ผลของวิตามินซีต่อไนตริกออกไซด์ซินเทสในเอนโดทีเลียมของหนูที่ถูกทำให้เป็นเบาหวานด้วยสเตรปโตโซโทซิน: การเปรียบเทียบในเชิงปริมาณโดยการวิเคราะห์อิมเมจ

นางสาว ภัทริน ศรีดุลยกุลย์

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EFFECT OF VITAMIN C ON ENDOTHELIAL NITRIC OXIDE SYNTHASE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS: QUANTITATIVE COMPARISON USING IMAGE ANALYSIS

Miss Pattarin Sridulyakul

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ภัทริน ศรีดุลยกุลย์: ผลของวิตามินซีต่อไนตริกออกไซด์ซินเทสในเอนโดทีเลียมของหนูที่ถูกทำให้เป็นเบาหวานด้วยสเตรปโตโซโทซิน: การเปรียบเทียบในเชิงปริมาณโดยการวิเคราะห์อิมเมจ (Effect of Vitamin C on Endothelial Nitric Oxide Synthase in Streptozotocin-Induced Diabetic Rats : Quantitative Comparison Using Image Analysis.) อ. ที่ปรึกษา: รศ. ดร. สุทธิลักษณ์ ปทุมราช, อ. ที่ปรึกษาร่วม: รศ. ดร. ภาวพันธ์ ภัทรโกศล; 99 หน้า ISBN 974-17-29985-5.

เพื่อทดสอบผลของการให้วิตามินซีเสริมต่อระดับไนตริกออกไซด์ซินเทสในเอนโดทีเลียมในภาวะเบาหวาน โมเดลของสัตว์ทดลองที่ใช้ทำการศึกษาคือหนูที่ถูกทำให้เป็นเบาหวาน โดยวิธีฉีดสารสเตรปโตโซโตซินเข้าทางหลอดเลือดดำเพียงครั้งเดียวในขนาดความเข้มข้น 50 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว หนูสเปรย์-ดอลเลย์ เพศผู้ น้ำหนัก 200-250 กรัม ได้ถูกแบ่งแบบสุ่มเป็น 3 กลุ่มคือ กลุ่มควบคุม (CON) กลุ่มเบาหวาน (DM) และกลุ่มเบาหวานที่ได้รับวิตามินซี (DM+Vit. C) การให้วิตามินซีเสริม ทำโดยให้สัตว์ทดลองดื่มน้ำ ซึ่งผสมวิตามินซีในขนาดความเข้มข้น 1 กรัมต่อน้ำ 1 ลิตร อย่างอิสระ

ทำการทดลองหลังจากสัตว์ทดลองได้รับการฉีดสารละลายซิเตรทบัฟเฟอร์หรือสเตรปโตโซโตซินไปแล้ว 12 และ 24 สัปดาห์ วันที่ทำการทดลองค่าน้ำหนักตัว ระดับวิตามินซีในพลาสมา และระดับน้ำตาลในเลือดของสัตว์ทดลองทุกตัว จะถูกรวบรวมและประเมินผล จากนั้นทำการตัด เก็บ หัวใจ หลอดเลือด เอออตาร์ และปอด ทันทีเพื่อนำมาใช้ในการวิเคราะห์โดยวิธีเวสเทินบลอท

จากผลการทดลองพบว่าในหนูกลุ่มเบาหวานทั้งที่ 12 และ 24 สัปดาห์ ระดับน้ำตาลในเลือดสูงขึ้นอย่างมีนัยสำคัญทางสถิติแต่น้ำหนักตัวและระดับวิตามินซีในพลาสมามีค่าลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ระยะเวลาเดียวกัน อย่างไรก็ตามในหนูเบาหวานที่ได้รับวิตามินซีระดับวิตามินซีในเลือดสูงกว่าเมื่อเปรียบเทียบกับหนูเบาหวาน อย่างไรก็ตามระดับวิตามินซีในพลาสมาในหนูเบาหวานที่ได้รับวิตามินซีมีค่าสูงกว่าหนูเบาหวานอย่างมีนัยสำคัญทางสถิติ ($p<0.01$)

การหาแถบแบนด์โปรตีนไนตริกออกไซด์ซินเทสจากเอนโดทีเลียม ที่ได้จากวิธีเวสเทินบลอทโดยใช้โมโนโคนอล แอนติบอดี้ที่จำเพาะต่อ ไนตริกออกไซด์ซินเทสจากเอนโดทีเลียม จะถูกนำมาวิเคราะห์หาปริมาณ โดยใช้ซอฟแวร์โกบอลแลป อิมเมจ ปริมาณไนตริกออกไซด์ซินเทสจากเอนโดทีเลียมได้มาจากการแปลงค่าจำนวนของจุดสีในแต่ละแถบแบนด์ของไนตริกออกไซด์ซินเทสจากเอนโดทีเลียม จากการวิเคราะห์ภาพ โดยเปรียบเทียบกับสมการมาตรฐาน $Y = 5.9 x 10^{3}X$ จากนั้นนำค่าความเข้มข้นของไนตริกออกไซด์ซินเทสจากเอนโดทีเลียมที่แปลงค่าแล้วมาคิดเป็นร้อยละ โดยเทียบกับ 5 ไมโครกรัมของจำนวนโปรตีนทั้งหมด จากการทดลองพบว่าในหนูเบาหวานทั้งใน 12 และ 24 สัปดาห์ มีการลดลงของปริมาณโปรตีนไนตริกออกไซด์ซินเทสจากเอนโดทีเลียม เฉพาะที่สกัดจากหัวใจ แต่ไม่พบในปอด เป็นที่น่าสนใจว่าการเสริมวิตามินซีในหนูเบาหวาน ทั้งที่ 12 และ 24 สัปดาห์ มีผลทำให้ค่าโปรตีนไนตริกออกไซด์ซินเทสในเอนโดทีเลียม สูงกว่าอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับในหนูเบาหวานที่ไม่ได้รับวิตามินซี ($p<0.001$)

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นถึงการนำวิธีการวิเคราะห์ผลภาพสามารถมาประยุกต์ใช้ในการแปลงผลที่ได้จากวิธีการเชิงคุณภาพ เช่น จากการทำเวสเทินบลอทมาเป็นวิธีการเชิงปริมาณ ความไวของเครื่องมือที่วัดจากโปรตีนมาตรฐานพบว่าไวพอในการบอกความแตกต่างของค่าการเปลี่ยนแปลงของโปรตีนในระดับไมโครกรัม และค่าความถูกต้องของเครื่องมือ อยู่ในช่วง ±15.76% จากผลการทดลองชี้ให้เห็นว่า การสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากระดับน้ำตาลในเลือดสูงในภาวะเบาหวาน มีผลทำให้ปริมาณโปรตีนไนตริกออกไซด์ซินเทสจากเอนโดทีเลียมในเนื้อเยื่อหัวใจ แต่ไม่มีผลต่อเนื้อเยื่อปอดของหนูเบาหวาน

ดังนั้นเราจึงตั้งสมมุติฐานว่าการกระตุ้น การสร้างโปรตีนไนตริกออกไซด์ซินเทสจากเอนโดทีเลียม อาจจะเกิดจากค่าความแตกต่างของความแรงการไหลของเลือดที่สูงใน หัวใจ และต่ำในปอด อย่างไรก็ตามการให้วิตามินซีเสริมสามารถป้องกันการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากภาวะเบาหวาน โดยเฉพาะอย่างยิ่งสามารถป้องกันการทำลายโปรตีนไนตริกออกไซด์ซินเทสจากเอนโดทีเลียมในระบบไหลเวียนเลือดของร่างกายที่มีความแรงของการไหลสูง ดังนั้นอาจกล่าวได้ว่าวิตามินซีอาจเป็นสารที่นำมาใช้ป้องกันการเกิดโรคแทรกซ้อนของหัวใจและหลอดเลือดในภาวะเบาหวานได้อย่างดีเยี่ยม

หลักสูตร วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต………………………………………………………
สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา……………………………………………..
ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……………………………………..

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KEYWORD: VITAMIN C/ STREPTOZOTOCIN-INDUCED DIABETIC RATS/ ENDOTHELIAL NITRIC OXIDE SYNTHASE/ IMAGE ANALYSIS

PATTARIN SRIDULYAKUL: EFFECT OF VITAMIN C ON ENDOTHELIAL NITRIC OXIDE SYNTHASE IN STREPTOZOTOCIN-INDUCED DIABETIC RATS : QUANTITATIVE COMPARISON USING IMAGE ANALYSIS THESIS ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., 99 pp. ISBN 974-17-29985-5.

To examine the effects of supplemented vitamin C on endothelial nitric oxide synthase (eNOS) in diabetes mellitus, the animal model of streptozotocin (STZ)-induced diabetic rats (a single intravenous injection of STZ ; 50 mg/kg BW) was used. Male Spraque-Dawley rats weighing 200-250 g were divided randomly into three groups of control (CON), diabetes (DM) and diabetic supplementation with vitamin C (DM+Vit.C). The supplementation of vitamin C was performed by allowing the animals freely assessed to drinking water added 1 g/L of ascorbic acid (Sigma, Chemical Co., USA).

The experiment were performed at 12 and 24 weeks (wks) after injection of citrate buffer solution STZ. On the day of experiment, body weight (BW), plasma vitamin C, blood sugar (BS) were evaluated for all animals. Isolated heart, aorta and lung used for Western blot analysis were immediately collected from every rats.

The results showed that both groups of 12 and 24 wks DM groups have the significantly increase in blood glucose (BS), but decrease in BW and plasma vitamin C levels as compared to their age-match control groups. However, the plasma vitamin C levels was significantly increased in DM+Vit.C group as compared to DM group ($p<0.01$).

The eNOS protein bands obtained by Western blot analysis using monoclonal antibody against eNOS were quantified by Global Lab Image software analysis. The values of pixel numbers within each eNOS band from image analyzed were directly converted to amount of eNOS proteins of each sample by the standard equation, $Y = 5.9 \times 10^{3}X$. After that all converted concentration of eNOS proteins were then normalized using the correlation by 100% equal to 5 μg total protein. It was found that the diabetic state caused the reduction of eNOS protein expression in the heart but not in the lung for both 12 and 24 weeks. Interestingly, it also found that lung have significantly higher eNOS protein levels in DM+Vit.C as compared to DM for both 12 and 24 wks ($p<0.001$).

In conclusion, the present study has demonstrated that the application of digital image analysis can be used for converting qualitative method, such as Western blotting, to quantitative method. By using standard protein, the sensitivity of its application is well enough for differentiate the changes of protein content in microgram level. And the accuracy of this application is within ± 15.76 %. From the present results indicated that the endothelial dysfunction induced by diabetic hyperglycemia has been resulted to the decrease in eNOS protein level in heart but not in lung of DM rats. Therefore, we hypothesized that it might due to the difference of high-and low-flow, that mediated eNOS protein synthesis, in heart and lung, respectively. However, this finding suggests that vitamin C supplementation could prevent the diabetic-induced endothelial impairment, especially to prevent eNOS protein damage in high flow systemic circulation. Therefore, it is suggested that vitamin C might be a great chemopreventive agent for diabetic cardiovascular complications.

Program: Medical Science Student's signature………….…………..

Field of study: Medical Science Advisor's signature………………………...

Academic year: 2002 Co-advisor's signature……………………..

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LIST OF ABBREVIATIONS

GPX = Glutathione peroxidase
GSH = Glutathione
GSSG-RD = Oxidized glutathione reductase
$H^\bullet$ = Hydrogen radical
HAEC = Human aortic endothelial cell lysate
HbA_1 = Hemoglobin A_1
HMW = High Molecular Weight
HNO_2 = Nitrous acid
H_2O_2 = Hydrogen peroxide
HOCI = Hydrochlorous acid
$HOO^\bullet$ = Hydroperoxyl
HRP = Horseradish peroxidase
HUVEC = Human umbilical vein endothelial cell culture
IDDM = Insulin dependent diabetes mellitus
i.e. = id est (that is)
iNOS = Inducible nitric oxide synthase
IP_3 = Inositol 1,4,5- triphosphate
$LO^\bullet$ = Lipid alkoxyl
$LOO^\bullet$ = Lipid peroxyle
LOONO = Lipid alkyl peroxynitrites
MDA = Malondialdehyde
ME = Mercaptoethanol
mg = Milligram
min = Minutes
ml = Milliliter
mm = Millimeter

LIST OF ABBREVIATIONS (Continue)

μg = Microgram

NADPH = Nicotinic acid adenine dinucleotide phosphate

nm = Nanometer

NO = Nitric oxide

NO_2 = Nitrogen dioxide

N_2O_3 = Dinitrogen trioxide

O_2 = Oxygen

$O_2^{\bullet}$ = Superoxide anion

O_3 = Ozone

OD = Optical density

$OH^{\bullet}$ = Hydroxyl

$ONOO^{\bullet}$ = Peroxynitrite

PBS = Phosphate-buffer saline

PGI_2 = Prostacyclin

PIP_2 = Phosphatidylinositol-4, 5-biphosphate

PKC = Protein kinase C

PVDF = Polyvinylidene difluoride

rGSH = Reduced glulathione

RNS = Reactive nitrogen speices

ROS = Reactive oxygen species

RS = Reactive species

RT = Room temperature

SD = Standard deviation

SDS-PAGE = Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM = Standard errors of mean

LIST OF ABBREVIATIONS (Continue)

SOD = Superoxide dismutase

STZ = Streptozotocin

TBARS = Thiobarbituric acid reactive substance

TXA_2 = Thromboxane A_2

VSMC = Vascular smooth muscle cell

CHAPTER I

INTRODUCTION

Diabetes mellitus has been well characterized for its increased risks of cardiovascular complications, including atherosclerosis, hypertension, and heart failure, etc. Several studies have been suggested that endothelial dysfunction might be the major underlining cause of such complications as well as other cardiovascular heart diseases. Endothelial dysfunction (ED) has also been described for its roles on both macro-and microangiopathy (Aydin A et al., 2001, Chan NN et al., 2000). The impairment of endothelial-dependent vasodilatation, such as acetylcholine (Ach) vasorelaxation, has been defined as one type of diabetic induced ED both in isolated arteries, and in diabetic rats (Kario K et al., 1995; Michael T et al., 1993; Wong KK et al., 1996). The decrease in endothelial derived nitric oxide (NO) has been used for explained as such abnormality of Ach-response. Either decreased NO synthesis or increased NO degradation has been documented as its possible reasons. Several investigations have given the potential supports for oxidative stress to represent as a key factor for the decreased NO. In particular, at least three possible mechanisms that could be resulted to free radical generation as hyperglycemia insulted. As which, those mechanisms are glycation reaction, polyol pathway, and glucose autooxidation (Vanderjagt DJ et al., 2001). There is increasing evidence suggesting that the increased production and/or ineffective scavenging of such reactive oxygen species (ROS) may play a crucial role in determining tissue injury (Aydin A et al., 2001). Recently, increased presence of ROS has also been implicated in the pathogenesis of type 1 diabetes (Santini SA et al., 1997).

Oxidative stress is controlled by antioxidant various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenger components (Aydin A et al., 2001). Antioxidants are generally classified as endogenous antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)/ oxidized glutathione reductase (GSSG-RD) systems), those produced internally by animals and humans, and as exogenous antioxidants (glutathione (GSH), β–carotene, and vitamin E and C), those which the body is not able to produce and which must be provided from external sources. A number of pathologic studies has shown that the production of endogenous antioxidants is not sufficient to protect against excessive oxidative stress. A variety of exogenous antioxidants such as β–carotene, vitamins E and C has proven beneficial in attenuating oxidative stress-associated changes (Radak Z, 2000).

Vitamin C or ascorbic acid (AA) is a water-soluble antioxidant, able to scavenge free radical and inhibit lipid peroxidation (Ashton T et al., 1999). However, levels of vitamin C in plasma and various tissues are decreased in diabetic patients and in animals with experimentally induced diabetes. Cellular deficiency of vitamin C has been implicated in some of the cellular pathology and complications of diabetes mellitus such as angiopathy. It has been suggested that vitamin C supplementation may help to prevent the development of some diabetic complications (Dai S and McNeill JH, 1995). Studies from our laboratory have reported that long term supplementation of vitamin C could markedly prevent the diabetic endothelial dysfunction including the ultrastructural changes of cerebral microcirculation (Jariyapongskul A., 2000).

However, a number of experimental data has been reported for the crucial vasoregulation of endothelial derived NO mediated by fluid sheer stress. The pathway of protein kinase might be considered for its difference from Ach-vasorelaxation. Especially, due to the lack of quantitative method in order to identify the changes of eNOS-protein levels, therefore the effects of Vitamin C to long-term diabetes on the endothelial derived NO mediated by fluid shear stress have not yet been well defined.

Therefore, the major objective of present study is aimed to determine the effects of vitamin C supplementation on eNOS expression in streptozotocin-induced diabetic rats by using the application of our image software in order to clarify the changes of eNOS-proteins.

The hypothesis of this study is vitamin C can protect the diabetic induced reduction of eNOS protein expression.

CHAPTER II

REVIEW LITERATURE

DEFINITION OF DIABETES MELLITUS

Diabetes mellitus, one of the most important world health problem, is characterized by alterations in carbohydrate, fat and protein metabolism which best characterized as a state of chronic hyperglycemia (World Health Organization, 1985), secondary to absent or markedly diminished insulin secretion and/or to ineffective insulin action.

The result of this metabolic disorder, the cell lacks of fuel, makes the body recognize not enough food resulted in triggering a sense of increase hunger, polyphagia (Cotran RS, 1999). The glucose level in blood increases, excess glucose circulates through the kidney and appears in the urine. The body knows when the urine is too loaded with high concentrated glucose, the body will try to dilute it by allowing large amount of fluid to flow through the kidney; polyuria (Cotran RS, 1999). When the fluid is lost, a sense in the thirst center is triggered making the individual drinks more fluid; polydipsia (Cotran RS, 1999). Fundamental to all types of diabetes is impairment of insulin secretion by the pancreatic beta cells. Chemical streptozotocin (STZ), alloxan and vacor and from pancreatitis or surgical pancreatectomy can cause loss of beta cells. In 1995, an International Expert Committee of the American Diabetes Association proposed a classification system that can be divided into five groups as follows:

(1) insulin-dependent diabetes (IDDM) or type I diabetes,

(2) non insulin-dependent diabetes (NIDDM) or type II diabetes,

(3) gestational diabetes mellitus (GDM),
(4) impaired glucose tolerance (IGI) and impaired fasting glucose (IFG),
(5) other specific types of diabetes

INSULIN-DEPENDENT DIABETES MELLITUS (IDDM)

This from of diabetes, previously encompassed by the terms insulin-dependent diabetes, type I diabetes, or juvenile-onset diabetes, result from a cellular-mediated autoimmune destruction of the β-cells of the pancreas (Atkinson MA. And Maclaren NK, 1994). Insulin dependence implies that the administration of insulin is essential to prevent spontaneous ketosis, coma, and death (Srikanta S. et al., 1983). IDDM is also the result from interaction between environmental factors and an inherited predisposition to the disease. The most important of inheritance of susceptibility to IDDM appears to reside in the HLA major histocompatibity complex (Atkinson MA. And Maclaren NK, 1994). The environmental factors that might lead to IDDM, including viral infections, mycobacterial infection and chemical toxin in foods (Atkinson MA. And Maclaren NK, 1994). Nevertheless, IDDM appears to be heterogeneous in terms of the genetic, environmental and autoimmune factors that participate the disease.

Diabetic complication

The long term insulin deficiency of diabetes mellitus can develop chronic complications affecting on multiple organ system. In the eye, retinal capillary damage leads to retinal edema and exudates, new-vessel formation and haemorrhage, while cataracts develop at an accelerated rate; these changes can cause severe visual impairment; retinopathy.

