and 3.81±0.60 %, respectively) ($p<0.001$)**.** Because of in DM rat, it involves mainly with the enhance ROS production form its hyperglycemia condition; ***we believe that vitamin C as its anti-oxidant property could react with $O_2^-$and/or H_2O_2, which, in turn, led to protect BH_4 from oxidation by ROS resulting in enhance BH_4 bioavailability.*** With this point of view, several studies have shown that vitamin C may be important in maintaining BH_4 levels in the setting of vascular oxidative stress. For the oxidation of BH_4, Huie and Padmaja, 1993 demonstrated that O_2^- can react directly with BH_4 with the rate constant of this reaction (K_{O2^-}) **$3.9x10^5$ mol · L^{-1} · s^{-1}**, which, in turn, lead to biopterin (BH_2)--an oxidize form of BH_4 ($BH_4 + O_2^- \rightarrow BH_2 + H_2O_2$). However, it is still lower than the rate constant of the reaction between NO and O_2^- (K_{NO})-- **$6.7 x10^9$ mol · L^{-1} · s^{-1}**, indicating BH_4 is unlikely to be acting merely as an antioxidant at physiological concentrations. Moreover, for the BH_4 oxidation, the experiment *in vitro* (Milstin and Katusic. 1999) and *ex vivo* (Laursen *et al.,* 2001) indicated that

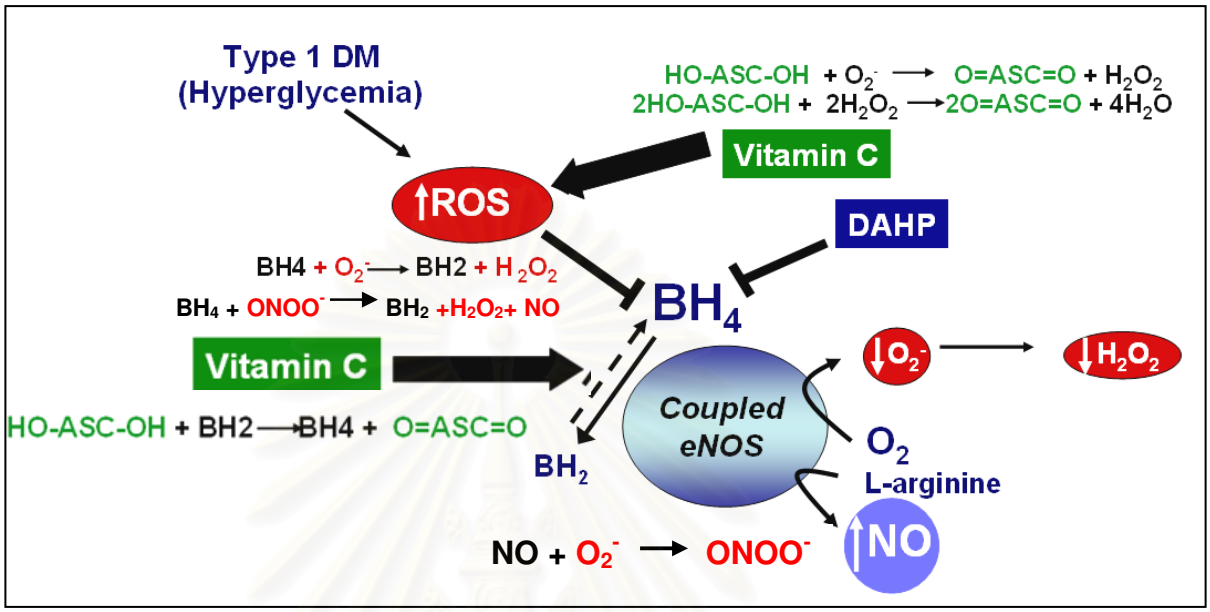
$ONOO^-$ generated from the reaction between NO and O_2^- can oxidize BH_4 within minutes, at physiologically relevant concentrations, and led to the BH_3^- radical (nonprotonated), and hence to BH_2 ($BH_4 + ONOO^- \rightarrow BH_3^- \rightarrow BH_2 + H_2O_2 + NO$). Also supporting by recent electron paramagnetic resonance experiments, they demonstrated that $ONOO^-$ oxidized BH_4 with a rate constant (K_{ONOO^-}) estimate to be **6 x10^3 mol · L^{-1} · s^{-1}** (Kuzkaya *et al.,* 2003).

