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ROLES OF VITAMIN C AND EXERCISE TRAINING ON ENDOTHELIAL DYSFUNCTION IN DIABETIC RATS

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จุดมุ่งหมายของการวิจัยนี้ คือ เพื่อศึกษาผลการป้องกันของการเสริมวิตามินซี และการฝึกออกกำลังกายต่อการสูญเสียหน้าที่ของเซลล์เอนโดทีเลียมในเบาหวาน หนูขาวเพศผู้ถูกแบ่งออกเป็น 5 กลุ่มได้แก่ กลุ่มควบคุม กลุ่มเบาหวาน กลุ่มเบาหวานที่ได้รับวิตามินซี กลุ่มเบาหวานที่ได้รับการออกกำลังกาย และกลุ่มเบาหวานที่ได้รับทั้งวิตามินซีและการออกกำลังกาย หนูถูกทำให้เป็นเบาหวานโดยการฉีดสารสเตรปโตโซโตซินในขนาดความเข้มข้น 50 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว กลุ่มที่ได้รับวิตามินซีถูกให้โดยผสมวิตามินซีในน้ำดื่ม ความเข้มข้น 1 กรัมต่อลิตร และกลุ่มที่ได้รับการออกกำลังกายถูกกำหนดให้วิ่งบนลู่กล 5 ครั้งต่อสัปดาห์ ด้วยความเร็ว 15 เมตรต่อนาที เป็นเวลา 30 นาที

ผลการทดลอง พบว่า ทั้งที่ 12 และ 24 สัปดาห์ หลังจากได้รับการฉีดสารสเตรปโตโซโตซิน หนูเบาหวานจะเกิดภาวะน้ำตาลในเลือด ไกลโคซิเลทฮีโมโกลบิน ความดันโลหิต น้ำหนักของหัวใจ และไตรกลีเซอไรด์ในเลือดที่สูง และมีระดับวิตามินซีในเลือดที่ต่ำอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตาม ระดับวิตามินซีในเลือดมีค่าสูงขึ้น ประกอบกับไกลโคซิเลทฮีโมโกลบิน และความดันโลหิตที่ต่ำลงอย่างมีนัยสำคัญในหนูเบาหวานที่ได้รับวิตามินซี ในขณะที่หนูเบาหวานที่ได้รับการออกกำลังกาย มีอัตราการเต้นของหัวใจที่ต่ำกว่าเมื่อเปรียบเทียบกับหนูกลุ่มควบคุม และมีการลดความผิดปกติของความดันโลหิตและน้ำหนักของหัวใจเมื่อเปรียบเทียบกับหนูกลุ่มเบาหวาน เพื่อทดสอบผลการเสริมวิตามินซี และการฝึกออกกำลังกายต่อหน้าที่ของเซลล์เอนโดทีเลียม ปฏิสัมพันธ์ระหว่างเม็ดเลือดขาวกับเซลล์เอนโดทีเลียมของหลอดเลือดดำรองในลำไส้ และการตอบสนองของหลอดเลือดแดงรองในลำไส้ต่อสารขยายหลอดเลือด ถูกศึกษาโดยใช้เทคนิคทางอินทราไวทัล ฟลูออเรสเซ้นท์ ไมโครสโคปี้ พบว่า ภาวะเบาหวานเป็นสาเหตุที่ทำให้เกิดการเพิ่มการเกาะติดของเม็ดเลือดขาวต่อเซลล์เอนโดทีเลียม และลดการตอบสนองของหลอดเลือดแดงรองต่ออะเซทิลโคลีนซึ่งเป็นสารขยายหลอดเลือดที่ทำงานผ่านเซลล์เอนโดทีเลียมทั้งที่ 12 และ 24 สัปดาห์ การเพิ่มขึ้นของการเกาะติดของเม็ดเลือดขาวนั้นถูกทำให้ลดลงโดยการเสริมวิตามินซีและการฝึกออกกำลังกาย นอกจากนั้น การลดลงของการตอบสนองของหลอดเลือดต่อสารอะเซทิลโคลีนถูกบรรเทาโดยวิตามินซี อย่างไรก็ตาม การฝึกออกกำลังกายไม่มีผลต่อการขยายตัวของหลอดเลือดต่อสารที่ทำงานผ่านเซลล์เอนโดทีเลียม