Chronic renal failure can occur because of capillary damage in the glomerulus associated with accumulation and modification of basement membrane and mesangial matrix; nephropathy. In diabetic nerve, tissue functional impairments and structural were changed including segmental demyelination, associated with changes in the vasa nervorum, leading to sensorimotor and autonomic dysfunction; neuropathy. Accelerated atheroma formation in large and medium sized arteries are responsible for premature, frequent and aggressive coronary artery, cerebrovascular and peripheral vascular disease. The final pathologic feature shared by all major diabetic complications is cellular hypertrophy or hyperplasia (Cotran RS, 1999; Porte D, 1997).

ENDOTHELIAL DYSFUNCTION IN DIABETES MELLITUS

Endothelial dysfunction (ED) plays a crucial role in the pathogenesis of diabetic vascular disease. Several studies have demonstrated that the large and resistance arteries of diabetic animals are presence of endothelial dysfunction, which is characterized by the impairment of endothelium-dependent vasodilation (Oyama Y et al., 1986; Mayhan W et al., 1991; Miyata N et al., 1992; Tesfamariam B et al., 1989).

Endothelium

The endothelial cell is an inner layer lining inside blood vessels which plays an important role in the modulation of vascular tone, platelet retardation and leukocyte adhesion, inhibition of vascular smooth muscle migration and proliferation and being a barrier to low density lipoprotein (Moncada S and Higgs A, 1993; Forstermann U et al., 1994). In the part of vascular tone regulation, endothelial cell can produce vasodilators which are nitric oxide (NO), prostacylin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF) and vasoconstrictors which are endothelin (ET), cyclooxygenase-dependent mediators such as thromboxane A_2 (TXA_2), endoperoxide (PHG_2, PGG_2) (Vane JR et al., 1990).

Nitric Oxide (NO)

Nitric oxide (NO) is a free radical molecule enzymatically produced by a number of human cells. It diffuses easily through the tissue, with functional radius of few layers of adjacent cells (Kelm M 1999; Moncada S and Higgs A, 1993). NO plays a critical role in a wide variety of physiological functions, including neuronal transmission, vascular relaxation, immune modulation and cytotoxicity (Kapur S et al., 1997). NO is synthesized by a complex enzymatic process in different nitric oxide synthase (NOS) isoforms. NOS is homodimeric flavo-hemoprotein that catalyzes the 5-electron oxidation of L-arginine to NO+L-citrulline and requires numerous cofactors including calmodulin, NADPH, flavins, heme and tetrahydrobiopterin (Brune B et al., 1998). There are three NOS isoenzymes, neuronal NOS (also known as nNOS, type I, NOS-I and NOS-1) first found in neuronal tissue, inducible NOS (also known as iNOS, type II, NOS-II and NOS-2) which is inducible in a wide range of cells and tissues and endothelial NOS (also known as eNOS, type III, NOS-III and NOS-3) first found in vascular endothelial cells.

These isoforms have in the past been also differentiated on the basis of their constitutive (eNOS and nNOS) (duration:seconds to minutes) versus inducible (iNOS) (duration:hours to days) expression, and their calcium-dependence (eNOS and nNOS) or independence (iNOS and eNOS) (Brune B et al.,1998; Haendeler J et al., 1999). Therefore, this study is focused on the eNOS. All three isoforms, each of which is presumed to function as a homodimer during activation, a carboxy-terminal reductase domain and an amino-terminal oxygenase domain (Alderton WK et al., 2001) (see Figure 1).

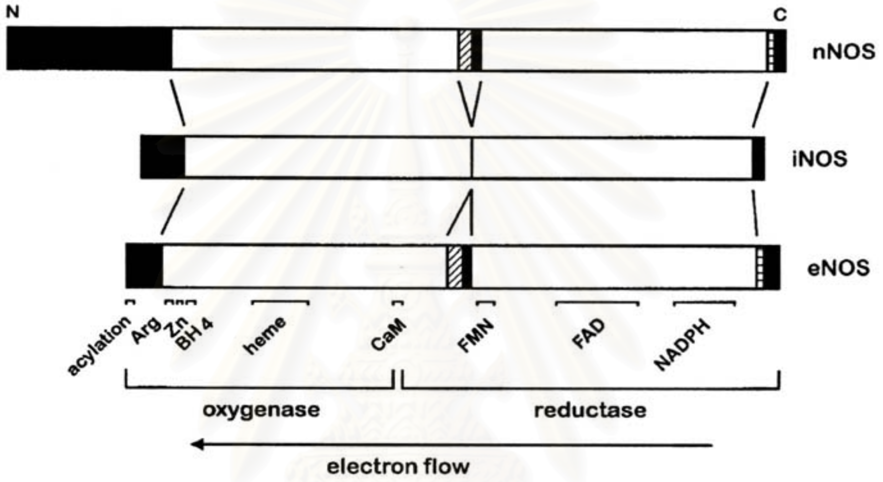


Figure 1 Domain structure of human nNOS, eNOS and iNOS. (From Govers R and Rabelink TJ, 2001)

Endothelial NOS (eNOS)

eNOS has a unique N-myrislylation consensus sequence that may explain its membrane localization and molecular weight is about 140 kDa and was originally characterized in blood vessel endothelium, cardiac myocytes and specialized cardiac conduction tissue (including sinoatrial and atrioventricular nodal tissue), as well as in some formed elements of blood including monocytes and platelets (Balligand JL et al., 1995; Han X, 1996). It plays an important role in regulation of vascular tone (Busse R and Mulsch A 1990; Bredt DS et al., 1991). Physiologically eNOS can be activated various agonists such as acetylcholine (Ach), bradykinin (BK) via an increase in the intracellular concentration of free Ca^{2+} and in the absence of extracellular Ca^{2+} which is activated by fluid shear stress through the Akt-dependent phosphorylation pathway (Dimmeler S et al., 1999; Alderton WK et al, 2001) then NO, it is produced and diffused across to vascular smooth muscle cell (VSMC) to cause vasorelaxation through cyclic guanosine 3’,5’ monophosphate (cGMP) process (Chan NN, 2000).

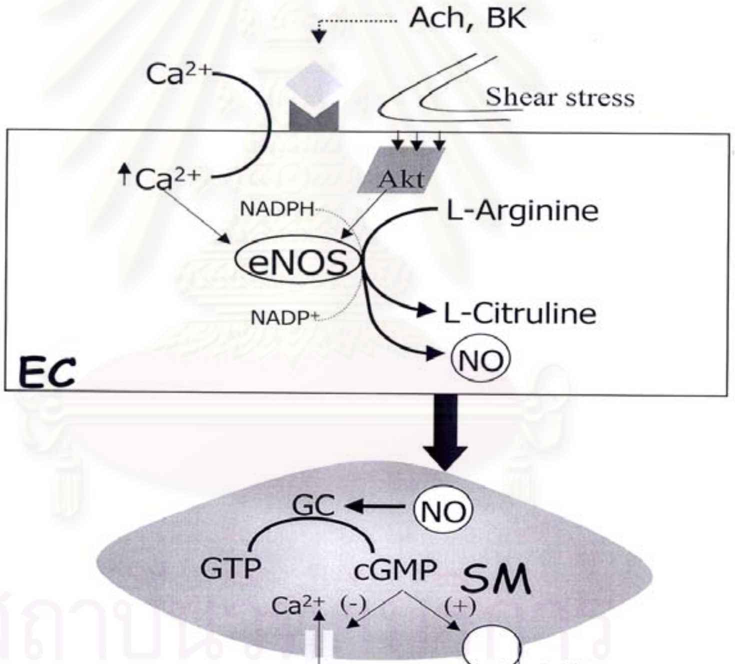


Figure 2 Mechanism of nitric oxide production activated by endothelial nitric oxide synthase through agonist and shear stress pathway.

(Modified from Dimmeler S et al., 1999; Alderton WK et al., 2001)

Endothelial dysfunction

ED exists in many arterial diseases and is characterized by deterioration of endothelial vasodilator function. It can manifest either by decreasing secretion of vasodilatory mediators, increasing production of vasoconstrictors, increasing sensitivity to vasoconstrictors and low resistance of VSMC to endothelial vasodilators (Vapaatalo H, 2001). Moreover, ED is also characterized by vasospasm, inflammation, platelet aggregation, thrombosis, abnormal vascular proliferation, and leukocyte adhesion resulting in atherosclerosis and hypertension. It is important to realize the values of indicators for endothelial functional tests to quantify the severity of disease in individual subjects. Indicators or markers which can be related in ED are NO metabolites, functional test of endothelial-dependent vasodilation (EDV), circulating markers of endothelial function, and adhesion molecules (Vapaatalo H, 2001).

Endothelial dysfunction and diabetes mellitus

The endothelial function may be impaired by risk factors for cardiovascular disease such as hypertension, hyperlipidemia, and especially diabetes.

Accumulating evidence suggests that insulin-dependent and non insulin-dependent diabetes mellitus are associated with impaired endothelial function (Johnstone MT et al., 1993; Williams SB et al., 1996). The impairment of EDV, such as Ach, has been defined as one type of diabetic induced ED both in isolated arteries, and in diabetic rats. (Johnstone MT, 1993; Kario K et al., 1995; Wong KK. 1996).

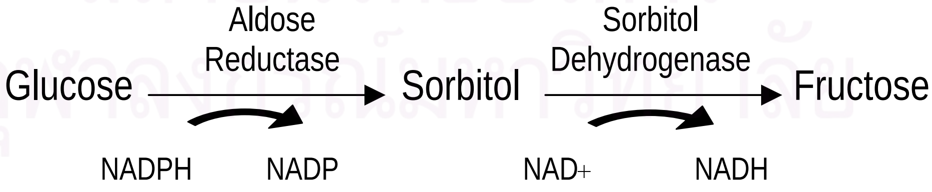
The decrease in endothelial derived NO has been used for explaining the abnormality of Ach-response. Either decreased NO synthesis or increased NO degradation has been documented as its possible reasons. Several investigations have given the potential supports for oxidative stress to represent as a key factor for the decreased NO. (Booth G et al., 2001; Matsuoka H, 2001; Heitzer T et al., 2001).

Mechanisms of endothelial dysfunction in diabetes mellitus.

In particular, at least three possible machanisms that could result in free radical generation as hyperglycemia . As which, those mechanisms are polyol pathway, nonenzymatic glycosylation, and glucose autooxidation. (Vanderjagt DJ et al., 2001; Baynes JW 1991, Kashiwagi A et al., 1996; Giugliano D and Ceriello A, 1996)

1. The polyol pathway

The polyol pathway is governed by aldose reductase which is found in tissues such as nerve, retina, lens, glomerulus and blood vessel wall, in which glucose uptake does not required insulin (Pickup J and Gareth W, 1997). The presence of the polyol pathway (Figure 3), sorbitol is formed from glucose under the influence of aldose reductase and is further metabolized to fructose by polyol dehydrogenase. Aldose reductase has a low affinity for glucose and is operative at low catalytic rate at physiologic glucose concentrations (Porte D, 1997). At normal glucose level, most of the glucose is metabolized to glucose–6-phosphate by hexokinase. When glucose levels rise, however, aldose reductase has a much higher levels for glucose than hexokinase, will make an increasing proportion of the glucose and promoting formation of sorbitol (Keen H, 1999). Sorbitol does not diffuse easily across cell membrane and may accumulate sufficiently within certain cells to cause osmotic damage and swelling. It was suggested that the effect of sorbitol within lens is probably aetiologically important in the development of diabetic cataracts (Lee A and Chung S, 1999). Moreover, the elevation of concentrations of sorbitol in peripheral nerves, schwann cell, are important as a cause of diabetic neuropathy (Keen H, 1999). Furthermore, it was indicated that the polyol pathway associated with the generation of oxygen free radicals. A local excess of those molecules in the vascular system can induce profound endothelial cell dysfunction leading to macro and microangiopathy in diabetes. The association of polyol pathway to increased oxidative stress was described by the perturbation of radical scavenger function, GSH redox cycle, in endothelial cell. Since the polyol pathway is based on aldose reductase enzymes, which can utilized the substrates, a wide variety of sugar-derived carbonyl compounds and reduced these by nicotinic acid adenine dinucleotide phosphate (NADPH) to respective sugar alcohols. If the polyol pathway as continuously and markedly activated, NADPH would be further used for those enzyme reactions, which might also accentuate depletion of NADPH and then further impair H_2O_2 degradation by the gluthathione redox cycle resulting in increased the generation of reactive oxygen species (Kashiwagi A et al., 1994).



(from Barnett AH,1991)

Figure 3 The polyol pathway.

2. Nonenzymatic glycosylation

Glucose can form nonenzymatic glycosylation products such as glycosylated hemoglobin via a nucleophilic addition on glucose to the amino groups of proteins and possibly DNA (Hunt JV et al., 1990; Mullarkey CJ, 1990). This refers to the process by which glucose chemically attaches to the amino group of proteins without the aid of enzymes. Glucose forms chemically reversible glycosylation products with protein (named Schiff bases) that may rearrange to form more stable Amadori-type early glycosylation products, which are also chemically reversible (Cotran R, 1999). The production of these intermediate glycosylated compounds eventually can lead to the formation of advanced glycosylation end-products (AGEs) in a chemical reaction that is irreversible (Hunt JV etal, 1990; Mullarkey CJ, 1990) (Figure 4). These glycosylated proteins can cause changes in cellular functions or generate free radicals that may contribute to further cross-linking and alterations in cellular functions. The major factors that govern formation of these glycosylated products are the level of glucose and the duration of exposure to glucose. Therefore, the AGEs will be formed and accumulated primarily in those macromolecules with a prolonged half-life. For example, collagen, other proteins are found in the basement membrane, and perhaps DNA are particularly disposed to the formation of AGEs because of their slow turnover rates. Vascular and neural tissues also may be particularly susceptible to the accumulation of nonenzymatic glycosylation products because of their slow turnover (Hunt JV et al., 1990; Mullarkey CJ, 1990).

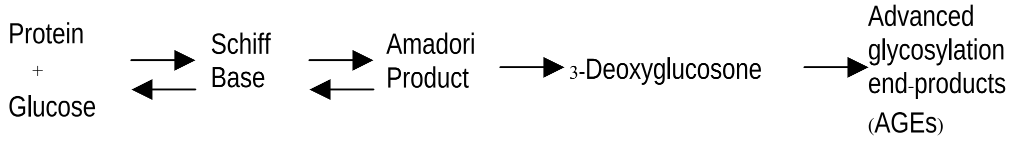


Figure 4 Nonenzymatic glycosylation of proteins.
(Modified form Pickup J and Gareth W, 1997)

A familiar example of a protein glycated in this way is glycated hemoglobin A_1 (HbA1). Considerable attention has been given recently to the post-transcriptional glycosylation of proteins in diabetes, particularly with respect to hemoglobin. Chromatography of adult hemoglobin yields a major fraction (more than 90% of the total) of hemoglobin A in front of which are three fast fractions, HbA_{1a}, HbA_{1b} and HbA_{1c}–the glycosylated hemoglobins (Keen H, 1999). These three hemoglobins accumulate during the life span of the red blood cell. HbA_{1c} comprises 4% to 6% of HbA, with the other fractions conprising 1-2% each. These glycosylated hemoglobins are formed nonenzymatically at a rate dependent on the ambient glucose concentration.

HbA_{1c} has been best characterized. Glucose combines with the N-terminal valine of the β-chain of HbA to yield an aldimine. This spontaneously undergoes the Amadori rearrangement to yield a ketoamine-the terminal product being 1-amino, 1-deoxyfructose (Keen H, 1999).

3. Glucose autooxidation

The term glucose autooxidation describes the capability of glucose to enolize, thereby reducing molecular oxygen and yielding oxidizing intermediates (Giugliano D and Ceriello A, 1996). It has been suggested that glucose autooxidation and nonenzymatic glycation, together termed glycoxidation, are the major contributors to the increase in free radicals in diabetes (Lee A and Chung S, 1999). Glycoxidation products may be considered biomarkers of carbohydrate-dependent damage to protein and indicators of the extent of underling chemical modification, oxidation, and cross-linking of tissue protein caused by reducing sugars. Furthermore, because these products accumulate in collagen normally as a function of age and at an accelerated rate in diabetes, diabetes may be legitimately described, at the chemical level, as a disease characterized by accelerated aging of collagen by both glycative and oxidative mechanisms. Individual differences in the accumulation of glycoxidation products in collagen (2-to 3-fold ranges at ages 60-80 yr in both diabetic and nondiabetic populations) suggest a wide variation in individual susceptibility to damage, an observation that might yield insight into the basis for individual differences in susceptibility to development of complications (Baynes JW, 1991).

OXIDATIVE STRESS

Oxidative stress has been defined as a disturbance in the balance between antioxidants and pro-oxidants (free radicals and other reactive oxygen and nitrogen species), with increased levels of pro-oxidants leading to potential damage (Sies H, 1991; Halliwell B, 1997). Severe prolonged oxidative stress is harmful, and may contribute to the development of ED.

Reactive oxygen and nitrogen species

The most known reactive species are free radicals. A free radical is any atom or molecule that contains one or more unpaired electrons

(Halliwell B and Gutteridge JMC, 1999). An unpaired electron is an electron that occupies an orbital alone, but electrons usually associate in pairs in orbitals of atoms and molecules. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive. Not only free radicals but also non-free radical compounds can cause a redox imbalance (oxidative damage). Free radicals are generally more reactive than non-radicals due to their unpaired electron, but different types offree radicals vary widely in their reactivity (Halliwell B and Chirico S, 1993; Slater TF, 1984).

Both free radicals and reactive non-free radical compounds are collectively called reactive species (RS). RS are divided into reactive nitrogen species (RNS)-derivates on the basis of nitrogen and reactive oxygen species (ROS)-derivates on the basis of oxygen.

ROS are superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$), lipid peroxyle ($LOO^{\bullet}$), lipid alkoxyl ($LO^{\bullet}$) and hydroperoxyl ($HOO^{\bullet}$) as free radicals, and hydrogen peroxide (H_2O_2), hydrochlorous acid (HOCI), ozone (O_3), singlet oxygen (O_2) and hydroxy alkenals as oxygen-based reactive non-radicals.

RNS are nitric oxide (NO), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^{\bullet}$) as free radicals and nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3) and lipid alkyl peroxynitrites (LOONO) as nitrogen-based non-radicals.

There are also hydrogen radical ($H^{\bullet}$), the carbon-centered radical ($R^{\bullet}$) and trichlromethyl radical ($CCI_3^{\bullet}$) (Halliwell B and Chirico S, 1993).

If two free radicals meet, they can join their unpaired electrons and make a covalent bond. Most molecules in the body are not radicals. Hence, any reactive free radical generated is likely to react with a non-radical. When a free radical reacts with non-radical, a free-radical chain reaction results and new radicals are rapidly formed.

Attack of reactive radicals on membranes or lipoproteins starts lipid peroxidation, which is particularly implicated in the development of atherosclerosis (Halliwell B and Gutteridge JMC, 1999). If hydroxyl radicals are generated close to DNA, they can attack the purine and pyrimidine bases and cause mutations (Dizdarglu M, 1991).

Free radicals do not only exert disadvantageous effects, but are also formed deliberately in the body for useful purposes and have important physiological functions. One of the well-defined roles of free radicals is when activated phagocytic cells produce superoxide anion radicals and hydrogen peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses (Halliwell B and Gutteridge JMC, 1999).

In a biological system, free radical attack takes place in the presence of an unbalanced ratio between free radicals and antioxidants.