Indeed, the rate constant for the reaction between vitamin C and O_2^- (K_{AH2}) was **8.2 x 10^7 mol · L^{-1} · s^{-1}**, which is approximately 200 and 10,000 times more than the rate constant of O_2^- reacted to BH_4 (K_{O2^-})and than $ONOO^-$ reacted to BH_4 (K_{ONOO^-}), respectively (Nishikimi *et al*., 1975; Nandi and Chatterjee. 1987).

Moreover, Table 4.14, we fond that the combination of vitamin C and high dose of BH_4 donor (DAHP+$BH_4$0.1mM+Vit.C-treated DM group) significantly enhanced Ach-induced vasodilation compared to DAHP groups treated-vitamin C or BH_4 alone. Interestingly, this group also showed a significant increase in vascular response to Ach ~2-time compared to DAHP group treated-vitamin C in combination of modulate dose of BH_4 (DAHP+$BH_4$0.01mM+Vit.C-treated DM group). It indicated that in the DM model, suffered from both hyperglycemia-induced ROS overproduction and also DAHP-mediated BH_4 deficiency, the administration of combined vitamin C and BH_4 at higher dose can improve eNOS uncoupling to generate NO production. We, therefore, strongly recommend not only supplement vitamin C but also BH_4 at the effective dose to diabetic patient who suffer from long-time severe hyperglycemia in order to reverse or reduce the risk of diabetes-induced endothelial dysfunction.

Therefore, as the overall conclusion, it may be remark here that in the control vascular endothelium, BH_4 bioavailability is not limited for any vasodilators-evoked NO release. Therefore, there is no uncoupled eNOS, no *O_2^- production*, and then no $ONOO^-$ formed. ***However, in diabetes,*** *O_2^-* production could be enhanced by several pathways (as mentioned earlier). NO production may remain unaffected initially, but Ach-evoked NO is reduced because of scavenging interactions with O_2^-, forming **increased $ONOO^-$ mostly due to its highest rate constant**. $ONOO^-$ and other ROS could then oxidize BH_4, via the BH_3 radical to BH_2 and biopterin, therefore, reducing the bioavailability of BH_4 and consequently ***promotes eNOS uncoupling***. eNOS now generates O_2^-, rather than NO, which

contributes to vascular oxidative stress and further reduces NO bioavailability. Through this sequence event, therefore, it may imply for ***the possible role of vitamin C in reversing diabetes-induced-endothelial dysfunction in associated with*** NO and



BH_4 bioavailability via the following mechanism shown in Figure 5.6

Figure 5.6. The diagram represents the proposed mechanism of vitamin C to protect against oxidative degradation of BH_4 by directly scavenging O_2^- and/or H_2O_2 and regenerating BH_4.

The Proposed Mechanism(s) for Our Findings on Anti-Oxidant Property of Vitamin C to Reverse Diabetes-Induced Endothelial Dysfunction

Up to our knowledge, we believe that our data should be the first time that demonstrates a major role for vitamin C to reverse diabetes-induced endothelial dysfunction in rat mesenteric microcirculation. In particular, our results have shown that 6-wks delayed supplementation of vitamin C had a potential benefit for reversing the established diabetes-induced endothelial dysfunction as its early phase (at week 6^{th} after STZ injection). Regarding the mechanisms of how this phenomenon occur, we would like to make remark that the abnormalities endothelial function after the STZ injection for 6-wks could be restored by supplementation of vitamin C, effectively for microvascular function, even though it fails to reduce plasma glucose concentration. Our present study has provided us for more information on how vitamin C can reverse endothelial dysfunction in diabetic rats. Targeting vitamin C may provide therapeutic strategies for reducing the risk of vascular disease in diabetic patients. Therefore vitamin C should be recommended as a new conceptual therapeutic agent for diabetic care.