วิตามินซีและการออกกำลังกายถูกพบว่ามีผลดีต่อภาวะออกซิเดทีฟ สเตรส และแอนตี้ออกซิแดนซ์ หนูเบาหวานมีระดับมาลอนไดอัลดีไฮด์สูงและระดับเอนไซม์ซุปเปอร์ออกไซด์ดิสมิวเทสต์ต่ำอย่างมีนัยสำคัญกว่าหนูกลุ่มควบคุม อย่างไรก็ตาม มาลอนไดอัลดีไฮด มีระดับต่ำลงเมื่อเทียบกับหนูเบาหวาน และไม่พบความแตกต่างของเอนไซม์ซุปเปอร์ออกไซด์ดิสมิวเทสต์ระหว่างหนูกลุ่มเบาหวานที่ได้รับวิตามินซี กลุ่มเบาหวานที่ได้รับการออกกำลังกาย และกลุ่มควบคุม นอกจากนี้ ยังพบว่าการลดลงของโปรตีนไนตริกออกไซด์ซินเทสในหนูเบาหวาน ถูกป้องกันด้วยการเสริมวิตามินซี เป็นที่น่าสนใจว่า พบบทบาทการป้องกันร่วมของการเสริมวิตามินซีและการฝึกออกกำลังกายในหนูกลุ่มเบาหวานที่ได้รับทั้งวิตามินซีและการฝึกออกกำลังกาย โดยแสดงถึงประโยชน์ที่มากขึ้นทั้งต่อความสุขสมบูรณ์ของหัวใจและหลอดเลือด และต่อหน้าที่ของเซลล์เอนโดทีเลียม

โดยสรุป การศึกษาครั้งนี้ชี้ให้เห็นว่าการสูญเสียหน้าที่ของเอนโดทีเลียมในหนูเบาหวานนั้นเกี่ยวข้องกับการเพิ่มการเกาะติดของเม็ดเลือดขาว และการลดลงของการขยายตัวของหลอดเลือดต่อสารที่ทำงานผ่านเซลล์เอนโดทีเลียม อย่างไรก็ตาม การเสริมวิตามินซีร่วมกับการฝึกออกกำลังกายสามารถป้องกันผลเสียเหล่านี้ได้ โดยการลดความไม่สมดุลของออกซิแดนซ์และแอนตี้ออกซิแดนซ์ที่เกิดจากภาวะเบาหวาน ดังนั้น การเสริมวิตามินซีร่วมกับการออกกำลังกายอย่างสม่ำเสมอ น่าจะเป็นการรักษาที่มีประโยชน์และประหยัด ในการป้องกันภาวะแทรกซ้อนทางหัวใจและหลอดเลือดที่เกิดจากการสูญเสียหน้าที่ของเซลล์เอนโดทีเลียมในโรคเบาหวาน

สาขาวิชา.....สรีรวิทยา (สหสาขาวิชา).....
ปีการศึกษา.....2545.....

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
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DAROONWAN CHAKRAPHAN : ROLES OF VITAMIN C AND EXERCISE TRAINING ON ENDOTHELIAL DYSFUNCTION IN DIABETIC RATS THESIS ADVISOR : ASSOC.PROF. SUTHILUK PATUMRAJ, Ph.D., THESIS CO-ADVISORS : ASSIST.PROF. BUNDIT THIPAKORN, Ph.D. AND PROF. VIRGINIA H. HUXLEY, Ph.D. 186 pp. ISBN 974-17-2987-1

The aim of this present study was to determine the protective effects of vitamin C supplementation and exercise training on the diabetic endothelial dysfunction (ED). Male Spraque-Dawley rats were divided into five groups of control (Con), diabetes (DM), diabetes with supplemented vitamin C (DM+Vit.C), diabetes with exercise- trained (DM+Ex) and diabetes with supplemented vitamin C and exercise- trained (DM+Vit.C+Ex) groups. Diabetes was induced by intravenous injection of streptozotocin (STZ;50 mg/kgBW). Vitamin C was given in drinking water with the concentration of 1 g/L. The exercise training protocol consisted of treadmill running 5 times/week with velocity 13-15 m/min for 30 min.