Antioxidants

Protection against free radicals attack can be achieved by prevention of free radical formation, blocking of chain reactions or repairing the oxidatively damaged biomolecules (Halliwell B and Gutteridge JMC, 1999). There are a number of antioxidants present in the body and derived from the diet. Based on the location, they can be divided into intracellular and extracellular antioxidants (Gutteridge JMC, 1995; Rice-Evans C and Burdon R, 1993). Intracellular enzymatic antioxidants are SOD, CAT and GPX that convert potential substrates (superoxide anion radicals and hydrogen peroxide) to less reactive forms in the body (Gutteridge JMC 1995; Rice-Evans C and Burdon R, 1993). Main non-enzymatic cellular antioxidant is rGSH.

Several extracellular antioxidants such as proteins (transferrin, lactoferrin, albumin, ceruloplasmin) and urate prevent free radical reaction in the body sequestering transition metal ions by chelation in plasma. Albumin, bilirubin and urate may also scavenge free radicals directly. Furthermore, plasma has a considerable peroxyl radical scavenging ability, which is mainly determined by its content of ascorbic acid (Gutteridge JMC, 1995; Rice-Evans C and Burdon R, 1993; Frei B et al., 1989).

Some antioxidants are located both intra-and extracellulary, such as α-tocopherol (vitamin E), which is the major lipid-soluble antioxidant, present in cellular membranes and in plasma lipoproteins. It is an effective chain-breaking antioxidant that protects polyunsaturated lipids from peroxidation by scavenging peroxyl radicals (Halliwell B and Gutteridge JMC, 1999).

Oxidative stress and endothelial dysfunction in diabetes mellitus

Several evidences have indicated that the generation of oxidative stress may play an important role in the pathology of diabetic vascular complication (Baynes JW, 1992). Therefore the oxidative stress induced by hyperglycemia is implicated as a source of altered endothelial-dependent vasodilation in diabetes (Tesfamariam B and Cohen RA, 1992). Furthermore, the other report found that the endothelial-dependent vasodilation in the aorta of STZ-induced diabetic rats was due to accumulation of O_2^-. (Hattori Y and Kawasaki H, 1991) which confirmed previous report that oxidative stress inactivated endothelium-derived relaxing factors (Gryglewski RJ et al., 1986) and selectively attenuated EDV. (Pieper GM and Gross G, 1988).

Vitamin C

Vitamin C, also known as ascorbic acid or ascorbate, is an essential water-soluble vitamin (Manore M and Thompson J, 2000).

Structure

Vitamin C comprises essentially two compounds, L-ascorbic acid , a strong reducing agent, and its oxidized derivative L-dehydroascorbic acid (DHA). Although most vitamin C in body fluids and tissues is in its reduced form, both ascorbic acid and DHA have biological activity, and are interconvertible by an oxidation and reduction reaction (Figure 5). Some of the enzymes responsible for these interconversions include glutathione dehydrogenase and ascorbate oxidase (Washko PW et al., 1992).

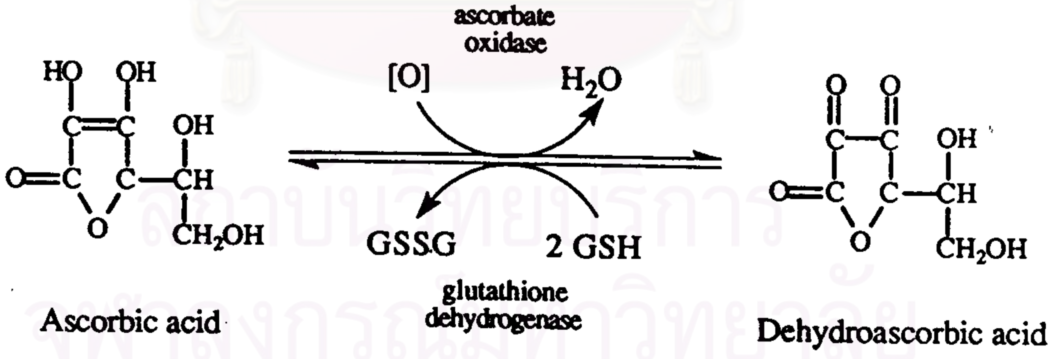


Figure 5 Interconvertibility of ascorbic acid by oxidation and reduction.
(From Tapan KB, 1996)

Absorption, Transport and Storage

The absorption of vitamin C in humans occurs in the buccal mucosa, stomach and small intestine. After absorption, vitamin C rapidly equilibrates in intra- and extracellular compartments. Although no particular organ acts as a storage reservoir for the vitamin, tissues such as the pituitary and adrenal glands, eye lens and leukocytes are concentrators of vitamin C. Vitamin C exists in blood and tissues mainly in the reduced form; its oxidized form is generally less than 10% (Tapan KB, 1996).

Vitamin C is not stored in single tissue deposits as is vitamin A. Rather it is more generally distributed throughout the body tissues, maintaining a tissue saturation level. Any excess is excreted in the urine. The tissue levels relate to intake, and the size of the total body pool adjusts to maintain balances (Williams SR, 1994). The overall metabolism of vitamin C is affected by the level of its intake. At a physiological level (30 mg), less than 10% is excreted in the urine as ascorbic acid and more than 90% as metabolites, whereas a reverse is seen when a large dose level of vitamin C (1-2 g) is ingested. The capacity of kidney tubules for reabsorption saturates at plasma concentrations of vitamin C below 0.8 mg/dl and most is lost in the urine within 24 hours (Tapan KB, 1996).

Functions

Vitamin C has several important functions as related to physical activity. The vitamin has long been known to be necessary for normal collagen synthesis. Vitamin C is needed for the formation of the vitamin-like compound, carnitine. The neurotransmitters, norepinephrine and epinephrine, also require vitamin C for their synthesis. Vitamin C seems to be needed for the proper transport of nonheme iron, the reduction of folic acid intermediates, and for the proper synthesis and/or release of the stress hormone, cortisol. Finally, vitamin C acts as a powerful water-soluble antioxidant. The vitamin seems to exert antioxidant functions in plasma and probably interfaces at the lipid membrane level (Wolinsky I and Driskell JA, 1997).

Ascorbate may be involved in reducing damage to the cell from radicals. A simplified mechanism (Figure 4) shows the $OH^{\bullet}$ reacting with a component in the cell, abstracting (pulling off) a hydrogen radical ($H^{\bullet}$). The product is a radical, but it is one that is more stable than $OH^{\bullet}$. Ascorbate may donate a $H^{\bullet}$ to this product, thus repairing it before further deterioration can occur. Here, the ascorbate is converted to a semidehydroascorbate, a relatively stable radical, which can be enzymatically reduced back to ascorbate. Moreover, it is thought that ascorbate can react with the vitamin E radical and regenerative vitamin E in its original (Brody T, 1994).

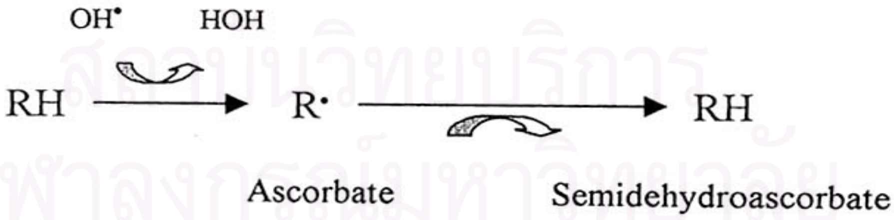


Figure 6 Possible use of ascorbate in reducing damage from radicals. (Modified from Brody T, 1994)

The suggested antioxidant actions of vitamin C include the following (Manore M and Thompson J, 2000):

- Stabilizes the hydroxyl radical
- Quenches singlet oxygen
- Reduces the oxidized form of vitamin E
- Reduces nitrosamines to harmless species
- May help protect the lungs from ozone and cigarette smoke

Vitamin C and diabetes

Abnormal endothelial function has been observed in patients with conditions predisposing to the development of diabetes. The mechanisms of endothelial dysfunction in diabetes individuals are not clear, but there is strong evidence that inactivation of nitric oxide by increased oxygen-derives free radicals could be responsible (Lekakis JP et al., 2000). There is evidence to suggest that oxidative stress is increased in human with diabetes and in animal models of diabetes (Giuglano D and Ceriello A, 1996). Moreover, evidence for oxidative stress in diabetes includes observations of decreased antioxidant plasma concentrations in both diabetes subjects and animal models of diabetes (Kashiba M et al., 2000).

Vitamin C is a naturally occurring major antioxidant that is essential to the scavenging of toxic free radicals in both plasma and tissues (Kashiba M et al., 2000). El-Missiry MA (1999) suggested that vitamin C has a protective effect on alloxan-induced damage by maintaining the activity of cellular antioxidants. It bluntes the increased lipid peroxidation in alloxan diabetic rats by protecting antioxidants enzymes. Nazirogly M et al. (1999) studied the effect of selenium, vitamin C and E on the lipid peroxidation, GPX and rGSH activities in the lens of STZ rats. They suggested that vitamin C and E and selenium can protect the lens against oxidative damage but the effect of vitamin C appears to be much greater than that of vitamin E and selenium. There are studies, however, which suggest that vitamin C levels in plasma and tissues have been reported to be significantly lower than normal in diabetic animals and humans (Lindsay RM et al., 1998). Chronic hyperglycemia may impose an intracellular deficit of vitamin C through competitive inhibition of membrane transport of vitamin C by the elevated plasma glucose (Dai S and McNeill JH, 1995). It was found that vitamin C concentrations of the brain, heart, lung, liver, kidney, and plasma of the diabetic rats decreased significantly after 8 weeks compared with those of the control group (Sun F, 1999). Vitamin C deficiency has been implicated in some of the complications of diabetes such as angiopathy (Dai S and McNeill JH, 1995). Therefore, vitamin C supplementation may be beneficial to diabetes. In 1997, Siman CM and Eriksson UJ reported that vitamin C supplementation yielded increased α-tocopherol concentration in the placenta and caused a reduction of the high concentrations of thiobarbituric acid reactive substance (TBARS) in serum of pregnant diabetic rats. According to Ting HH (1996), they found that abnormal endothelial function in type 2 diabetes has been restored by treatment with vitamin C supplementation. Since, diabetes mellitus in man and in experimental animals are associated with elevated plasma lipid level, in particular triglycerides, it was also showed that the production of hyperlipidemia in diabetes had prevented by vitamin C supplementation (Dai S and McNeill JH, 1995).

As regards the literature review from above, it might be summarized the idea as that the generation of ROS (oxidative stress) may play an important role in the etiology of diabetic complications.

CHAPTER III

MATERIALS AND METHODS

1. Animal preparation

Male Spraque-Dawley rats (National Laboratory Animal Center of Salaya Campus, Mahidol University,Thailand) weighing 200-250 g , 7-week old were divided randomly into three groups:

1. Control group (N=20) The rats in this groups were normal rats that received a single intravenous injection of citrate buffer (50 mg/kg BW.)
2. Diabetic group (N=20) was induced by a single intravenous injection of STZ (50 mg/kg BW) that lead to hypoinsulinemia and hyperglycemia.
3. Diabetic treated vitamin C group (N=20) was induced the same way as described for the diabetes group. Supplementation of rats with vitamin C (L-ascorbic acid, 99%, Sigma Chemical Co., USA) started 48 hours after administration of STZ. Vitamin C was prepared daily by dissolving in drinking tap water at concentration of 1 g/L and the experimental rats were freely accessed to this vitamin C drinking water (Dai S and McNeill JH, 1995).

All animals were fed with regular dry rat chow and allowed freely assess to drinking water. In this study each group was further divided into 2 subgroups according to the time of collecting the specimens i.e, at the 12 weeks (n=10) and 24 weeks (n=10).

2. Diabetic induction

To induce diabetes mellitus, STZ (Sigma Chemical Co., USA) was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co., USA) and immediately single injected into the tail vein of 8-hour fasted rats, at a dose of 50 mg/kg BW. Blood glucose (BS) was determined by using glucometer (Advance Glucometer, Bochringer Mannheim, Germany). Samples were analyzed by applying a drop of blood to a prepared strip. Rats treated with STZ that did not exhibit an elevation of BS level at 48 hours greater than 200 mg/dl were excluded from the study. In addition, diabetic condition was also confirmed by rat’s manifestation of polyuria, polyphagia, and polydipsia.

4. Tissue harvest

The experiments were performed at 12 and 24 weeks after injection of citrate buffer or STZ. On the day of experiment, rats were weight (BW) and anesthetized with an intraperitoneal injection of sodium pentobarbital (50mg/kg BW). Blood samples were withdrawn from the carotid artery for the measurements of BS and plasma vitamin C level by using glucometer. (Advance Glucometer, Bochringer Mannheim, Germany) and enzyme-assisted spectrophotometric method, respectively. Both BS and plasma vitamin C were analyzed by Research center, Ramathibodi Hospital, Mahidol University. Then the right lung, heart and aorta were isolated, cleaned of extraneous tissue, and washing in ice-cold phosphate-buffer saline (PBS) and stored at -70^{o}C until analyses.

5. Protein extraction

Tissue samples were cut with medium-sized scissors on ice block, then tissue was placed in test tube containing homogenization buffer (10 mM Tris-HCl buffer (pH 7.4), 255 mM sucrose, 2 mM EDTA, 12 µM leupeptin, 4 µM pepstatinA, 1 µm aprotinin. And 2 mM phenylmethylsulfonyl fluoride (PMSF)), then homogenized in an ice bath using the homogenizer by turning a rotor at 12,000 rpm for 3 min.

Homogenates were centrifuged at 2,000 rpm at 4^{o}C for 40 min to remove tissue debris. After that, the supernatant was collected and aliquoted into sterile microcentrifuge tubes, kept -70°C until used. All samples will be further assayed for the amount of protein and Western blot analysis.

6. Protein assay

One of the theoretical difficulties with measuring protein concentration is the chemical basis of the method. All the proteins have in common the peptide bond between each amino acid residue, and yet it is not easy to find a versatile and sensitive methods which measure exclusively the peptide bond. Most methods give different results with different proteins depending on their amino acid composition, and some reagents also react with certain amino acid side-chain. Since the 1950 when Lowry and coworker modified the method originally devised by Folin and Ciocalteu, the Lowry method (Lowry OH et al., 1951) has become the main method for protein determination in our experiment.

The principle lies in the reactivity of the peptide bond of protein with the copper II ion (divalention) under alkaline conditions and reduced to copper I ion (monovalention). Copper I ion and the radical groups of tyrosine, tryptophan, and cysteine amino acid side chains of protein (the protein-copper complex) react with the folin phenol reagent (phosphomolybdic-phosphotungstic acid) to produce an unstable product that heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acid to turn the color to a blue and analyzed by visible spectroscopy which is able to measure light absorption or transmission. Unknown protein concentration was quantitated by comparing the blue color of its own to the color values drived from a standard curve of a standard protein bovine serum albumin (BSA).

6.1 Lowry Method

This assay is designed to quantify 1 to 100 μg/ml protein. A standard curve is an absorbance (A) at 670 nm versus BSA (Sigma Chemical Co., USA) concentration (μg/ml) confirm to Beer’s Law (it’s a straight line passing through the origin). Determine the line of best fit for the data by linear regression (y=ax+c) along with an r^2 value. Then use the linear regression equation to determine the protein content of samples based upon absorbance as follows: -

Set up a series of standards (in duplicate) which contain: 10, 25, 50, 75, 100 μg/ml of BSA indicated on data sheet.

The BSA was prepared as stock at concentration of 1 mg/ml.

Standard Curve Preparation.

Tube	BSA (μl)	DW (μl)	BSA (μg/ml)	A670
1	0	500	0	
2	50	450	10	
3	125	375	25	
4	250	250	50	
5	375	125	75	
6	500	0	100	

Note: DW = Double distilled deionized water

6.2 Sample preparation

Thaw samples on ice and mix by vortexing. Using a micropipette, accurately pipette 40 μl of each sample stock into the appropriate microcentrifuge tube, add 1,160 μl of homogenization buffer (1:30 dilution), and mix well.

6.3 Chemical substances and reagents

1. Solution A (alkaline tartrate reagent)

$NaCO_3$	10.0	g
$Na_2C_4H_4O_6.2H_2O$	0.1	g
NaOH	1.2	g
DW	500	ml

2. Solution B (0.5% copper sulfate)

$CuSO_4.5H_2O$	0.5	g
DW	100	ml

3. Solution C

   Solution A: solution B 50:1

4. Solution D (1 N folin phenol reagent)

   2 N folin ciocalteu phenol reagent : DW 1:1

5. Standard protein solution

   BSA 1 mg/ml

   Range 10-100 µg/ml

6.4 Procedure

Five standards dilution in duplicate (10,25,50,75,100 µg/ml) were prepared by dissolving the stock standard BSA solution (1 mg/ml) as indicated in 6.1. 0.5 ml of each standard dilution, sample dilution and blank tubes (DW) were added into the clean 13 x 100 mm test tubes. Then 5 ml of solution C in each tube were mixed by vortexing and incubated at room temperature (RT) for at least 30 min. After that, quickly added 0.5 ml of solution D to each tube, vortexed immediately, and allowed to stand 30 min at RT. The absorbance of each solution was read and recorded at 670 nm against a reagent blank by using 1 cm cuvettes. The excel spreadsheet and the linear regression equation of a standard curve were used for the Lowry assay to calculate the concentration of protein in each sample.

7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The most widely used method for qualitative analysis of a protein mixture is SDS-PAGE using the buffer system of Laemmli UK (1970). With this method, it is possible to determine both the purity and the relative molecular weight of an unknown isolated protein or proteins. In the process, proteins migrate in response to an electrical field through pores in the gel matrix and separate based on molecular size after sample proteins are solubilized by boiling at 100^0C, 5 min in the presence of anionic detergent, SDS detergent and 2-Mercaptoethanol (2-ME). The 2-ME is a disulfide reducing agent, and serves to reduce and disulfide bridges holding together the tertiary structure of the protein. The anionic SDS detergent binds strongly to the protein thus disrupting its secondary, tertiary and quaternary structure, resulting in a linear polypeptide chain coated with negatively charged SDS molecules. The binding efficiency of the SDS is generally one SDS molecule for every two amino acid residues. Under this condition, the polypeptide chains are unfold and assumed a rod-like structure and have negative charge, resulting in a constant charge to mass ratio. Then proteins move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. Separation is determined by size and therefore when compared to standards of known molecular weight, the relative molecular mass can be estimated. Purity is determined by the presence of a band associated with the desired protein and the absence of bands associated with contaminating proteins.

The SDS gel is comprised of a main separating gel and a stacking gel. The proteins, which have been mixed with a loading buffer, containing an ionizable tracking dye bromophenol blue, are loaded into wells formed in the stacking gel. A current is passed through the gels and the proteins migrate through the stacking gel and are concentrated into a solid band at the separating gel. When the proteins enter into the separating gel, the negatively charged protein-SDS complexes migrate toward the anode. Their migration in the gel relative to each other is the same based on their uniform negative charge. Separation, therefore, occurs as a result of the molecular sieving properties of the gel. The larger the protein, the more its mobility is retarded by the frictional resistance of the gel the smaller the molecule the further its mobility in the gel. The bromophenol blue is completely unretarded in the gel due to its small size relative to proteins and it is thus used to monitor the progress of the electrophoresis. The current is turned off once the tracking dye has migrated to the bottom of the gel. The gel is removed from between the plates and the stacking gel discarded. The separating gel is stained by shaking it in a solution of Coomassie Brilliant Blue for a few hours and then destained by shaking overnight to remove the unbound dye background. The proteins are visible as blue bands on a clear background.