And finally based on the overall results and discussion above, we would like to propose the possible mechanisms how vitamin C could exhibit the reverse effect on diabetes-induced endothelial dysfunction in rat mesenteric microcirculation (Figure 5.7). In brief, in the early phase (6-wks) of DM, hyperglycemia target area--endothelial cell is effected by several mechanisms (ie, polyol pathway, nonenzymatic glycosylation, glucose autooxidation and protein kinese C pathway) that enhanced ROS production resulting in endothelial dysfunction as characterized by depletion of vasodilation and elevation of leukocyte adhesion. Present data shows that 6-wks delayed supplementation of vitamin C reverses endothelial dysfunction in diabetic rats. Underlying mechanisms include the effect of vitamin C, as an antioxidant, on scavenging ROS, therefore, preserving NO from ROS interaction. (determined through H_2O_2 content and NO content, respectively). On BH_4 bioavailability, it is postulated that anti-oxidant property of vitamin C not only can prevent the oxidation of BH_4 to BH_2 but also can reduce the BH_2 to regenerate BH_4, suggesting that vitamin C may re-couple eNOS (mediated via Ach-induced vasodilation and H_2O_2 content).

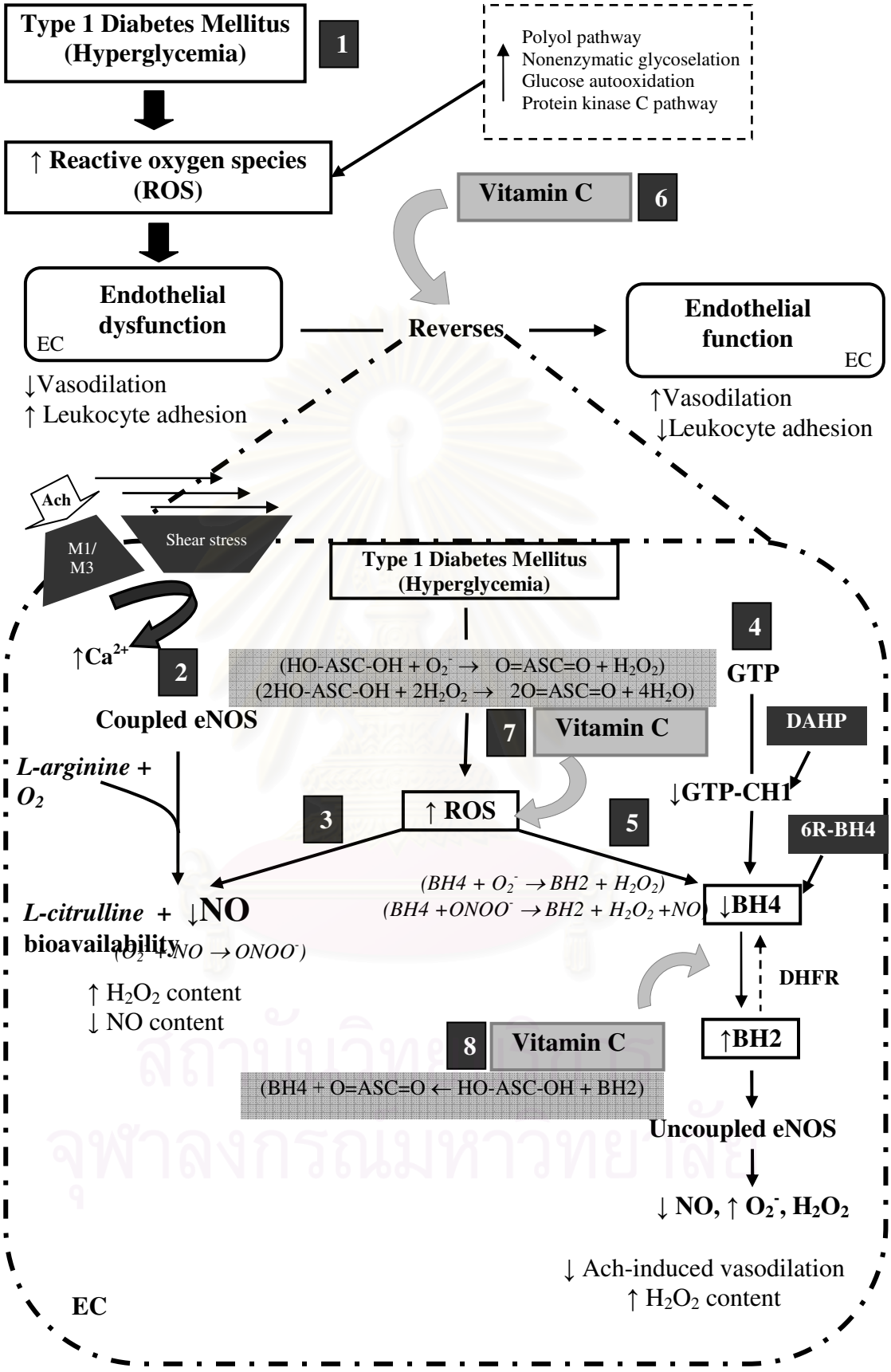

Type 1 Diabetes Mellitus (Hyperglycemia)
1
Polyol pathway
Nonenzymatic glycoselation
Glucose autooxidation
Protein kinase C pathway
↑ Reactive oxygen species (ROS)
Vitamin C
6
Endothelial dysfunction
EC
↓Vasodilation
↑ Leukocyte adhesion