The results showed that either 12 and 24 weeks (wk) after the STZ injection, blood glucose (BG), glycosylated hemoglobin (HbA_{1C}), arterial blood pressure (BP), heart weight (HW) and plasma triglyceride (Trig) were significantly higher and lower in plasma vitamin C levels in DM rats. In DM+Vit.C rats,the plasma vitamin C levels and HbA_{1C} were significantly increased ($P<0.05$). However, BP were decreased in DM+Vit.C rats. Whereas DM+Ex rats had reduced the abnormalities of BP and HW when compared with DM rats and had a significant decrease in heart rate compared to Con rats. To examine the effects of vitamin C supplementation and exercise training on ED, leukocyte-endothelial cell (EC) interaction in mesenteric venules and vascular reactivity response to vasodilators in mesenteric arterioles were monitored using intravital fluorescence microscopy. It was found that the diabetic state enhanced leukocyte adhesion and impaired vasodilatory response to the EC-dependent vasodilator, Ach, either 12- and 24- wk. The increase of leukocyte adherance was attenuated by supplemented with vitamin C and exercise training. The impaired vascular reactivity to Ach was found to be attenuated by supplemented vitamin C. However, it failed to improve the impairment of EC-dependent vasodilation by exercise training.

In addition, vitamin C and training were also shown to have favorable effects on oxidative stress and antioxidant status. DM rats had significantly higher in malondialdehyde (MDA) level and lower activity of superoxide dismutase (SOD) than Con rats. However, the MDA levels in DM+Vit.C and DM+Ex were significantly lower than those of DM rats and there were no significant differences in SOD activity among DM+Vit.C and DM+Ex compared to Con group. Moreover, the decreased eNOS protein in DM rats was prevented by vitamin C supplemented. Interestingly, we found the combined protective roles of supplemented with vitamin C and exercise training in DM+Vit.C+Ex group as well. They showed the more beneficial influence both in cardiovascular fitness and endothelial function in diabetic rats.

In conclusion, our observations indicated that the endothelial dysfunction of diabetic rats is associated with increased leukocyte adhesion and impaired endothelium-dependent relaxation. However, vitamin C supplementation combined with exercise training could prevent these deleterious effects by ameliorating the diabetic-induced imbalance of oxidants/antioxidants. Therefore, vitamin C supplementation and regular exercise training might be an effective and inexpensive therapeutic modality in preventing diabetic cardiovascular complications which were crucially induced through the endothelial dysfunction in diabetes.

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Student's signature.....
Advisor's signature.....
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LIST OF ABBREVIATIONS

AA	=	Ascorbic acid
Ach	=	Acethylcholine
AGES	=	Glycosylation end-products
BW	=	Body weight
BG	=	Blood glucose
BSA	=	Bovine serum albumin
°C	=	Degree celsius
cAMP	=	Cyclic adenosine monophosphate
CAT	=	Catalase
cGMP	=	Cyclic guanosine 3', 5' monophosphate
cNOS	=	Constitutive nitric oxide synthase
DHAA	=	Dehydroascarbic acid
dl	=	Deciliter
DM	=	Diabetes Mellitus
DW	=	Double distilled deionized water
ECL	=	Enhanced chemiluminescence
EC	=	Endothelial cell
ED	=	Endothelial dysfunction
e.g.	=	Exempli gratia (for example)
eNOS	=	Endothelial nitric oxide synthase
Ex	=	Exercise
FITC	=	Fluoresceir isothiocyanate
GLUT-1	=	Glucose transporter-1
GPX	=	Glutathione peroxidase
GR	=	Glutathione reductase
GSH	=	Glutathione peroxides

LIST OF ABBREVIATIONS (Continued)

$H^{\bullet}$	=	hydrogen radical
H_2O_2	=	Hydrogen peroxide
HbA_{1C}	=	Glycosylated hemoglobin
IDDM	=	Insulin-dependent diabetes mellitus
i.e.	=	id est (that is)
iNOS	=	Inducible nitric oxide synthase
IP_3	=	Inositol 1,4,5- triphosphate
MDA	=	Malondialdehyde
ml	=	Milliliter
mg	=	Milligram
μm	=	Micrometer
nmole	=	Nanomole
NADPH	=	Nicotinic acid adenine dinucleotide phosphate
NIDDM	=	Non insulin-dependent diabetes mellitus
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
$O_2^{\bullet}$	=	Superoxide anion, Superoxide radical
OD	=	Optical density
$OH^{\bullet}$	=	Hydroxyl radicol
PBS	=	Phosphate-buffer saline
PKC	=	Protein kinase C
PUFA	=	Polyunsaturated falty acid
ROS	=	Reactive oxygen species
SEM	=	Standard error

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	=	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electropharesis
SNP	=	Sodium nitroprusside
SOD	=	Superoxide dismutase
STZ	=	Streptozotocin
TBARS	=	Thiobarbituric acid reactive substance
Vit.C	=	Vitamin C
VSMC	=	Vascular smooth muscle cell
wk	=	Weeks

CHAPTER I

INTRODUCTION

Diabetes mellitus is associated with increased risks of hypertension, atherosclerosis and disorders of the microcirculation. Increasing evidence have suggested that vascular endothelial dysfunction may play a major role (Palmer AM., 1998) as an underlying cause of those diabetic cardiovascular complications.