7.1 Assembly of apparatus

The reagents used in preparing the gel should be removed from the refrigerator and allowed to warm and degas for one hour prior to the preparation of the gel. While this is happening, set up the “sandwich” plates for casting the gel itself. A “sandwich” consists of one outer is a rectangular back plate with the rounded bottom corners, measuring 10x11.5 glass plate and one inner is same size but with a notch 1.1 cm deep and 10.4 cm long separated by plastic spacers of equal thickness

(0.5 mm). In order to prepare a flawless gel, one containing has no air bubble or debris, the glass plates must be perfectly cleaned with liquid detergent, rinse with distilled water and then dry with absolute ethanol. The plates were wiped with gauze. To protect the bubble airs, the edge side of outer glass plates were sealed by gasket. After that, the casting clamps were used to mount the outer and inner glass plates facing together.

7.2 Preparation of Slab gel

The polyacrylamide gel was prepared using N,N'-methylenebisacrylamide as cross-linker in the amount corresponding to 2.6% of the weight of acrylamide.

The separating gel contain 7.5% acrylamide (see Appendix A) was carefully poured between the plates so that it flows down the slide between plates and fills from bottom to top with no air bubbles. The height of the separating gel was adjusted by the comb. Those height of the gel should be approximately 1 cm below the bottom of the comb. The top layer was filled with a small amount of DW using a syringe for aiding the formation of a smooth interface. Add water to a height of 2-3 mm. The polymerize was allowed to incubate at RT for at least one hour. After the separating gel is solid (polymerize) then the 5% stacking acrylamide (see Appendix A) gel would be prepared. The water was drained off and excess liquid was removed with a piece of Whatman 3 MM paper. The correct comb (number of wells, thickness same as spacers) with ten teeth was immediate gently inserted between the surfaces of lower gel to create lanes for adding sample. It should be made sure that no air bubbles form around the teeth of the comb, as they will impede the migration and separation of the proteins. Then leaving the gel for one hour at RT prior to electrophoresis were performed.

7.3 Preparation of sample

While the stacking gel is polymerizing, prepare the sample by dilute to 1 μg/μl with homogenization buffer (see Appendix A). Use spreadsheet to calculate volumes. The samples are prepared to a final volume of 10 μl by adding 5 μl of each protein to 5 μl sample buffer (see Appendix A) containing a tracking dye (bromophenol blue), preferably mix in capped microcentrifuge tubes. Heating the samples for five min to 100^{o}C in a boiling water bath were performed to denature the protein. Do not cap the tubes as they will explode from the pressure created during boiling. The treated protein solution could be kept at 4^{o}C until loaded on gel. Unused sample can be stored at -20 ^{o}C and boiled again before using. The markers and eNOS protein standards do not need to be boiled as recommended by the Amersham pharmacia biotech company, UK.

7.4 Electrophoresis

After polymerization is complete, gently remove the comb, being careful not to rip any of the lanes and remove the bottom spacer, mount the gel in the electrophoresis apparatus so that the notch in the inner glass plate was next to and lined up with the notch on the upper buffer chamber which had been filled with Tris-glycine electrophoresis buffer, pH 8.3 (see Appendix A). Remove any bubbles that become trapped at the bottom of the gel between the glass plates. Air bubbles will interfere by causing disruptions in the electrical circuit and an uneven electrophoresis. This is done with a bent hypodermic needle attached to a syringe.

10 μl which equal to 5 μg of total protein extracts of each of samples were loaded into the bottom lanes by using pipette tips. When loading the wells, be sure that the tip of the gel loading is between the plates and directly over the desired well before dispensing the sample to prevent contamination of neighboring wells. Add 10 μl of the High

Molecular Weight (HMW) Calibration Kid marker (Amersham pharmacia biotech, UK) was added and 10 μl of eNOS protein standard; Human aortic endothelial cell lysate (HAEC) which stock solution is 1μg/μl (Transduction Laboratories, UK), and positive control (lung tissue extracts from male Wista rats, n=5), to separate lanes. The unused lanes were filled with an equal volume of sample buffer. Do not add samples to the two outside lanes of the gel since these lanes may become distorted during electrophoresis. If possible, avoid adding HMW marker and protein standard to outside lanes as well, keeping the outside lanes filled with sample buffer only. The electrophoresis apparatus was attached to an electric power supply. The power supply was turned on at 150 volt. Small bubbles should start to be produced and rise off the electrode wire at the bottom of the lower chamber. If no bubbles appear then there is an electrical circuit problem. The gel was run until the dye front reaches the bottom of gel, approximately one hour. Then the power supply was turned off. The orientation of the gel was marked by cutting a corner from the bottom of the gel. After that the gel from the glass plate was removed into a staining dish or transfer buffer for transfer protein membrane.

7.5 Staining and Destaining

Polypeptides separated by SDS-PAGE can be simultaneously fixed with methanol: glacial acid and stained with Coomassie Brilliant Blue (R-250, Sigma Chemical Co., USA) (see Appendix A). The gel was covered with Coomassie staining solution and placed on the shaker and allowed the gel to stain for at least two hours. Decant off the dye and add destaining solution to cover the gel were performed. Excess background stain was removed by soaking the gel in several changes of destaining solution (see Appendix A) until the background color was clear. This may take several hours. The proteins will appear as dark blue bands on a colorless background.

7.6 Drying

When destaining was complete, the gel was washed briefly in DW, transferred onto a cellophan sheet which is arrange on glass plate and place another piece of sheet on surface of the gel. The piece of cellophan sheet should be large enough to accommodate all of the gels that are to be dried at the same time. Then seal around the gel with clamps. Gently smooth out any air bubbles between sheets, which will interfere when drying. Leave the gel at RT for overnight.

7.7 Molecular Weight Determination

The molecular weight of an unknown protein was estimated by comparing its mobility to the mobilities of known standards run on the same gel. A standard curve was constructed by plotting the relative mobilities of standard protein markers versus their log molecular weights on semilogarithmic graph paper.

Relative mobility of the polypeptides was calculated according to the following formula;

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

The standard protein marker used in this study was the HMW Calibration Kit for SDS electrophoresis (Amersham pharmacia biotech, UK)was composed of Myosin, 220 kDa ; α_2-Macroglobulin, 170 kDa ; β-Galactosidase, 116 kDa ; Transferrin, 76 kDa ; and Glutamic dehydrogenase, 53 kDa. The standard curve is illustrated in Figure 7.

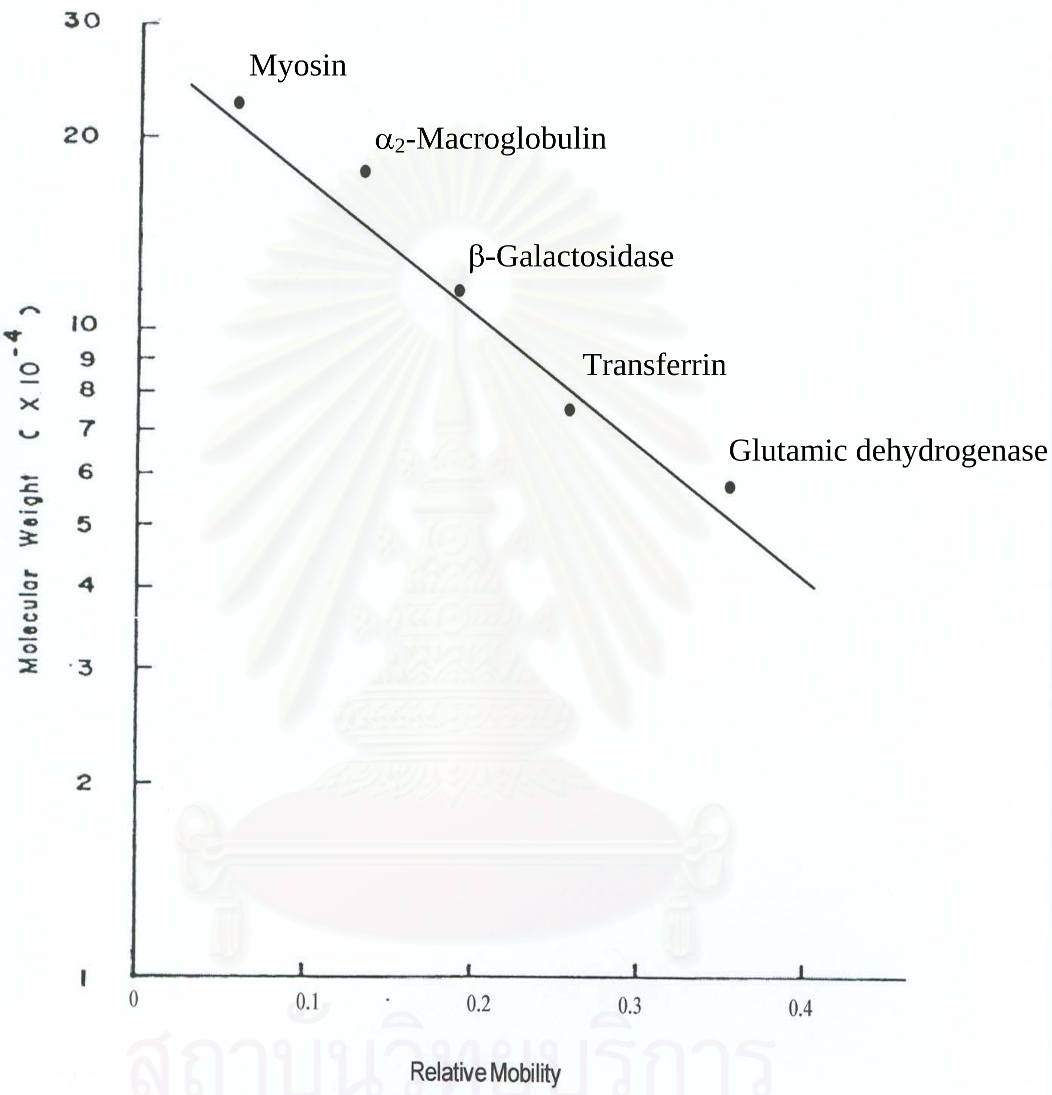


Figure 7 A calibration curve for molecular weight estimation by SDS-PAGE.

8. Immunoblotting technique

In this study, immunobloting was used to detect the separated proteins in SDS-PAGE.

Western blot analysis is the most commonly used immunochemical technique, which is Towbin, in 1979, developed for studying protein function and localization. Unlike the blotting techniques of Southern and Northern, which are transfered of nucleotides.

In a Western blot, proteins are electrophoretically separated on an acrylamide gel, then transferred to a membrane detected with one or more antibodies. The antibody detection technique may be direct (an enzyme-conjugated tag-specific antibody) or indirect (first with an unconjugated tag-specific antibody, and with an enzyme-conjugated antibody). Then suitable enzyme substrates must produce a signal on the membrane at the site of the enzyme-conjugated antibody by using chemiluminescent (signal recorded on X-ray film) or chromogenic visualization (signal recorded on the membrane).

In this study, eNOS antigen extract was used as an antigen source.

8.1 Protein transfer

The set of protein transfer unit is composed of: Sponge based for electroblotting, Whatman 3 MM paper and transfer buffer (detail in Appendix A). After electrophoresis method, which has been done as described before, then an SDS-PAGE gel was placed into the set of protein transfer unit covering the SDS-PAGE gel with polyvinylidene difluoride (PVDF) membrane. Then cut off the lower right corner of PVDF membrane to identify the gel and membrane orientation, and must be prewetted in absolute methanol for a few seconds, rinsed in DW. During the protein transfer it has to be careful to exclude any air bubbles between layers, which will interfere with transfer. The sandwich was placed between support pads provided with the transfer apparatus and inserted into the transfer device so that the membrane is closest to the side of the positive electrode (anode). The sandwich was transferred for one hour at 100 volt. Then a current passed at right angles to the gel, which causes the separated proteins to electrophoresis out of the gel and into the PVDF membrane. This membrane is called the “blot”. After transfer, the membrane is removed from the sandwich and rinsed briefly in TBS (see Appendix A). The membrane can be stored dry, can be stained with Amido black stain solution (see Appendix A) to visualize transferred protein, or can be used directly in next step (blocking).

8.2 Blocking

After, the protein was transferred to the PVDF membrane, then it can be used for probing. The sensitivity of Western blotting depends on reducing this background of nonspecific binding by blocking potential binding site with irrelevant protein. Place the PVDF membrane in a container, and add blocking solution (5% non-fat dry milk, 1% BSA), incubate for two hours at RT with gentle agitation on a platform shaker. After blocking, the membrane was washed 1 time 15 min, then 4 times 10 min with 120 ml of TTBS (see Appendix A).

8.3 eNOS antibody detection

The dilution of the primary antibody was prepared in TTBS. For eNOS detection, mouse eNOS monoclonal antibody (Transduction Laboratories, UK) at 1:2500 (4 μl in 10 ml) was used. Then the membrane was placed in a heat-sealable plastic bag. It was added 0.1 ml of primary antibody solution per square cm of membrane and incubate overnight at 4^{o}C. After incubation the plastic bag was cut and the primary antibody solution was discarded. Washing the membrane was performed with 120 ml of TTBS in the period of 15 min for 1 time and 10 min for 4 times. The secondary antibody was prepared by TTBS with 1% BSA. For eNOS antibody mentioned above, use goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) conjugate (Bio-Rad, USA) at 1:10,000 (1 μl in 10 ml). The membrane from the final wash in TTBS was transferred to a heat-sealable plastic bag contain 0.1 ml of fresh secondary antibody solution per square cm of membrane. Then the membrane was incubated for two hours at RT with gentle agitation on a shaker. After that the bag was cut the bag and the membrane was moved to a container, wash in 120 ml of TTBS 1 time 15 min, 4 times 10 min, and 1 time 60 min with gentle agitation on a platform shaker.

8.4 Enhanced chemiluminescence (ECL) detection

The ECL detection system is a light emitting non-radioactive method for detection of immobilized specific antigens, conjugated directly with HRP-labelled antibodies. The chemiluminescent reaction of cyclic diacylhydrazides such as luminol has been widely used in chemical analysis and extensively studied. HRP is often used to catalyze the oxidation of luminol in the presence of hydrogen peroxide (H_2O_2). Immediately following the oxidation, the luminol is in an excited state, which may decay to the ground state via a light emitting pathway.

Prepare ECL solution (see Appendix A) by combining solution A and solution B sufficient to cover membrane (use 6 ml of each solution per membrane). In a dark room, washed membrane was drained of excess buffer from and place in a fresh container. Then the detection reagent was directly added to the membrane on the surface carrying the protein; do not leave the membrane to dry out. After incubating for precisely one min at RT, the excess detection reagent was drained off and the membrane was gently placed, protein side up, on a piece of Saran Wrap. A piece of Saran

Wrap was placed over the membrane, smoothing out any creases of bubbles that may develop between membrane and Saran Wrap. It is necessary to work quickly once the membrane has been exposed to the detection solution. The membrane was placed, protein side up, in X-ray film cassette. The lights were turned off and a sheet of autoradiography film carefully placed (Hyperfilm^{TM}-ECL, Amersham pharmacia biotech, UK.) on the top of the membrane, the cassette was closed and exposed for 1 min (this depend on the amount of target protein on the membrane). If background is high the membrane may be rewashed with TTBS and re-detected with ECL solution.

9. Image Analysis

According to one of the major objectives of this study was to make qualitative technique of Western blotting to become the quantitative outcome by using the application of image software analysis.

Global Lab Image/2 (GLI/2) is an object-oriented scientific imaging software product that powerful, expandable, able to definite of contrasting areas, and improved precision in measurements, using the Edge Finder and Histogram tool to quantitate eNOS bands. Results can be printed, exported to Excel spreadsheets and expressed in percent compared with control (100%).

9.1 Image requirements

The following are the recommended requirements for using GLI/2 program: scanJet 6200C scanner (Hewlett Packard), CD writable, picture 1200 dpi. BMP or JPEG files.

9.2 Step for quantitate eNOS band

In this section, an example shows how to quantify eNOS band by using GLI/2 program.

1. Begin by starting New Viewport (if it is not open). From the file menu, select New Viewport or from the Toolbar, select 🗋 icon.
2. Next step will be opened a File Manager Tool as mention below (Appendix B, see Figure J). In the following example is the image file of Western blotting of heart-tissue eNOS protein taken from HAEC; image eNOS standard. (see Figure 8).

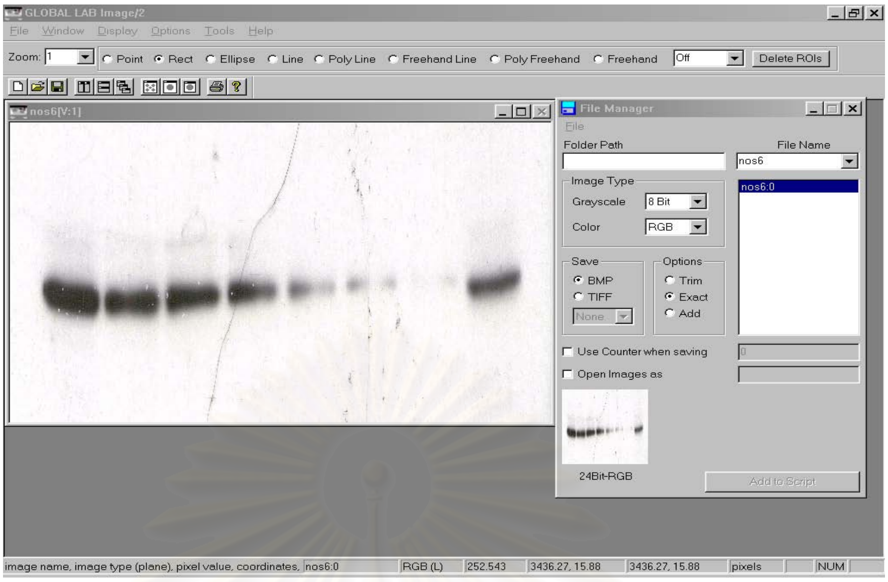


Figure 8 Using “open” command to loading the file of eNOS scanned previously.

3. Create a rectangle that covers around a positive eNOS band (P^+) by, click Rect in the ROI Menu Bar and click Draw in the ROI actions. (see Figure 9).

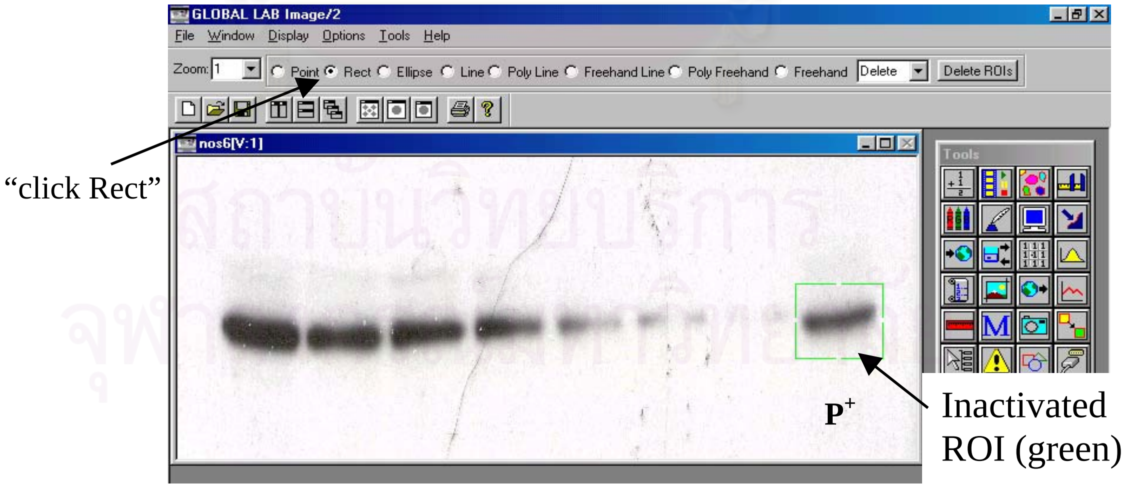


Figure 9 Using a rectangle window to select the eNOS band that want analyze, one by one.