Reverses
Endothelial function
EC
↑Vasodilation
↓Leukocyte adhesion
Ach
M1/ M3
Shear stress
Type 1 Diabetes Mellitus (Hyperglycemia)
↑Ca2+
2
Coupled eNOS
(HO-ASC-OH + O2- → O=ASC=O + H2O2)
(2HO-ASC-OH + 2H2O2 → 2O=ASC=O + 4H2O)
4
GTP
DAHP
7
Vitamin C
L-arginine + O2
↓GTP-CH1
3
↑ ROS
5
6R-BH4
(BH4 + O2- → BH2 + H2O2)
(BH4 +ONOO- → BH2 + H2O2 +NO)
L-citrulline + ↓NO bioavailability
(O2- + NO → ONOO-)
↓BH4
DHFR
↑ H2O2 content
↓ NO content
8
Vitamin C
↑BH2
(BH4 + O=ASC=O ← HO-ASC-OH + BH2)
Uncoupled eNOS
↓ NO, ↑ O2-, H2O2
↓ Ach-induced vasodilation
↑ H2O2 content
EC

Figure 5.7. The proposed mechanism for the reverse effect of vitamin C supplementation on diabetes-induced endothelial dysfunction obtained from this present study could display as following:

1) Hyperglycemia results in endothelial dysfunction which is mediated by increased reactive oxygen species (ROS) through several mechanisms.

2) Endothelial NO synthase (eNOS) catalyzes the production of nitric oxide (NO) and L-citrulline from the L-arginine, using molecular oxygen (O_2) through the stimulation of shear stress and agonists such as acetylcholine (Ach) to up-regulate calcium (Ca^{2+}) in the endothelial cell (EC).

3) NO is rapidly oxidized by superoxide (O_2^-), leading to reduce NO bioavailability and forming peroxynitrite ($ONOO^-$), another ROS with potentially toxic effect.

4) Tetrahydrobiopterin (BH_4) is synthesized de novo from guanosine triphosphate (GTP) by the enzymes GTP-cyclohydrolase I (GTP-CH 1).