Endothelial dysfunction has been suggested to be an early event in diabetic vascular disease (Chan NN.,2000). It is considered an intrinsic element in the pathogenesis of diabetic angiopathies (Aydin A.,2001). The development of endothelial cell dysfunction is characterized by an impairment in vasorelaxation and increased adhesiveness of the endothelial cell lining. Moreover, the dysfunctional endothelium predisposes vascular tissues to vasoconstriction, platelet-thrombus formation and inflammatory cell infiltration (Gibbon GH.,1996).

The association between diabetic complications and oxidative stress have been fairly well supported. Currently, the potential contribution of increased oxidative stress to the development of endothelial dysfunction in diabetes has received much of interest. The impairments of vasodilatory responses to the endothelium-dependent vasodilator acetylcholine (ACh) in arteries isolated from diabetic subjects (Michael T., 1993) and from the streptozotocin (STZ)-induced diabetic rats (Keegan A.,1995 , Wang SP.,1996) have been reported. The abnormal ACh response is likely to result from either decreased nitric oxide (NO) synthesis or increased NO degradation. Several mechanisms thought to play a pivotal role oxidative stress. Among the many

suggestions regarding the origins of oxidative stress in diabetes, are free radical reactions related to glycation of proteins, consumption of NADPH through the polyol pathway, glucose autooxidation, hyperglycemia-induced pseudohypoxia, and activation of protein kinase C (VanderJagt DJ.,2001). In addition, oxidative stress has been implicated increasingly in the accelerated atherosclerosis, a microvascular complications of diabetes. Oxidative stress can result in widespread lipid, protein and DNA damage; of the events oxidative modification of LDL cholesterol was believed to be central in the pathogenesis of endothelial dysfunction (Laaksonen DE.,1996). Further the increased production and/or ineffective scavenging of such reactive oxygen species may play a crucial role in determining the extent of tissue injury (Aydin A.,2001). Recently, increased presence of reactive oxygen species (ROS) has also been implicated in the pathogenesis of type 1 diabetes (Santini SA.,1997).

Oxidative stress is controlled by a variety of antioxidant cellular defense mechanisms consisting of enzymatic and nonenzymatic scavengers (Aydin A.,2001). Antioxidants are classified generally as endogenous antioxidants [e.g. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)/oxidized glutathione reductase (GSSG-RD)], those produced internally by animals and humans, and as exogenous antioxidants [glutathione 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 pathologies studies have shown that the production of endogenous antioxidants are not sufficient to protect against excessive oxidative stress. A variety of exogenous antioxidants such as β–carotene, vitamins E and C have 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.,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.,1995). Studies from our laboratory have reported that long term supplementation of vitamin C could markly prevent the diabetic endothelial dysfunction including the ultrastructural changes of cerebral microcirculation (Jariyapongskul A.,2000).

The cardiovascular adaptations caused by exercise training result in improved circulatory performance. It is known that the conductance of peripheral circulation is increased as a result of chronic exercise and could be due to both structural and functional changes in the microcirculation (Sun D.,1994). Exercise training is known to have beneficial effects on diabetes. One of those is an increase in glucose uptake and metabolism (Enevoldse LH.,2000). Recent work support the hypothesis that physical training improves endothelial function, particularly in patients with high cardiovascular risk including diabetes (Higashi Y.,1999). There is growing understanding that as a consequence of the increased vascular shear stress derived from repetitive exercise, endothelial cells respond by increasing their production of nitric oxide and other vasodilatory products. In addition, it has been reported that a marker of endothelial damage was found to decrease after 3 months of exercise training (Rigla M.,2001). Moreover, training has also been shown to have favorable effects on oxidative stress and antioxidant status.

Increased activity of antioxidant enzymes has been reported in muscles with high oxidative capacity. Accordingly, regular exercise or exercise training may also have a protective effect on oxidative stress in diabetes (Laaksonen DE.,1996).