4. In the rectangle ROI, click inside the ROI with the right mouse button. The newly activated ROI turns red (see Figure 10). Previously inactivated ROI is green.

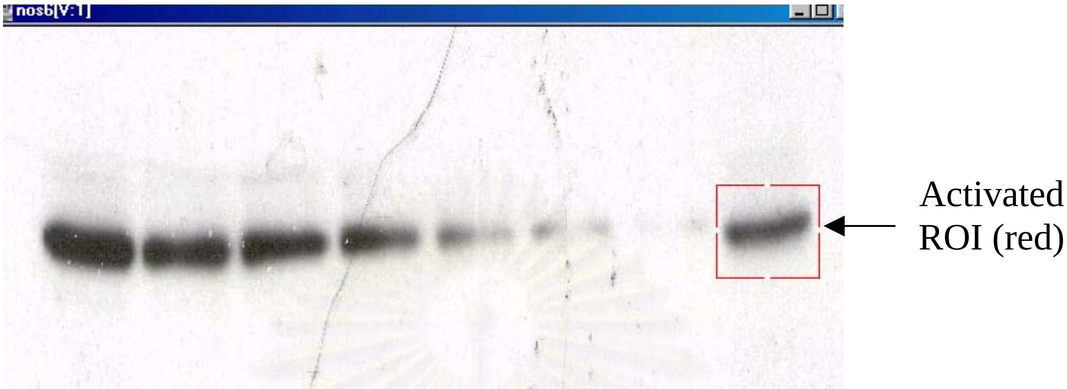


Figure 10 After selecting, then activating by "click" on rectangle window again.

5. Open the Histogram Tool as described in detail in Appendix B (see Figure 11). Click Add Histogram button to add a histogram to the graph (see Figure 12).

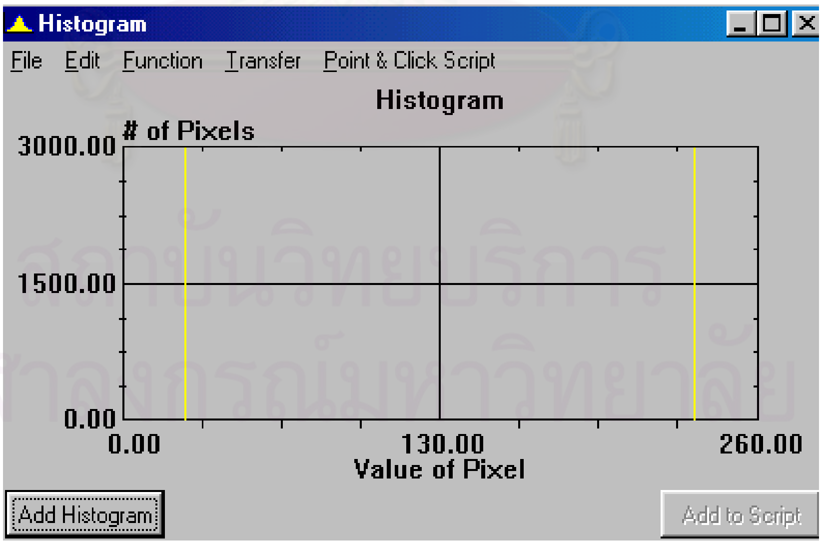


Figure 11 The figure shows the "Histogram Tool" used for analysing the digital image of eNOS band.

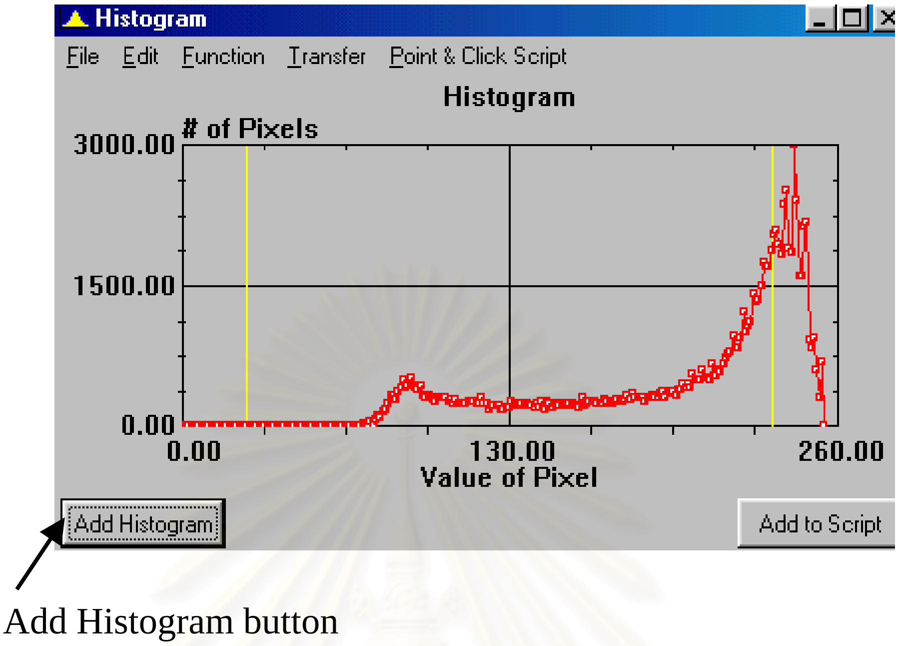


Figure 12 The figure shows the use of “Histogram” to analyze digital Image of eNOS band.

6. Select the Function Menu/Show statistics (see Figure 13)

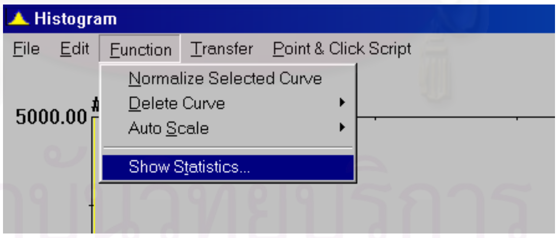


Figure 13 “Histogram” shows statistic values.

The statistics shown (see Figure 14) are calculated with regard to the range of pixel values. Then record Mean value out put (the average pixel value in the selected range) for set specifying of the maximum threshold limits that appropriate for this image (eNOS standard) in the Edge Finder Tool step.

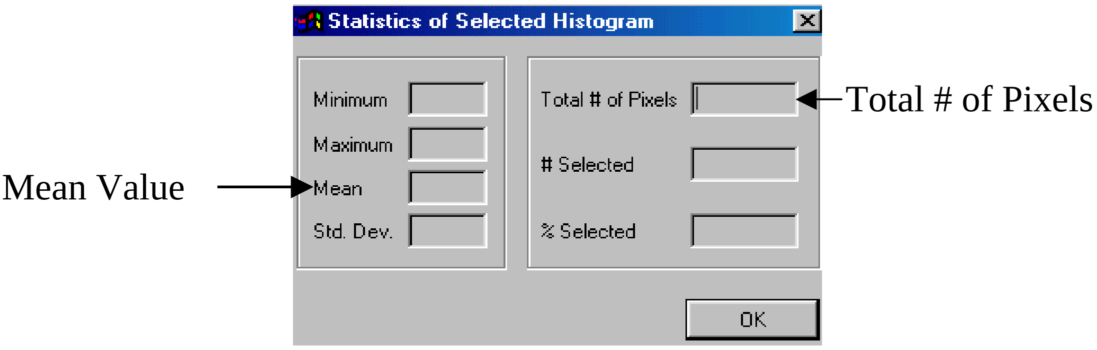


Figure 14 Shown the result of“Histogram” statistic calculation.

7.Create a rectangle cover each eNOS bands in image nos6 (see Figure 15).

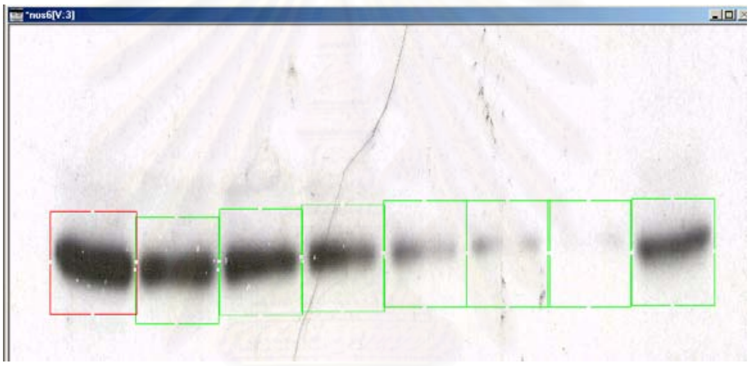
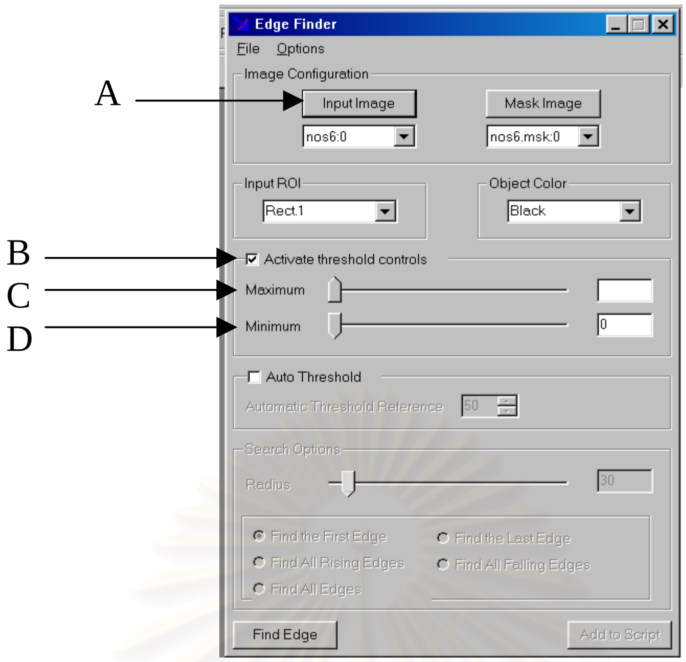


Figure 15 Demonstration of create rectangles for every bands of eNOS standards.

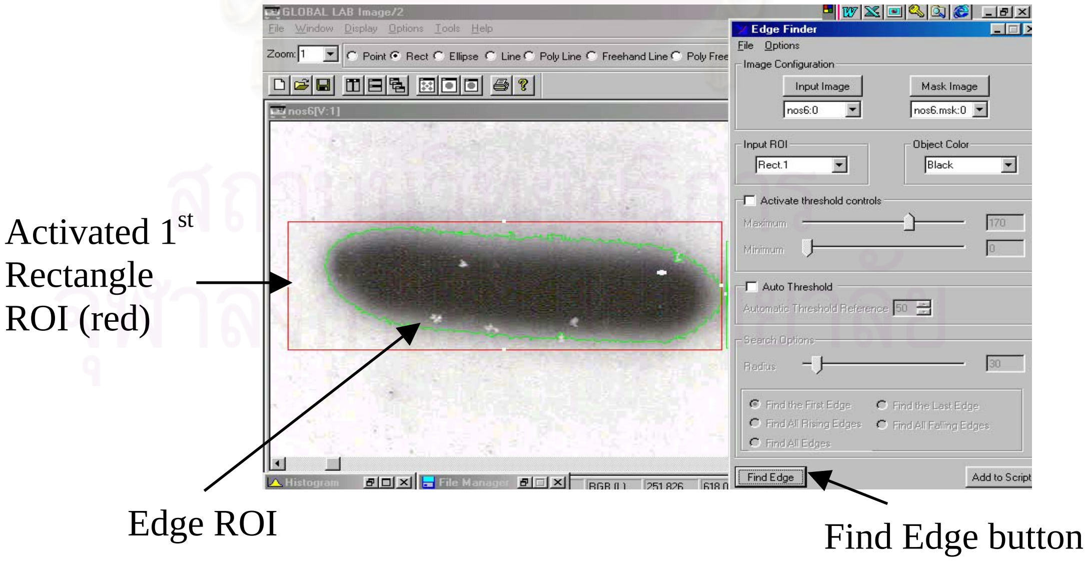
8. Open the Edge Finder Tool at mention below (Appendix B). Click in the viewport on the image eNOS standard with left mouse button. Image eNOS standard is now active viewport, then click input image (A) to load image eNOS standard as input image (see Figure 16). Select the first rectangle ROI which wants to generate edges by click inside ROI to activate it (turn red) (see Figure 15). Select the Activate Threshold Controls (B) check box and use the Maximum (C) and Minimum (D) slider controls to adjust the threshold limits. The upper limit is equal Mean value of Histogram statistic in step 6 and lower limit is 0 value.



Input Image: nos6:0
Mask Image: nos6.msk:0
Input ROI: Rect.1
Object Color: Black
☑ Activate threshold controls
Minimum: 0
☐ Auto Threshold
Automatic Threshold Reference: 50
Radius: 30
● Find the First Edge

Figure 16 Edge Finder Tool

9. When all the setting are correct, click Find Edge button (E) to finds edges that within a first rectangle ROI (activated ROI) (see Figure 17). Then activate another rectangle ROI and click Find Edge, repeat this step to all rectangles ROI (see Figure 18).



Zoom: 1
● Rect
Input Image: nos6:0
Mask Image: nos6.msk:0
Input ROI: Rect.1
Object Color: Black
☐ Activate threshold controls
Maximum: 170
Minimum: 0
☐ Auto Threshold
Automatic Threshold Reference: 50
Radius: 30
● Find the First Edge

Figure 17 Edge Finder button

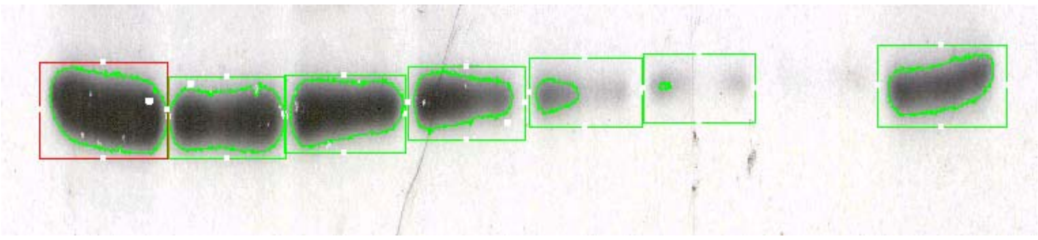


Figure 18 The image eNOS standard; Edge Finder Tool; Edge that within all rectangles ROI.

10. Open the Histogram Too (if one is not already open) as mention above in step 5. Activate first Edge ROI by click in it with the right mouse button (see Figure 19).

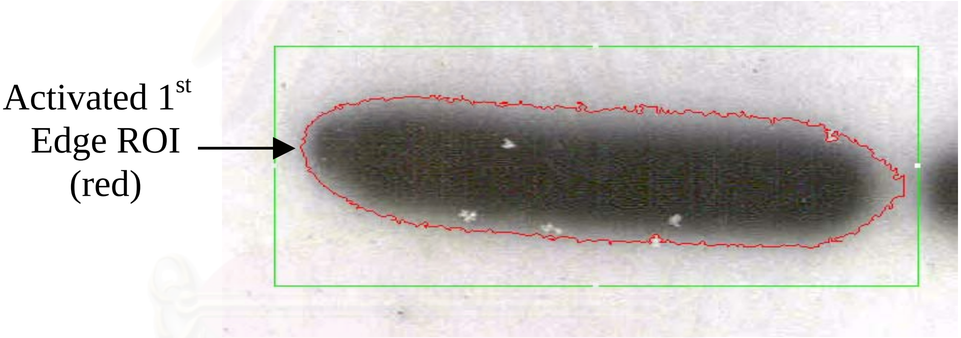


Figure 19 The image eNOS standard; Edge ROI; activate

11. Click Add Histogram, then histogram was created using the first Edge ROI from image eNOS standard (see Figure 20).

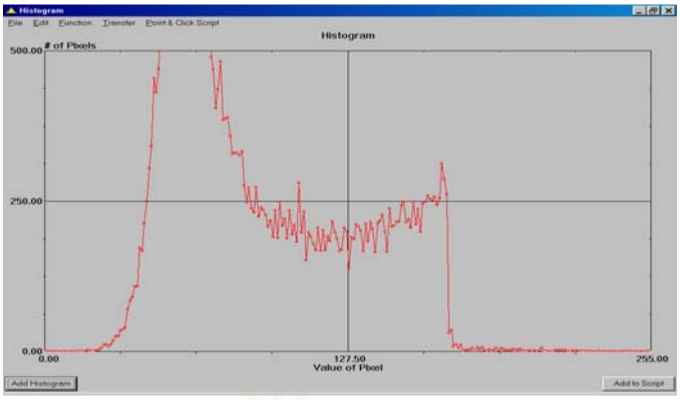


Figure 20 The image eNOS standard; Edge ROI; Histogram

12. Select the Function Menu/Show statistics (see Figure 13), then the result is a Total # of Pixels (see Figure 14). After that the result of all selected histogram data (curve data and statistics data) can send to the Excel spreadsheet by select Transfer/DDE Transfer/Send Data to Excel (see Figure 21).

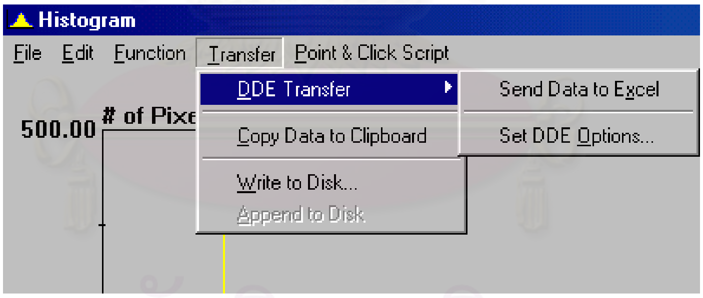


Figure 21 The image eNOS standard; Edge ROI; Histogram; Transfer

13. Repeat steps 10-12 for all Edge ROI from image eNOS standard.

.

9.3 Standard curve of eNOS protein.

The endothelial nitric oxide synthase (eNOS) protein level was evaluated by Western blot analysis. An intense band developed on, from gel, transfered to PVDF membrane, and exposed to ECL films was scaned (scanJet 6200C scanner, Hewlett Packard), and quantitated using an image analysis (GLI/2 software, area x density of band). To optimize the condition of eNOS analysis, a standard curve generated by six bands from one gel made with serial concentrations of 2,4,5,6,8,10 μg/μl from HAEC, which stock solution is 1 μg/μl (Transduction Laboratories, UK), was done. It was found to be linear over a wide range (Figure 22). Positive control of 5 μg of protein prepared from lung tissue form Wista rat (n=5) was run in each blot to use as correcting factor for each run.

The linear correlation equation is $Y = 5.9 \times 10^3 X$ with highly significant ($p<0.001$) as shown in Figure 23. The positive control 5 μg of protein have eNOS expression as # selected 37027 pixel (Table 1).

In each Western blot experiment, 5 μg of protein per well were used. Sample of HMW marker of eNOS, proteins extracted from HAEC, positive control, and tissue extract from each sample were together given for every experiment. Then image analysis were used for evaluate the number of pixels for each eNOS-bands as described previously. Calculation of eNOS protein in each sample was evaluated from the standard equation of $Y = 5.9 \times 10^3 X$ as shown in example A.