5) BH_4 is susceptible to oxidation by ROS such as O_2^-/ $ONOO^-$, forming dihydrobiopterin (BH_2) , which, in turn, to promote eNOS uncoupling leading to generate O_2^- and/or H_2O_2 rather than NO. When BH_2 levels are supraphysiologic, regeneration of BH_4 form BH_2 is facilitated by dihydrofolate reductase (DHFR). 2,4-diamino-6-hydroxy-pyrimidine (DAHP) is inhibit GTP-CH1. The exogenous 6R-BH4 is used in order to re-confirm the decreased BH4 bioavailability.

6) Our obtained data shows that 6-wks delayed supplementation of vitamin C reverses endothelial dysfunction in diabetic rats.

7) Vitamin C as a anti-oxidant properties could scavenge ROS, therefore, protect not only NO but also BH_4 form the oxidation wirh ROS.

8) Furthermore, vitamin C was able to regenerate BH_4 form BH_2, therefore, it could enhance NO bioavailability and re-coupling eNOS to function again.

CHAPTER VI

CONCLUSION

In the present study, we can characterized the alteration of endothelial function and directly detect the alteration of ROS, in particular H_2O_2, NO and BH_4 bioavailability using the intravital fluorescent videomicroscopy together with the novel fluorochromes including Acrydine orange, FITC-Dextran, DHR 123, and DAF-2DA. Such a beneficial technique, the reverse and protective effect of vitamin C supplementation and its underlying mechanisms on diabetes-induced endothelial dysfunction in mesenteric microcirculation *in vivo* were examined. Our obtained data are concluded as follows;

1. Complete destruction of pancreatic islet cells was induced by intravenous injection of rats with high dose STZ (50 mg/kg.bw). The loss of pancreatic insulin content triggered a severe hyperglycemia by 48 hr of DM rats, which maintained throughout the 6 weeks and prolonged until 12 weeks of experimental period, which can be recognized by the appearance of polyphagia, polydipsia, polyuria and weight lose. At 6^{th} week and 12^{th} week of the experimental period in DM rats, we found the significantly increase in blood glucose, HbA_{1C} and MAP levels, and decrease in plasma vitamin C levels. Whereas the plasma vitamin C levels were significantly normalized to control levels after supplement of vitamin C 1g/L daily (DM+Vit.C_{6wks} and DM+Vit.C_{day2}).

2. The measurement of the leukocyte adhesion and the Ach-induced endothelium-dependent vasodilation demonstrated a fully established diabetes-induced endothelial dysfunction by 6 weeks and was still markedly prominent at 12 weeks of experimental period.

3. Interestingly, the delayed supplementation of vitamin C exerted a profound effect on reversing the established diabetes-induced endothelial dysfunction through the reducing leukocyte adhesion and increasing vascular response to Ach, an endothelium-dependent vasodilator, but normal response to SNP, a direct smooth muscle cell dilator.

4. In confirmation of our finding, early supplementation of vitamin C had a beneficial effect on preventing the diabetes-induced endothelial dysfunction to promote the decreased production of leukocyte adhesion as well as increase Ach-induced endothelium-dependent vasodilation, whereas the endothelium-independent vasodilation to SNP was unaffected by this supplementation.

5. Direct measurement of ROS contents using DHR 123 *in vivo* showed a marked increase on H_2O_2 in DM rats; this change was improved after delayed and early phase of vitamin C supplementation. This result provided an evidence of vitamin C on ROS-scavenging.

6. Real-time detection of NO contents using DAF-2DA *in vivo* demonstrated a greatly decrease on NO in DM rats. However, this abnormality was significantly improved by 20 min 2.6 mM vitamin C perfusion either in CON or DM group. So, it led us to suggest that vitamin C could enhance NO bioavailability.

7. Concerning underlying mechanism of vitamin C, these results of ROS and NO indicated that vitamin C could protect NO molecule from ROS leading to elevate NO bioavailability.