In conclusion, it might be said that both vitamin C and exercise training are likely to amerliorate diabetic-induced imbalances of oxidants and antioxidants. However, there is no report showing a synergistic protective role of vitamin C plus exercise training on diabetic endothelial dysfunction. Therefore, the present study was designed to establish whether dietary supplementation of vitamin C and/or exercise training have a biological effect in the protection against oxidative stress in diabetes. The purpose of present study is to determine

1. whether vitamin C supplementation reduces diabetic endothelial dysfunction

2. whether exercise training attenuates diabetic endothelial dysfunction

3. the combined influence of vitamin C supplementation action and exercise training on diabetic endothelial dysfunction, and

4. the possible mechanisms of the effects of vitamin C and exercise training on endothelial dysfunction in diabetes.

CHAPTER II

LITERATURE REVIEW

I. DIABETES MELLITUS

Definition

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. (The expert committee on the diagnosis and classification of diabetes mellitus.,2001).

Classification

The current classification and diagnosis of diabetes was developed by an international Expert Committee, working under the sponsorship of the American Diabetes Association (ADA) and established in May 1995.

The classification of diabetes mellitus can be divided into five groups as follows: (The expert committee on the diagnosis and classification of diabetes mellitus.,2001)

1. Type 1 Diabetes mellitus (IDDM)

Type 1 diabetes referred to previously as “insulin-dependent diabetes mellitus”, “type I diabetes mellitus”, or “juvenile-onset diabetes mellitus” is caused by an autoimmune cell-mediated destruction of the pancreatic beta cells leading to absolute insulin deficiency. Type I diabetes is dominated by polyuria, polydipsia, polyphagia and ketoacidosis. Despite an increased appetite, catabolic effects prevail resulting in weight loss and muscle weakness, effects consequence to

metabolic derangements, mainly hyperglycemia. Hyperglycemia in Type 1 diabetes results from a severe, absolute lack of insulin caused by a reduction in β–cell mass (Cotran RS., 1999).

2. Type 2 Diabetes mellitus (NIDDM)

Individuals with type 2 diabetes mellitus (previously termed "non-insulin-dependent diabetes mellitus," "type II diabetes mellitus," or "adult-onset diabetes") are characterized by insulin resistance and relative (rather than absolute) insulin deficiency. Unlike type 1 diabetes, there is no evidence that autoimmune mechanism are involved. Life style clearly plays a role, especially when obesity is considered. The two metabolic defects that characterize type 2 diabetes are (1) a derangement in β–cell secretion of insulin and (2) a decreased response of peripheral tissues to respond to insulin (insulin resistance) (Cotran RS.,1999).

3. Other specific types of diabetes

This category of diabetes includes a wide variety of unrelated disorders that can not be categorized as either type 1 or type 2 diabetes mellitus as follows ;

a. Genetic defect of the β-cell.
b. Genetic defect in insulin action.
c. Diseases of the exocrine pancreas.
d. Endocrinopathies
e. Drug or chemical-induced diabetes.
f. Infections.
g. Uncommon forms of immune-mediated diabetes
h. Other genetic syndromes sometimes associated with diabetes.

4. Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus refers to the onset or first recognition of diabetes mellitus during pregnancy, most commonly during the third trimester.

5. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG)

The terms IGT and IFG refer to a metabolic stage intermediate between normal glucose homeostasis and diabetes. This stage includes individuals who have IGT and individuals with fasting glucose levels ≥110 mg/dl but <126 mg/dl (IFG).

Diabetic Complications

The morbidity associated with long-standing and poor control diabetes of either type results from a number of serious complications. The important morphologic changes related to the many late systemic complications of diabetes are likely to be found in arteries (atherosclerosis), basement membranes of small vessels (microangiopathy), kidneys (nephropathy), retina (retinopathy), nerves (neuropathy) among other tissues. These changes are seen in both type 1 and type 2 diabetes (Cotran R.,1999).

Similarly, all types of diabetic vessels show are expanded extracellular matrix. In the retina and vasanervorum, this occurs primarily as basement membrane, while in the glomerulus, mesangial matrix is predominant. In diabetic arteries, collagen is the matrix component that increase most prominently in developing plaques. The final pathologic feature shared by all major diabetic complications is cellular hypertrophy/ hyperplasia. In the retina, endothelial cells proliferate, forming fronds of new vessels. In the glomerulus, increased tuft volume and capillary filtration surface area appear to reflect coordinated growth that is outpaced ultimately by mesangial expansion. In peripheral nerve

microvessels, the number of endothelial cell nuclei and the transverse endothelial area are both increased in diabetic patients, and are associated with the neuropathologic index of neuropathy severity. Arterial smooth-muscle cell proliferation accounts for a major portion of atherosclerotic plaque mass at all clinically significant disease sites, while subintimal neovascularization may further accelerate the rate of large vessel occlusion. Together, the cummulative effect of these shared pathologic processes is progressive narrowing of diabetic vascular lumina, causing inadequate perfusion of critical segments of target organs (Porte D.,1997).