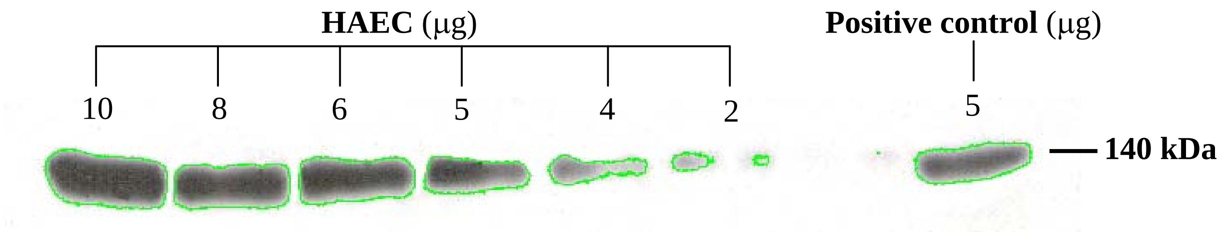


Figure 22 The serial concentration (2,4,5,6,8,10 µg/µl) of six eNOS bands and one eNOS band of positive control (5 µg/µl) from one gel.

Table 1. Number of pixels within the selected intensity range (#selected)

µg	10	8	6	5	4	2	Positive control (5µg)
No.of pixels (#selected)	54885	48849	44809	34578	15414	4198	37027

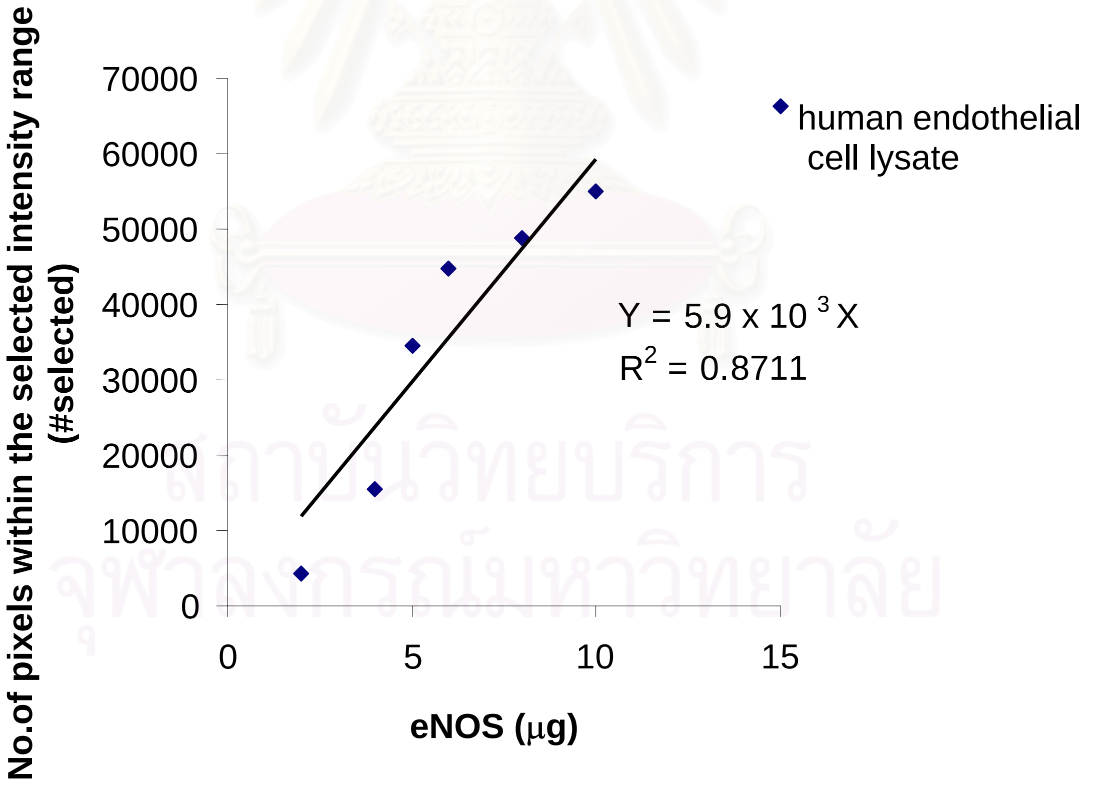
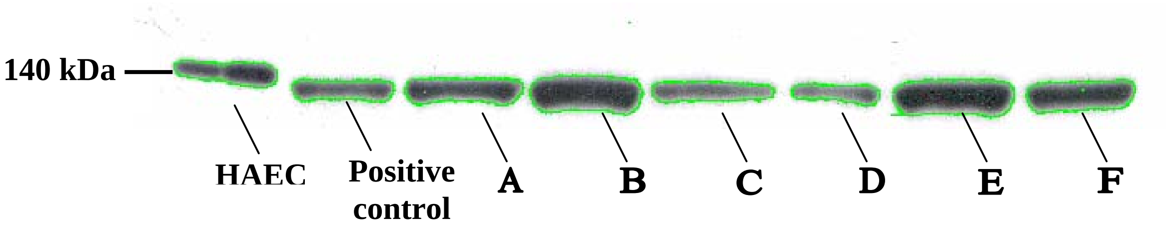


Figure 23 Standard curve for optimized condition eNOS protein level. Correlation between proteins extracted from HAEC (µg) and number of pixels within the selected intensity range (#selected).

Example A. Calculated eNOS band protein level



Positive control (# selected)	Sample band	No.of pixels (#Selected)	Y	X (μg)
29914	A	36938	45721.18	7.71764
	B	49857	61712.08	10.41688
	C	34605	42833.43	7.23020
	D	22437	27772.11	4.68787
	E	52870	65441.52	11.04640
	F	41506	51375.36	8.672064

Standard linear correlation Equation is $Y = 5.9 \times 10^3 X$

Positive control of Standard gel is 37037

$$Y = \frac{\text{Number of pixel within sample band (\#Selected)} \times 37027}{29914}$$

$$X = \frac{Y}{5.9 \times 10^3}$$

10 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 Turkey’s test. 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

This chapter composes of two major parts which were served to examine the effects of vitamin C on endothelial nitric oxide synthase in diabetic rat. The two major parts were listed in the following:-

Protocol 1: The effects of vitamin C supplementation on endothelial nitric oxide synthase.

:eNOS protein level expresses in the lung tissues
:eNOS protein level expresses in the heart tissues
:eNOS protein level expresses in the aorta tissues

Protocol 2: Accuracy of image analysis with GLI/2 software.

Rat with intravenous injection of STZ 50 mg/kg BW significantly resulted in polydipsia, polyphagia and polyglycemia within 48 hours and has shown persistent hyperglycemia throughout the experiment. In the present study, the criteria used for diabetic rats was the blood glucose level had to be higher than 200 mg/dl.

The results shown in Table 2 demonstrated that the diabetic rats (DM) and diabetic rats supplementation with vitamin C (DM+Vit.C) significantly loss of body weight and significantly increase blood glucose compared to the control rats (CON) at 12 and 24 weeks. Results of plasma vitamin C concentration in DM was significantly lower than CON at 12 and 24 weeks. However, both plasma Vit.C of 12 and 24 weeks DM+Vit.C were significantly higher than those of 12 and 24 DM groups.

The Standard equation

By using HAEC with serial concentration of 2,4,5,6,8 and 10 μg/μl, the Western blot of standard eNOS-protein bands were obtained as shown in Figure 22. Together with this performance for standard Western blotting of eNOS, the positive control of 5 μg was also used the same as other experiments. As previously described in “Method Section”, the software GLI/2 was used to analyze the total numbers of pixels within each standard. eNOS bands for each concentration wer used. The average of numbers of pixels of each concentration were then graphically plotted against their corresponding concentrations as shown in Figure 23. The linear correlation equation is $Y = 5.9 \times 10^3 X$ and highly significant ($p < 0.001$) as shown in Figure 23. The positive control 5 μg of protein have eNOS expression as # selected 37027 pixel with $R^2 = 0.8711$ (Table 1).

The means and the standard deviation of means that expressed in μg of protein were shown in Table 3 and Table 5 whereas those in percent compared with controls (100%) were also demonstrated in Table 4 , Table 6 , Figure 24 and Figure 25.

Protocol 1: The effects of vitamin C supplementation on endothelial nitric oxide synthase.

1) eNOS protein level expresses in the lung tissues

The results shown in Table 3, 4 and also in Figure 24 indicated that eNOS protein level both in 12 and 24 weeks of DM were significantly higher than CON. However, there was no significant increase of eNOS protein in lung as compared between DM+Vit.C and CON at both 12 and 24 weeks. Especially, at 24 weeks, the increase in eNOS level of DM lung was significantly, but not for DM+Vit.C group. Interestingly, the results have shown and implied in the way that eNOS protein in lung of DM would increase following the progression of the disease. However, the supplementation of vitamin C seems to prevent such increase in diabetic lung due to higher eNOS protein levels than CON.

2) eNOS protein level expresses in the heart tissues

Means and SEM shown in Table 5, 6 and Figure 25 are represented the quantitative eNOS proteins in hearts.

The results indicated that eNOS protein level both in 12 and 24 weeks of DM were significantly lower than those of CON. However, DM+Vit.C had shown their eNOS protein significantly higher than DM.

However vitamin C supplementation had shown their effect to prevent the decrease in eNOS protein in diabetic rat at 12 and 24 weeks.

3) eNOS protein level expresses in the aorta tissues

The eNOS protein level (μg) of the aorta tissues were shown in Figure 26. The result of this aortic sample was actually received from pooled aortic vessels of 10 rats for each group. Since, there was vary small amount of eNOS protein from each rat, therefore, we have to pool all 10 specimens together. From the Figure 26, eNOS protein of 10-rat aortas was calculated by using the standard equation $Y = 5.9 \times 10^3 X$. Therefore, the μg proteins of each group were obtained. However, the only one set of such eNOS bands were able to obtain from Western blotting. Therefore, the statistical analysis is not available in this experiment. The results were then comparable using ratio between DM/CON and DM+Vit.C, assembly, CON was equal to 1 folds. The results indicated that DM, eNOS (μg) was 0.194 (0.087/0.447) and 0.108 (0.074/0.0683) folds at 12 and 24 weeks, respectively. In DM+Vit.C, the results have shown that eNOS protein at 12 weeks was 1.05 (0.470/0.447) and 1.48 (1.012/0.683) folds of 24 weeks.

Table 2. Means ± SEM of body weight (g), blood glucose (mg/dl) and plasma vitamin C (mg/dl) of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) were shown for both 12 and 24 weeks of experimental periods.

Groups		Metabolic changes		
		Body weight (g)	Blood Glucose (mg/dl)	Plasma vitamin C (mg/dl)
CON	12 wk	425.666±4.659 (n=9)	93.125±7.70 (n=8)	1.295±0.151 (n=6)
	24 wk	489.818±10.978 (n=10)	98±5.102 (n=8)	1.17±0.109 (n=7)
DM	12 wk	182.7±7.871*** (n=10)	418.333±17.238*** (n=9)	0.621±0.019* (n=7)
	24 wk	294.65±8.073*** (n=10)	327.666±20.823*** (n=9)	0.511±0.04* (n=7)
DM+Vit C	12 wk	228±15.18 $^{***,\#\#}$ (n=9)	380.125±18.608^{***,ns} (n=8)	0.956±0.107$^{ns, NS}$ (n=6)
	24 wk	266.5±11.173^{***,ns} (n=9)	287.8±18.782^{***,ns} (n=10)	1.578±0.406$^{\#\#,ns}$ (n=8)

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

*** Significantly different as compared to CON ($p<0.001$)

$^{\#\#}$ Significantly different as compared to DM ($p<0.01$)

NS = no significantly different as compared to CON

ns = no significantly different as compared to DM

Table 3. Means ± SEM of endothelial nitric oxide synthase (μg) of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) in lung tissues were shown for both 12 and 24 weeks of experimental periods.

Group	Endothelial Nitric Oxide Synthase (μg)	
	12 wk	24 wk
CON	3.888 ± 0.425 (n=10)	5.350 ± 0.867 (n=8)
DM	8.295 ± 0.9432 * (n=8)	11.880 ± 1.793 ** (n=8)
DM+Vit.C	7.040 ± 0.773 $^{ns, NS}$ (n=10)	6.448 ± 0.644 $^{\#\#, NS}$ (n=8)

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

** Significantly different as compared to CON ($p < 0.01$)

Significantly different as compared to DM ($p < 0.01$)

NS : no significantly different as compared to CON

ns : no significantly different as compared to DM

Table 4. Means ± SEM of endothelial nitric oxide synthase (%) of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) in lung tissues were shown for both 12 and 24 weeks of experimental periods.

Group	Endothelial Nitric Oxide Synthase (%)	
	12 wk	24 wk
CON	77.76 ± 8.50 (n=10)	107.00 ± 17.34 (n=8)
DM	165.90 ± 18.86* (n=8)	237.60 ± 35.86** (n=8)
DM+Vit.C	140.80 ± 15.46$^{ns, NS}$ (n=10)	128.96 ± 12.88$^{\#\#, NS}$ (n=8)

$$\% \text{ eNOS} = \frac{\text{eNOS protein level of sample } (\mu g) \times 100}{5\ \mu g \text{ of total protein}}$$

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

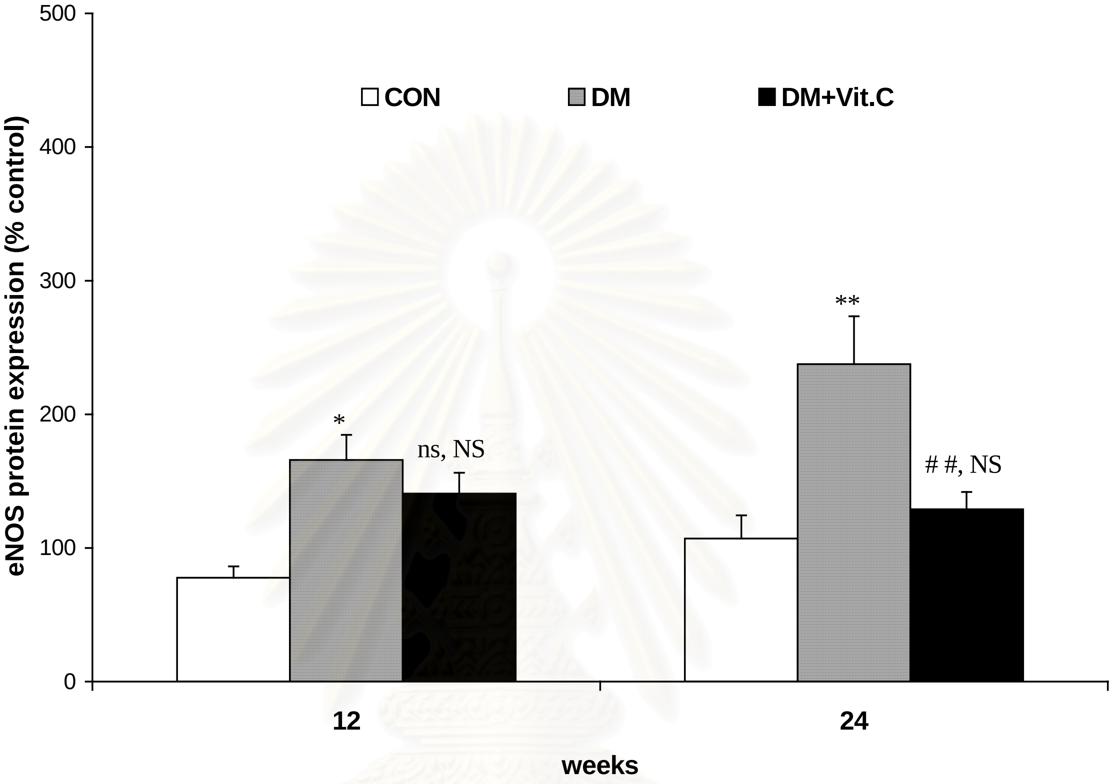
** Significantly different as compared to CON ($p < 0.01$)

Significantly different as compared to DM ($p < 0.01$)

NS : no significantly different as compared to CON

ns : no significantly different as compared to DM

Figure 24. Effect of vitamin C supplementation on the eNOS protein expression in lung tissues



CON ; non-diabetic control rats

DM ; diabetic rats

DM+Vit.C; diabetic rats supplementation with vitamin C

Values are means ± SEM.

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

** Significantly different as compared to CON ($p< 0.01$)

Significantly different as compared to DM ($p< 0.01$)

NS = no significantly different as compared to CON

ns = no significantly different as compared to DM

Table 5. Means ± SEM of endothelial nitric oxide synthase (μg) of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) in heart tissues were shown for both 12 and 24 weeks of experimental periods.

Group	Endothelial Nitric Oxide Synthase (μg)	
	12 wk	24 wk
CON	3.037 ± 0.161 (n=10)	4.812 ± 0.565 (n=6)
DM	0.754 ± 0.251** (n=7)	0.751 ± 0.439*** (n=8)
DM+Vit.C	4.590 ± 0.320$^{NS,\#\#\#}$ (n=6)	4.633 ± 0.641$^{NS,\#\#\#}$ (n=6)

** Significantly different as compared to CON ($p< 0.01$)

*** Significantly different as compared to CON ($p< 0.001$)

Significantly different as compared to DM ($p< 0.001$)

NS = no significantly different as compared to CON

Table 6. Means ± SEM of endothelial nitric oxide synthase (%) of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) in heart tissues were shown for both 12 and 24 weeks of experimental periods.

Group	Endothelial Nitric Oxide Synthase (%)	
	12 wk	24 wk
CON	60.74 ± 3.22 (n=10)	96.24 ± 11.30 (n=6)
DM	$15.08 \pm 5.02^{**}$ (n=7)	$15.02 \pm 8.78^{***}$ (n=8)
DM+Vit.C	$91.8 \pm 6.40^{NS,\#\#\#}$ (n=6)	$92.66 \pm 12.82^{NS,\#\#\#}$ (n=6)

$$\%\ \text{eNOS} = \frac{\text{eNOS protein level of sample } (\mu\text{g}) \times 100}{5\ \mu\text{g of total protein}}$$

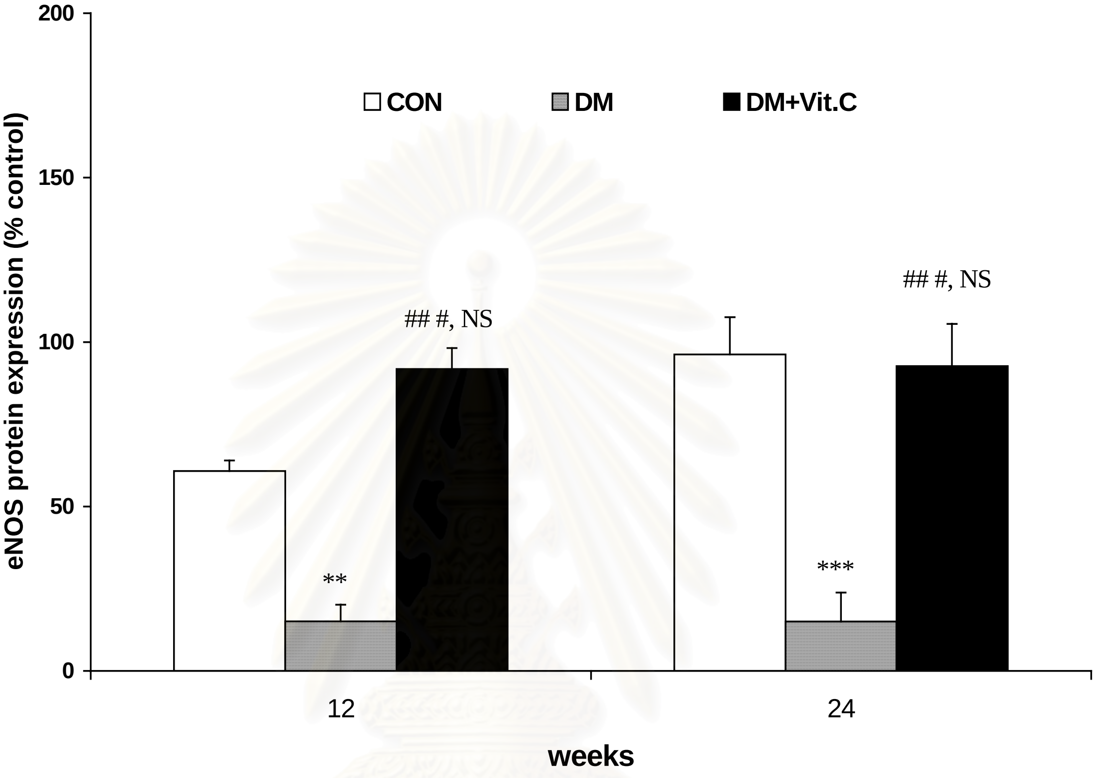
** Significantly different as compared to CON (p< 0.01)

*** Significantly different as compared to CON (p< 0.001)

Significantly different as compared to DM (p< 0.001)

NS = no significantly different as compared to CON

Figure 25. Effect of vitamin C supplementation on the eNOS protein expression in heart tissues



CON = non-diabetic control rats

DM = diabetic rats

DM+Vit.C = diabetic rats supplementation with vitamin C

Values are means ± SEM.