8. The acute changes in Ach-induced vasodilation and H_2O_2 contents were significantly increased after normal rat endothelial cell exposed to DAHP- induced BH_4 deficiency, resulting in an alter eNOS actvity--eNOS uncoupling. However, these abnormalities could significantly improved by topical application of 2.6 mM vitamin C for 1-min. It is believed that vitamin C might reduce the BH_2 to regenerate BH_4 and thereby preserving BH_4 bioavailability, indicating that vitamin C can "recouple" eNOS and enhance its enzymatic activity to produce NO rather than H_2O_2.

9. In DM rat endothelial cell exposed to DAHP-induced BH_4 deficiency showed worsen in the vascular response to Ach. Surprisingly, the 1-min topical application of 2.6 mM vitamin C significantly improved Ach-induced endothelium-dependent vasodilation. It is demonstrated that vitamin C has the capacity not only to regenerate BH_4 but also protect BH_4 form oxidation by ROS, resulting in available BH_4 in endothelial cells, leading to increase eNOS coupling.

10. Besides, the reduced BH4 bioavailability has also been re-confirmed by exogenous 6R-BH_4 application in both CON and DM group.

11. The combination of vitamin C and 6R-BH_4 strongly attenuated endothelial dysfunction induced by BH_4 depletion in both CON and DM group.

12. Therefore, our findings can positively support our hypothesis that the supplementation of vitamin C (1g/L daily) could reverse the established endothelial dysfunction in STZ rats by scavenging ROS production, increasing NO bioavailability, and enhancing BH_4 bioavailability.

13. Base on our finding, it is noteworthy that vitamin C had an intent powerful to reverse the early stage of endothelial dysfunction (only functional change not morphological change) in the small blood vessel of diabetes mellitus, leading to reduce the risk of vascular disease. Even though, diabetic patient is usually detected with the severe stage (irreversible endothelial dysfunction), dietary high dose vitamin C should be delaying the progression of diabetic complication. Therefore, vitamin C should be recommended as a new conceptual therapeutic agent for diabetic care.

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APPENDIX

PUBLICATIONS

การเผยแพร่ผลงานวิจัยจากวิทยานิพนธ์

- Sridulyakul P., Chakraphan D., Patumraj S. (2005) Vitamin C supplementation could reverse diabetes-induced endothelial cell dysfunction in mesenteric microcirculation in STZ-rats. *Oral presentation*, The 6th Asian Congress for Microcirculation (ACM'05) in conjunction with The 30th Annual Meeting of Japanese Society for Microcirculation (JSM'05). (Abstract)
- Sridulyakul P., Chakraphan D., Patumraj S. (2005) Diabetes induced endothelial dysfunction could be reversed by vitamin C supplementation. *Oral presentation*, The 34th Annual Acadamic Meeting of the Physiological Society of the Thailand. (Abstract)
- Sridulyakul P., Chakraphan D., Patumraj S. (2005) Ascorbic acid could reverse diabetes-induced endothelium dysfunction in STZ-rat mesenteric microcirculation. *Poster presentation*, The first International Conference on Natural Products for Health and Beauty. (Abstract)
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AWARDS

- Young Investigator Award. 5th Asian Congress for Microcirculation, 2003, Manila, Philippines.
- Fellowship award of Tokyo Biomedical Research Foundation, Japan (2007)

BIOGRAPHY

NAME	**Ms. Pattarin Sridulyakul**	
DATE OF BIRTH	April 25, 1978	
PLACE OF BIRTH	Bangkok, Thailand	
INSTITUTION ATTENDED	1997-2000	Bachelor of Science (Biology) with the second class honor, Srinakharinwirot University
	2000-2003	Master of Science (Medical science), Chulalongkorn University, GPA: 3.56
	2003-2007	Ph.D. (Physiology), Chulalongkorn University
POSITION & OFFICE	2005-Present	Lecturer, Department of Biology, Faculty of Science, Srinakarinwirot University, Bangkok,Thailand
RESEARCH GRANTS	The Graduate Scholarship from the 90th Anniversary of Chulalongkorn University Fund, Thailand Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkon University, Thailand	