However, the data from the Wisconsin study demonstrate a strong and consistent relationship between glycemia and the incidence and progression of microvascular and macrovascular complications in people with both type 1 diabetes and type 2 diabetes. These vascular complications are the principal cause of death and disability in diabetic patients.

Possible mechanisms for diabetic vascular complications

Diabetes mellitus is associated with vascular complications including macroangiopathy and microangiopathy. Diabetic microangiopathy is one of the main causes of blindness, terminal renal failure, and tissue necrosis leading to amputation (Chan NN.,2000). Hyperglycemia and abnormalities in lipid and lipoprotein metabolism are characteristic of both type 1 and type 2 diabetes. They are risk factor that may predispose the diabetic vasculature to deleterious consequences of diabetic complications (Panes J.,1996).

Hyperglycemia

Specific abnormalities of glucose metabolism in diabetes may exist and may be related causally to pathogenesis of macro and

microvascular complications (Kashiwagi A.,1996). Numerous investigations have concluded that causal factor primarily responsible for the development of most diabetic complication is probably prolonged exposure to hyperglycemia (Pickup J.,1997). However, the exact mechanism of hyperglycemia detrimental effect is not clear. Quite possibly, hyperglycemia mediates its adverse effects via multiple mechanisms, since glucose and its metabolites are utilized by numerous pathways (Kahn CR.,1994). It appears to involve the polyol pathway, nonenzymatic glycosylation, and glucose autooxidation. These pathways may contribute to increased oxidative stress which is a common pathway linking diverse mechanisms for the pathogenesis of complications in diabetes (Baynes JW.,1991 , Kashiwagi A.,1996 , Giugliano D.,1996).

1. The polyol pathway

The polyol pathway in tissues such as nerve, retina, lens is governed by glucose uptake does not required insulin (Pickup J.,1997). The presence of the polyol pathway (Figure 1), sorbital 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 levels of glucose, most of the glucose is metabolized to glucose–6-phosphate by hexokinase. When glucose levels rise, however, aldose reductase has a much higher Km for glucose than hexokinase, metabolites an increasing proportion of the glucose and promoting formation of sorbital (Keen H.,1999). Sorbital does not diffuse easily across cell membranes 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.,1999). Moreover, the elevated concentrations of sorbitol in peripheral nerves, Schwann cell, is 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 lead to macro and microangiopathy in diabetes. The association of the polyol pathway to increased oxidative stress was described by the perturbation of radical scavenger function, Glulathione redox cycle, in endothelial cells. Since the polyol pathway is based on aldose reductase enzymes, which can utilize a wide variety of sugar-derived carbonyl compounds as substrates and reduce these by nicotinic acid adenine dinucleotide phosphate (NADPH) to there respective sugar alcohols. If the polyol pathway were 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.,1996).

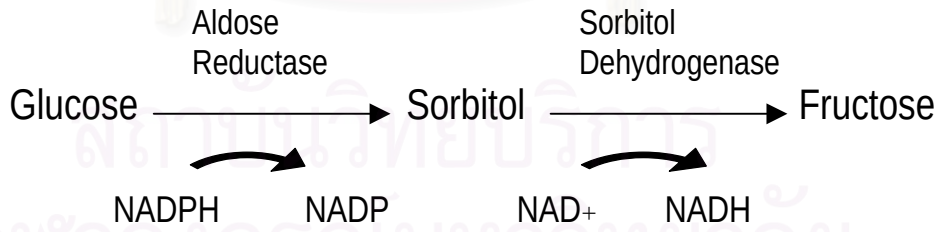


Figure 1 The polyol pathway.
(from Barnett AH.,1991)

2. Advanced products of 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 (Kahn CR.,1994). 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 can lead eventually to the formation of advanced glycosylation end-products (AGE) in a chemical reaction that is irreversible (Kahn CR.,1994) (Figure 2). 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 AGE products will from and accumulate primarily in those macromolecules with a prolonged half-life. For example, collagen, other proteins found in the basement membrane, and perhaps DNA, are particularly disposed to the formation of AGE products because of their shown turnover rates. Vascular and neural tissues also may be particularly susceptible to the accumulation of nonenzymatic glycosylation products because of their slow turnover (Kahn CR.,1994).