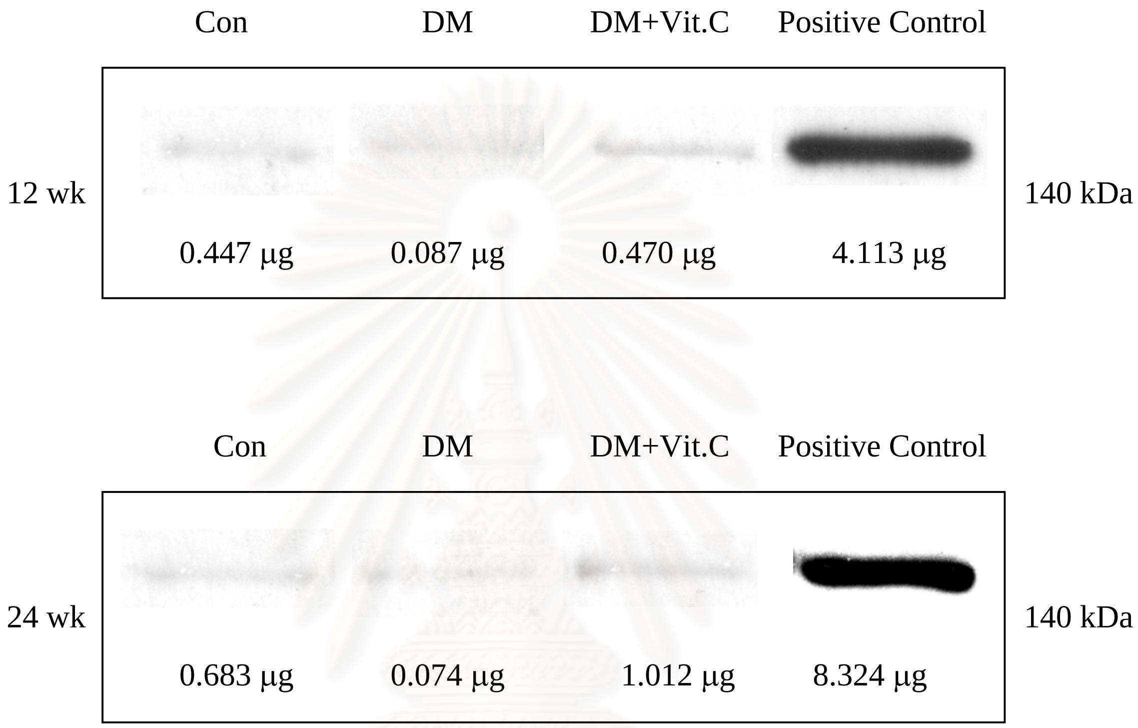
** Significantly different as compared to CON (p< 0.01)

*** Significantly different as compared to CON (p< 0.001)

Significantly different as compared to DM (p< 0.001)

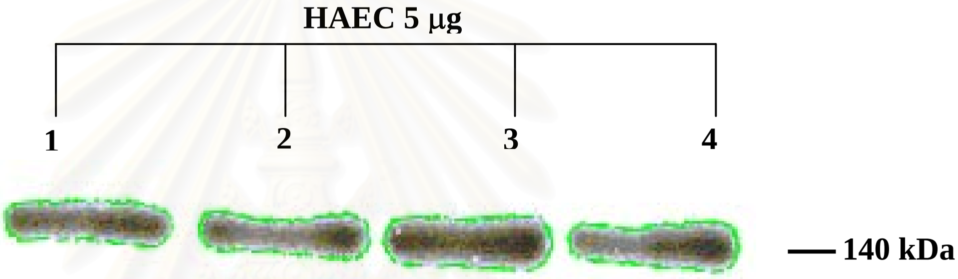
NS = no significantly different as compared to CON

Figure 26. The endothelial nitric oxide synthase (μg) band images of control rats (CON), diabetic rats (DM), diabetic rats supplementation with vitamin C (DM+Vit.C) in aorta tissues.



Protocol 2: Sensibility of image analysis with GLI/2 software.

The accracy of this application was detected by using HAEC with concentration of 5µg / µl that generated in 4 bands from one gel. Then the processing of image analysis were used for evaluate the quanlitative of eNOS-protein bands as described previously. From our finding, an accuracy value is within ± 15.755 %. The result indicated that the sensitivity of its application is well enough for differentiate the changes of protein content in microgram level.



	HAEC 5 µg			
	1	2	3	4
#Selected	30930	31655	39868	34696
X (µg)	5.211	5.343	6.73	5.857
Mean = 5.78775 = ± 15.755		Accuracy value (%)		

CHAPTER V

DISCUSSION

In diabetes, the major cause of mortality is from diabetic cardiovascular complications. Low levels of endogenous antioxidants, such as vitamin C, have been well defined for its association with such complications (Wen Y et al., 2000; Sargeant LA, 2000).

In Table 2, our study has shown that DM and DM+Vit.C have significantly loss of BW than their age.-match controls. Hyperglycemia was significantly confirmed at both experimental periods of 12 and 24 weeks. Besides, our results also shown that the chronic diabetes mellitus, the low levels of plasma vitamin C concentration has been developed as the disease progressed. Therefore, it might be postulated that vitamin C deficiency may play an important role in the development of diabetic complications, due to the imbalance of oxidative-antioxidative condition.

Why was vitamin C in diabetes decreased?

Ascorbic acid outside cells can be oxidized to DHA, which is transported through membranes by glucose transporters, especially GLUT-1 (Siman CM et al., 1997; NG LL et al., 1998). Therefore, chronic hyperglycemia may impose an intracellular deficit of ascorbic acid through competitive inhibition of membrane transport of ascorbic acid by the elevated plasma glucose (Dai S and McNeill JH, 1995*).* *In vivo* study, exposure to hyperglycemia will exacerbate the impaired DHA uptake, leading to loss of DHA through its hydrolysis in aqueous solution. It could be suggested that such defect of vitamin C transportion into the cell would bring the result in the increasing of intracellular ROS level.

Since antioxidant enzymes, SOD and glutathione were used to amerliorate the harmful at ROS. Therefore, the more increased in ROS level, the more decrease in SOD and glutathione levels

Hyperglycemia can increase ROS by three possible mechanisms

At this point, the question might come up that how diabetic model could generate ROS to increase. Recently, the possible mechanisms that are popularly used to explain how hyperglycemia could generate ROS as following :-

a. Polyol pathway leads to an increase in sorbitol flux and fructose synthesis which are converted from glucose, enhances aldose reductase activity (Kashiwagi A. et al., 1994)
b. Nonenzymatic glycosylation, glucose chemically attaches to protein, nonenzymatically, to form Amadori product (Cotran RS, 1999) can lead to the formation of AGE (Hunt JV etal, 1990; Mullarkey CJ, 1990).
c. Glucose autooxidation describes the capability of glucose to enolize, thereby reducing molecular oxygen and yielding oxidizing intermediates (Giugliano D, 1996)

The imbalance of ROS-antioxidant causes endothelial dysfunction and further diabetic cardiovascular complications

Several studies have suggested that hyperglycemia, which exists in diabetes, could be a significant factor for causing endothelial dysfunction both *in vivo* (Bohlen HG and Lash JM, 1993) and *in vitro* (Tesfamariam B and Cohen RA, 1992). Those findings have demonstrated on diminishing endothelium-dependent vasodilation in diabetic microvessel. The decrease in NO content as well as eNOS activity has been significantly documented for such abnormalily of endothelium-dependent vascular response.

The influence of hyperglycemia and diabetes on the synthesis and release of NO by cells and tissues have been the subject of intense interest in recent year. A number of studies have suggested that ***either guenching effect of oxidative stress on normally released NO or impaired NOS activity could be as possible mechanisms for those decrease in NO production.***

Diminished capacity of eNOS to generate NO has been demonstrated experimentally when endothelial cells are exposed *in vitro* , to hyperglycemic environment (Avogaro A et al., 1999. Cipolla MJ, 1999). In addition to variations in eNOS protein levels and the interaction of NO with superoxide, regulation of the eNOS enzyme appears to be abnormal in vascular disease states, resulting in reduced NO production despite the presence of eNOS protein.

What happened to eNOS proteins in diabetic heart?

In the present study, the evaluation of eNOS level by mean of digital image analysis, therefore, the quantitative value of eNOS protein could be numerically demonstrated out from the eNOS bands of ECL films obtained from Western blot analysis. The application of image software together with the high resolution of scanning image, and the calibration curve between number of pixels within the standard-eNOS bands and its corresponding concentration was performed and used for determining eNOS protein in our samples as described previously. The significant decrease in eNOS protein expression in the heart of DM was demonstrated for both 12 and 24 weeks. The previous study performed in the aortic ring of STZ-rats was also demonstrated the decrease in both eNOS protein expression and plasma nitrite-nitrate concentration (Park KS et al., 2000). Moreover, it has been reported that in endothelial cell culture the NO synthesis was reduced by high glucose concentration in a dose-dependent manner (Chakravarthy U et al., 1998).

The result of our study has further confirmed these findings. Therefore, we have extended the explanation for decreasing eNOS activity as that it was actually through the decrease of eNOS protein synthesis. In recent years, the role of endothelial cell dysfunction and oxidative stress for development of cardiovascular disease has been highlighted (Heinecke JW, 1998; Diaz MN et al., 1997). These findings prompted us to investigate endothelial function and oxidative stress in diabetes mellitus.

Hyperglycemia increases the auto-oxidation and protein glycation (the level of HbA_{1C} confirmed by previous study in our laboratory which was significantly inreased (data not shown), increasing oxidative factors (MDA level was increase significantly (data not shown)) and reducing the anti-oxidant levels as demonstrated by plasma vitamin C.

Oxidative stress may damage endothelial function through several mechanisms. ROS, especially $H^{\bullet}$ may injure the endothelial cell membrane. An interaction between ROS and endogenous vasoactive mediators formed in endothelial cells, has been demonstrated, i.e. superoxide anions reacting with endothelium-derived NO leading to inactivation of NO (Wever RM et al., 1998). Increased $O_2^{\bullet}$ production may account for a significant proportion of the NO deficit in ED from atherosclerotic vessels (Harrison DG, 1998). $O_2^{\bullet}$ reacts rapidly with NO, there by reducing NO bioactivity and producing $ONOO^{\bullet}$ (Gryglewski RJ, 1986; White CR et al., 1994). With interested result, we have shown that vitamin C could attenuate this endothelial dysfunction concomitant with the increase in heart eNOS protein level.

Effect of vitamin C supplementation on diabetic endothelial dysfunction

Recent studies shown that vitamin C administration improved ED in patients with diabetes (Lindsay RM et al., 1998 Ting HH et al., 1996) or coronary artery disease (Levine GN et al., 1996; Hornig B et al., 1998).

As demonstrated in experimental result, vitamin C supplementation was significantly reduced hyperglycemic state (the result shown in Table 2). The previous report was suggested that the beneficial effect of an acute rise in plasma vitamin C was to decrease ROS (Hornig B et al., 1998; Lindsay RM et al., 1998). Also we have demonstrated the increases in eNOS protein levels for both heart and aorta of DM+vit.C groups. Therefore the results suggests that a mechanism for the scavenging oxygen derived free radicals such as $O_2^{\bullet}$, $H^{\bullet}$ and other ROS by the antioxidant properties, vitamin C, within the endothelial vasculature could help to preserve endothelial function. As such eNOS protein synthesis were protected.

What happened to eNOS proteins in diabetic lung?

Interestingly, the heterogeneity of eNOS protein reduction by diabetic condition has been indicated in our present study. Since, we could quantify the amount of eNOS protein in both systemic circulation; heart tissue, and pulmonary circulation; lung tissue. Our result demonstrated that the reduction of eNOS protein progressive with the diabetic stage was only found in the heart and aorta, **not** in the lung.

In 1996, Mancusi et al. revealed no alteration in either mRNA or protein for eNOS in long-term human umbilical vein endothelial cell culture (HUVEC) under high glucose conditions.

Moreover, it has been reported that expression of mRNA and protein for eNOS were increased after culture with elevated glucose concentrations in human aortic endothelial cells for 5 days (Cosentino F et al., 1997). Surprisingly, both endothelial cell culture and lung endothelium are kinds of cells standing in the low pressure, low flow, and low shear stress environment. Therefore, it might be postulated that the role of extracellular matrix on shear stress-dependent eNOS expression might be a potential reason for those difference between systemic and pulmonary endothelial cells in hyperglycemia condition.

As the overall conclusion, our findings have shown the decrease in eNOS-protein level in diabetic heart at both 12 and 24 weeks of experimental periods. However, the decrease of diabetic heart eNOS protein could be able to prevent by vitamin C supplementation. Beside, our study also found that there was a heterogeneity of eNOS protein damaging by diabetic induced ROS. Since, the results of lung eNOS became significantly increase for both experimental periods of 12 and 24 weeks. We hypothesized that the difference between heart and lung eNOS might be able to explain by their different in location facing high- and low-flow, respectively. However, the further study is needed to clarify such diabetic induced changes of flow-mediated eNOS protein synthesis.

CHAPTER VI

CONCLUSION

In the present study, by using Western blotting and the application of image processing analyses, the effects of vitamin C supplementation on diabetic-induced ED were studied. The metabolic changes including BW, BS and plasma vitamin C and the eNOS protein level in different organ tissues were also determined. The experimental data were determined for each group of CON, DM and DM+Vit.C, for both periods of 12 and 24 weeks.

From the present results, the conclusions could be as follow;

1. The injection of STZ 50 mg/kg BW into Sprauqe dawley rats resulted in polydipsia, polyuria, polyphagia and shown persistent hyperglycemia throughout the experiment. The levels of BW was demonstrated in all groups of diabetic rats found to be significantly decreased when compared with CON ($p<0.05$).
2. The BS levels in DM and DM+Vit.C have been reported to be significantly increased when compared with CON.
3. The levels of plasma vitamin C in DM have been reported to be decreased when compared with CON. And there was no significant difference between CON and DM+Vit.C for both experimental period of 12 and 24 weeks. ($p>0.05$).
4. In heart tissue, eNOS protein level was found to be significantly decreased in DM for both 12 and 24 weeks. Interestingly, there are significantly higher in eNOS protein level for DM+Vit.C when compared with DM. ($p<0.001$).

5. In aorta tissue, eNOS protein level was able to demonstrate as shown in Figure 26. The result found that, eNOS (μg) found in DM+Vit.C at 12 (1.05 folds) and 24 weeks (1.48 folds), seemed to be comparable to CON (1 folds). In the DM, eNOS (μg) was 0.194 and 0.108 folds lower than that CON at 12 and 24 weeks, respectively. But no significantly result in the statistic analysis could be done because we have not enough samples to make more Western blot analysis. Therefore, we suggest that if one wants to continue study using aortic ring, the specimen of aortic ring might have to be pooled from more rats.

6. Although this present study has found the reduction of eNOS protein expression in the heart and aorta, but not in the lung.

Therefore, we are still lack of information to understand the regulation of flow-mediated NO synthesis through eNOS protein. Depending one of our objectives, quantify eNOS protein may help us to further elucidate our understanding on heterogeneous pathogenesis of diabetic induced ED. Moreover, the more we can evaluate the eNOS and NO bioavailability, the more we can open a new era for therapeutic potential for not only diabetes but also other cardiovascular disease.

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APPENDICES

APPENDIX A

Buffers and Reagents

TISSUE PREPARATION

0.1 M Phosphate Buffer Saline (PBS), pH 7.4

Na_2HPO_4	11.500	g
KH_2PO_4	2.584	g
NaCl	8	g
DW	1000	ml

SDS-PAGE

Stock acrylamide

Acrylamide	15	g
N,N-methylenebisacrylamide	0.4	g
DW	50	ml

Lower gel tris

Tris-base	3.643	g
SDS	0.080	g
DW	20	ml

Adjust to pH 8.8 with 12 N HCL and filter through o.45 µl membrane

Upper gel tris

Tris-base	1.204	g
SDS	0.080	g
DW	20	ml

Adjust to pH 6.8 with 12 N HCL and filter through o.45 µl membrane

Sample buffer (dye marker)

Tris-HCL	0.0985	g
SDS	0.4	g
Glycerol	1	ml
2-ME	0.5	g
Bromphenol blue	10	mg

Adjust to pH 6.8 using 1 N NaOH and add DW to 10 ml

2 % Ammonium persulfate

Ammonium persulfate	40	mg
DW	2	ml

Seperating gel 7.5 %

Stock acrylamide	0.85	ml
Lower gel tris	0.85	ml
DW	1.53	ml
TEMED	2.72	µl
2 % Ammonium persulfate	0.17	ml

Stacking gel 5 %

Stock acrylamide	0.332	ml
Lower gel tris	0.5	ml
DW	1.12	ml
TEMED	2	µl
2 % Ammonium persulfate	40	µl

Electrophosis buffer (running buffer)

Tris-base	1.2	g
Glycine	5.76	g
SDS	0.4	g
DW	400	ml

Coomassie Staining

Stain

Coomassie Brilliant Blue R	0.91	g

Acetic acid	45	ml
MeOH	215	ml
DW	240	ml

Destain

Acetic acid	90	ml
MeOH	430	ml
DW	480	ml

Amido black staining

Stain

Amido black	0.01	g
MeOH	45	ml
Acetic acid	10	ml
DW	45	ml

Destain

MeOH	90	ml
Acetic acid	2	ml
DW	8	ml

WESTERN BLOT REAGENTS

Protein Transfer Buffer , pH 8.3

Tris-base	1.93	g
Glycine	9.0	g
DW	1000	ml

20x Tris-Buffer Saline (TBS), pH 7.5

Tris-base	24.228	g
NaCl	175.32	g
DW	1000	ml

TTBS (0.05 % Tween 20)

Tween 20	0.5	ml
TBS	1000	ml

Blocking Solution (5 % non-fat dried milk, 1 % BSA)

Milk	5	g
BSA	1	g
TBS	100	ml

ECL REAGENTS

90 mM p-Coumaric acid stock solution

p-Coumaric acid	0.015	g
DMSO	1	ml

Store in the dark at 4 °C

250 mM Luminol stock solution

Luminol	0.043	g
DMSO	1	ml

Store in the dark at 4 °C

100 mM tris pH 8.0, sterilize by autoclaving

Tris	1.2114	g
BW	100	ml

Solution A: 5 ml	100 mM tris pH 8.0	5	ml
	90 mM Coumaric acid	22	µl
	250 mM Luminol	50	µl
Solution B: 5 ml	100 mM tris pH 8.0	5	ml
	3 % H_2O_2	30	µl

APPENDIX B

Image processing Global Lab Image/2 (GLI/2) software

1. Main application of Windows

The main Window of this program shown in Figure A.