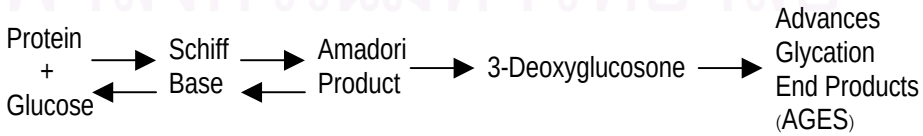


Figure 2 Nonenzymatic glycosylation of proteins.
(Modified form Pickup J.,1997)

A familiar example of a protein glycated in this way is glycated hemoglobin (HbA_1). Considerable attention has been given recently to the post-transcriptional glycosylation of proteins in diabetes, particularly with respect to haemoglobin. Chromatography of adult haemoglobins yields a major fraction (more than 90% of the total) of haemoglobin A in front of which are three fast fractions, HbA_{1a}, HbA_{1b} and HbA_{1c} – the glycosylated haemoglobins (Keen H.,1999). These three haemoglobins 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 haemoglobins are formed non-enzymatically 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 autoxidation

The term glucose autoxidation describes the capability of glucose to enolize, thereby reducing molecular oxygen and yielding oxidizing intermediates (Giugliano D.,1996). It has been suggested that glucose autoxidation and nonenzymatic glycation, together termed glycoxidation, are the major contributors to the increase in free radicals in diabetes (Lee A.,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 and indicators of the extent of underlying 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).

Dyslipidemia

Diabetes, particularly those with NIDDM, commonly have abnormalities of plasma lipids and lipoprotien concentrations, and dyslipidemia outweighs all of the other major cardiovascular risk factors (i.e., hypertension, glucose intolerance, obesity) in this patient poppulation. Individuals with poorly controlled IDDM also frequently present with a dyslipidemia, but the pattern differs from that in NIDDM (Defronzo RA.,1998).

Lipid and lipoprotein disturbances occur more frequently in NIDDM patients than is IDDM patients, and the characteristic dyslipidaemia is already present at the prediabetic stage of impaired glucose tolerance. The most common abnormality is hypertriglyceridaemia, often associated with low HDL cholesterol, while total and LDL cholesterol concentrations are similar to non-diabetic levels (see Table 2.1). The lipid and lipoprotein abnormalities in NIDDM are related closely to insulin resistance and elevated insulin concentrations and hyperinsulinaemia has been shown to precede the development of other metabolic abnormalities (Pick up.,1997).

In well-controlled IDDM patients, serum lipid and lipoprotein concentrations are similar to those in non-diabetic people. Indeed, some studies have reported lower levels of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) and higher levels of high density lipoprotein (HDL). However, disordered lipid and lipoprotein metabolism is common in poor glycaemic control, with increased concentrations of triglyceride-rich lipoproteins, chylomicrons and VLDL (see Table 1). Insulin deficiency is associated with increased hepatic production of apoprotein B-containing lipoproteins and ineffective lipoprotein clearance due to decreased activity of the insulin-dependent lipoprotein lipase. Severely insulin-deficient patients with ketosis may develop severe lipaemia with chylomicronaemia. These abnormalities are rapidly corrected with improved insulin therapy, through decreased hepatic lipoprotein production and increased lipoprotein lipase activity (Pick up J.,1997).

Table 1 Characteristic dyslipidaemias in diabetes.

(From Pickup JC.,1997)

	Serum lipid						
	Chol	Tg	VLDL	LDL	HDL	ApoB	ApoA-1
IDDM							
Good control	N / ↓	N / ↓	N / ↓	N / ↓	N / ↑		N/ ↑
Poor control	↑	↑	↑	N / ↑	N	↑	N
Nephropathy	↑	↑	↑	↑	↓	↑	↓
NIDDM							
Good control	N / ↑	↑	↑	N	↓	N	N / ↓
Poor control	↑	↑	↑	↑	↓	↑	↓

Chol, cholesterol; Tg, triglvecride; N. normal; ↓ , ↑ , lower , higher than normal, respectively.

Recently, Idzior-Walus B. et al. (2001) assessed the determinants and prevalence of hyperlipidaemia in 3159 type 1 diabetic patients. They found that plasma total cholesterol, high density lipoprotein cholesterol (HDL-C), and HDL subfractions were higher in women than in men, while plasma triglycerides were higher in men. Total cholesterol, low density lipoprotein cholesterol (LDL-C) and HDL-C and HDL-C subfractions were, as expected, significantly associated with age and HbA1c in both sexes. In aseparate study, it was found that after 12 weeks of a single injection of streptozotocin (STZ;45 mg/kgBW,i.p.) in rats, there was a significant increase in blood glucose, plasma cholesterol and triglyceride levels of diabetic animals (Ozansoy G.,2001).