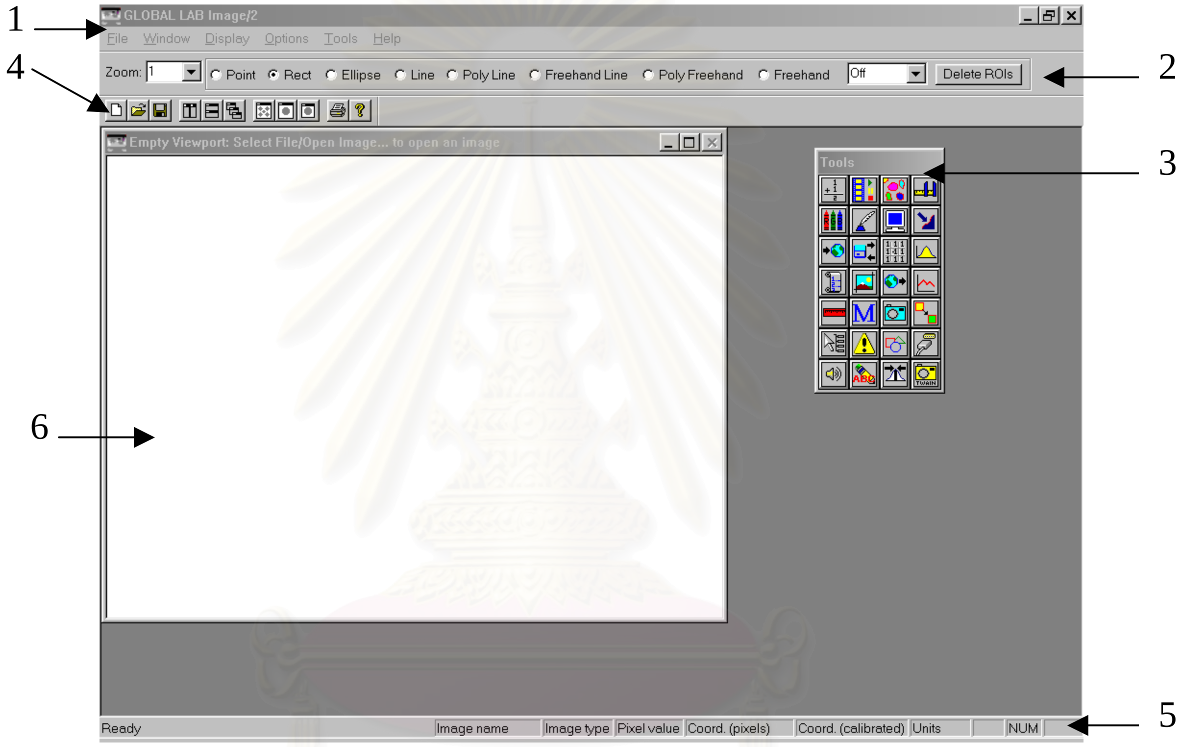


Figure A. Main Window of GLI/2

1. File Menu Options
2. ROI Type
3. Tool box
4. Toolbar
5. Status Bar
6. Viewport

1.1. File Menu Options

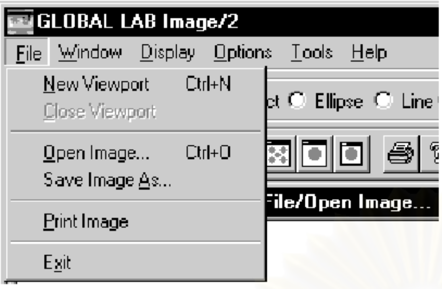


Figure B

New Viewport

Select this option to create a new viewport so that you can view an image. The new viewport becomes the active viewport.

Close Viewport

Select this option to close the active viewport and delete any ROIs attached to the viewport.

Open Image

Select this option to open an image from disk. The image must be stored in standard Windows bitmap format (noncompressed). The image can be opened as a binary, 8-bit grayscale, 16-bit grayscale, 32-bit grayscale, floating-point grayscale, 24-bit RGB (Red/Green/Blue), or 24-bit HSL (Hue/Saturation/Luminance) color image. By default, the image is opened as an 8-bit grayscale image.

Save Image As

Select this option to save the image in the active viewport as a standard Windows bitmap file.

Print Image

Select this option to print the image exactly as it is seen in the active viewport. Zoomed images print exactly as seen. Images are printed as large as possible while keeping their aspect ratios.

Exit

Select this option to close the application and all open tools.

1.2. ROI Type

The ROI type can be specified by using the ROI menu bar which refer to Figure C or Options / ROI Type from the main application. The ROI Manager tool shown in Figure D.

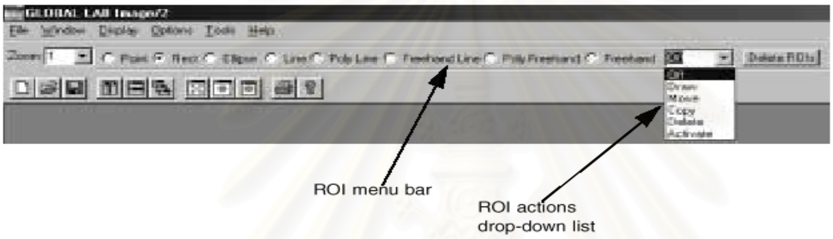


Figure C

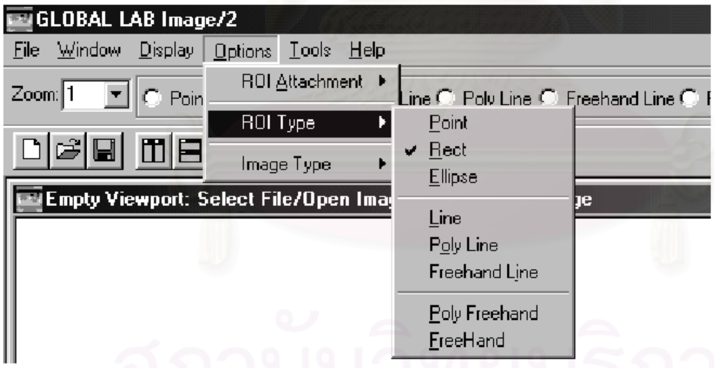


Figure D

An ROI is a region of interest. It it is the portion of an image to manipulate. This section contains additional information about ROIs.

GLI/2 provides eight different types of ROIs. Each ROI is created, moved, copied, selected, used, and deleted in the same way. The ROI type that has been selected determines the type of ROI that is created.

An ROI is a region of interest. It is the portion of an image that you want to manipulate. This section contains additional information about ROIs.

GLI/2 supports the following types of ROIs: Point, Rectangle, Ellipse, Line, and Poly line.

1.3. Toolbox

The Toolbox and Tools/show Toolbox from the main application that holds all the loaded tools were shown in Figure E and F, respectively. To use a tool in the Toolbox, click on the tool icon.

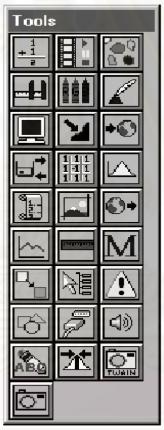


Figure E

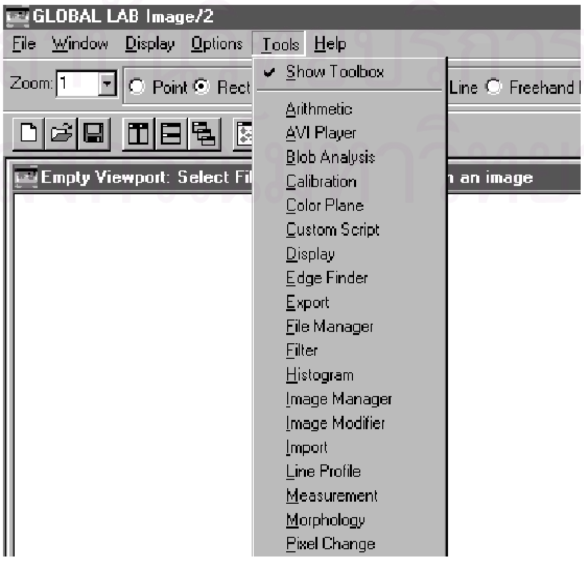


Figure F

1.4. Toolbar

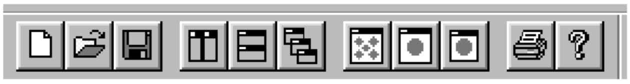


Figure G

The first three buttons on the toolbar correspond to the following menu options:File /New viewport, File/Open Image, File/Save Image As.

The next three buttons on the toolbar correspond to the following menu options: Window /Tile Vertical, Window /Tile Horizontal, Window/Cascade

The next three buttons on the toolbar correspond to the following menu options: Display/Image Display Mode/Size Image to Viewport, Display/Image Display Mode/Show Image Actual Size, Display/Image Display Mode/Fit Viewport to Image

The last two buttons on the toolbar correspond to the following: File/ Print Image menu option, Shows the About box for GLI/2

1.5. Status Bar

The status bar is displayed in the lower right corner of the main application window. An example status bar is shown in Figure H.

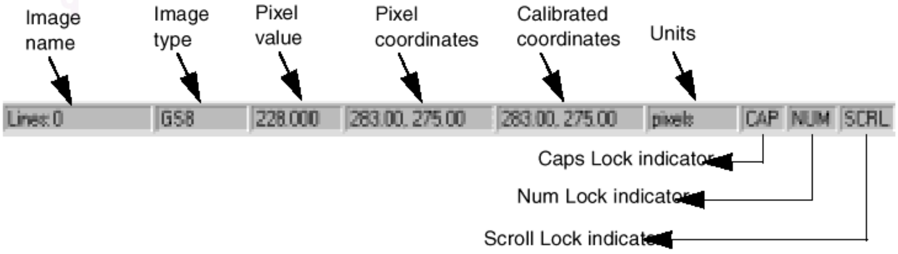


Figure H

The items shown in the status bar are described as follows:

Image name – the name of the image in the active viewport.

Image type – the type of image in the active viewport

Pixel value – the value of the pixel at the current cursor location.

Pixel coordinates – the location (x, y) of the pixel at the currentncursor location, where 0,0 refers to the lower-left corner of the image.

Calibrated coordinates – the location (x, y) of the pixel at the current cursor location in calibrated units (if the image has an attached calibration object).

Units – the unit of measure that GLI/2 uses to perform its calculations. By default, GLI/2 uses pixel measurements. If the image has an attached calibration object, GLI/2 displays the measurements in calibrated units.

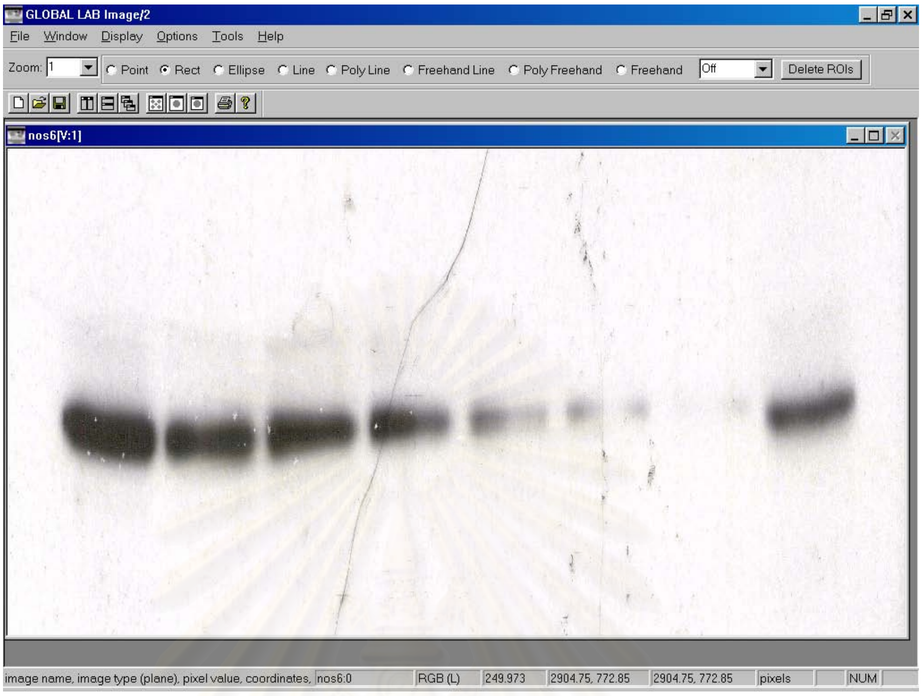
Caps Lock indicator – CAPS indicates that the Caps Lock key is ON (alphabetic characters on the keyboard are shifted to uppercase).

Num Lock indicator – NUM indicates that the Num Lock key is ON (the numeric keypad on the keyboard is activated).

Scroll Lock indicator – SCRL indicates that the Scroll Lock key is ON (the cursor control keys on the keyboard are affected).

1.6. Viewport

A viewport is a window in which to view an image. Each viewport contains a view and a title bar. The view portion of the viewport is the portion actually showing the image. The title bar contains information about the viewport. Viewports also have scrollbars that you can be used to move the image around if the image does not fit inside the viewport. Figure I shows open viewports with image eNOS standard.



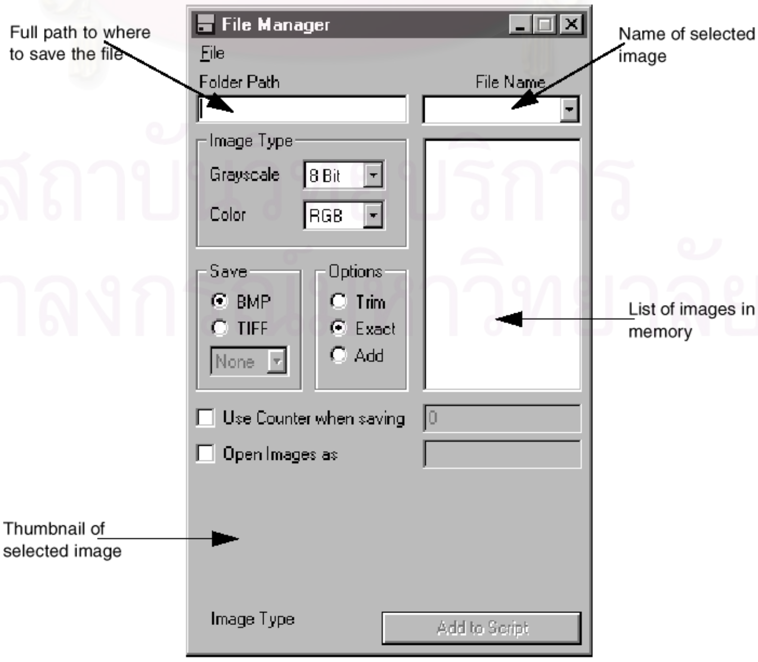
Zoom: 1
● Rect
Off

image name, image type (plane), pixel value, coordinates, nos6:0 | RGB (L) | 249.973 | 2904.75, 772.85 | 2904.75, 772.85 | pixels

Figure I

2. Using the File Manager Tool

To open a File Manager Tool, select the icon from the Toolbox or select File Manager from the Tools menu (see Figure J).



Grayscale 8 Bit
Color RGB

● BMP
○ TIFF
None

○ Trim
● Exact
○ Add

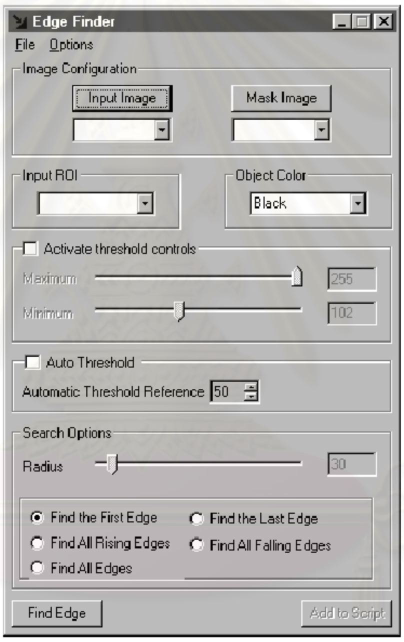
☐ Use Counter when saving 0
☐ Open Images as

Figure J

File Manager Tool allows to open many popular file formats. It also open a mixture of color and grayscale images of different image types without being concerned with file conversion.

3. Using the Edge Finder Tool

To open an Edge Finder Tool, select the icon from the Toolbox or select Edge Finder from the Tools menu (see Figure K).



Object Color: Black

☐ Activate threshold controls

Maximum 255

Minimum 102

☐ Auto Threshold

Automatic Threshold Reference 50

Radius 30

● Find the First Edge
○ Find the Last Edge
○ Find All Rising Edges
○ Find All Falling Edges
○ Find All Edges

Figure K

The Edge Finder Tool allows to extract points, edges, or contours from a binary image.

4. Using the Histogram Tool

To open a Histogram Tool, select the icon from the Toolbox or select Histogram from the Tools menu (see Figure L).

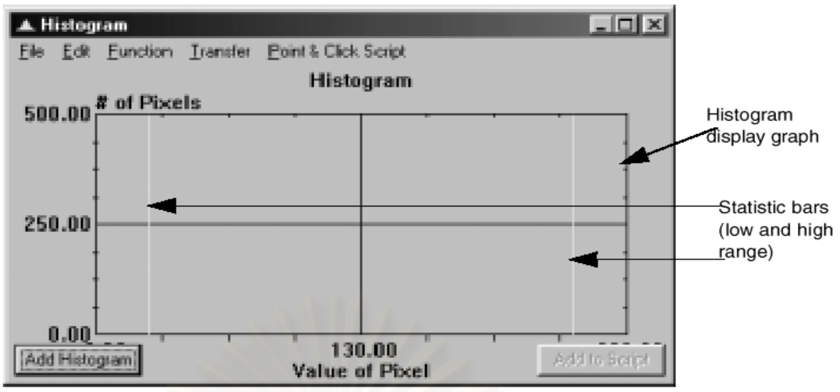


Figure L

The Histogram Tool allows to create histograms of images. Up to 100 histograms can be loaded to the same graph. The histograms can be added from multiple images and from multiple viewports. Histogram data can be transferred directly to the Microsoft Excel worksheet program.

APPENDIX C

Publications

1. Sridulyakul P., Bhattarakosol P., Patumraj S. Endothelial nitric oxide synthase expression compared between systemic and pulmonary circulation of streptozotocin-induce diabetic rats: Quantitative comparison using image analysis. Clinical Hemorheology Micro, 2003 (in press)
2. Sridulyakul P., Bhattarakosol P., Patumraj S. Endothelial nitric oxide synthase expression compared between systemic and pulmonary circulation of streptozotocin-induce diabetic rats: Quantitative comparison using image analysis. The 5th Asian Congress for microcirculation Manila Philippines, 2003. (Abstract)

Award

Young Investigator Award of The 5th Asian Congress for microcirculation Manila Philippines, 2003.

BIOGRAPHY

Miss Pattarin Sridulyakul was born on April 25, 1978 in Bangkok, Thailand. She received the degree of Bachelor of Biology of Science in 1999 from Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.

Experience and Positions

2000-Present Teacher: Physiology, Department of Biology, Faculty of Science, Srinakharinwirot University.

She has enrolled at Chulalongkorn University in graduate program for the degree of master of science in Medical Science in 2000.