As mentioned earlier, there is some evidence indicating an association of oxidative stress and hyperlipidemia in type 1 diabetes. There was a significant increase in plasma non-esterified cholesterol, triglycerides and phospholipids, as well as decrease in HDL-C accompanied by an increase in lipid peroxidation by product, malondialdehyde (MDA) (Ahmed I.,2001). It was suggested that the decreasing of circulating lipids may be effective strategy to minimize increased oxidative stress in diabetic plasma vasculature (Ozansoy G.,2001). Moreover, some studies have demonstrated that antioxidant supplementation may develop hyperlipidemia in type 1 diabetes. Since, oxidative stress and defective fatty acid metabolism may lead to impaired endothelial function in diabetes. Ford I. et. al. (2001) studied the effects of 2-weeks treatment with the antioxidant alpha-lipoic acid (ALA) on lipids and endothelial factors, in rats with STZ-induced diabetes. They found that compared with their nondiabetic littermates, untreated diabetic rats had increased serum triglycerides, cholesterol and von Willebrand

factor (vWF). Treatment with ALA was associated with marked and statistically significant decreases in triglyceride and vWF.

II. ANIMAL MODELS OF DIABETES MILLITUS

Several models of diabetes have been used in the previous investigations. There are many animal models with diabetes as presented in phenotypic forms of type 1 diabetes and type 2 diabetes. None of the species exhibits the full spectrum of functional or structural lesions associated with diabetes in humans, but each offers an opportunity for investigating certain clusturs of derangements that are common in diabetic humans, particularly in their formative stages. A synopsis of important metabolic endocrine abnormalities and pancreatic features is presented in Table 2 (Porte D.,1997).

Table 2 Classification and features of rodents species with diabetes
(From Porte D.,1997)

Species	Metabolic-Endocrine Abnormalities	Pancreatic Function
■ CYTOTOXIC DIABETES		
Most species administered streptozotocin or alloxan	Features similar to IDDM: Rodents survive without insulin support Up-regulation of insulin receptors	Almost complete loss of beta cells; inclination to pancreatic tumors on long-term maintenance
■ AUTOIMMUNE DIABETES		
BB rat	Spontaneous onset at~8 weeks, differing in colonies and conditions of maintenance	Insulitis and necrosis as in human IDDM but mechanism of islet beta-cell injury may differ
NOD mouse	Spontaneous onset with early insulitis with overt diabetes at 13 to 30 weeks; sex bias-most males exhivit insulitis not progressing to beta-cell destruction	As above; marked influence of environmental factors
■ VIRUS-INDUCED DIABETES	Changes similar to autoimmune diabetes but transient	Function compromised but no insulin dependency

Species	Metabolic-Endocrine Abnormalities	Pancreatic Function
■ GENETIC MUTANTS WITH DIABESITY C57BL/6J ob mice	Hyperinsulinemia, insulin resistance directing nutrients to lipogenesis, moderate hyperglycemia; neuroendocrine dysregulation; no response to satiety signals; defective thermogenesis; low insulin receptor function	Profuse and persistent insulin oversecretion, hyperplasia and hypertrophy, sensitivity to neurogenic stimuli
KK mice and yellow agouti A^{vy} + hybrids	Obesity promoted by high-energy diet, insulin resistance, hyperglycemia	Changes as in ob mice; sustained oversecretion, does not lead to beta-cell degranulation
NZO mice	Obesity, insulin resistance; insulinemia less severe than in ob	Loss of first-phase release but persistent oversecretion; impaired islet glucose metabolism
C57BL/Ks db mice	Hyperinsulinemia with obesity, then hypoinsulinemia and ketosis; hyperphagia and faulty satiety receptors; resistance with low insulin receptor response; carbohydrate-rich diet shortens survival	Labile islets; hypertrophy and enhanced replication followed by beta-cell degeneration probably due to hyperglycemia
Zucker fa and ZDF/Drt-fa rats	Hyperphagia, hyperinsulinemia, and resistance similar to ob mice; remarkable obesity which develops even on restricted diets; hypothalamic insulin resistance; hyperlipidemia; diabetes sexually dimorphic	Insulin hypersecretion persists in isolated islets; separate gene or neurogenic factor effects
WDF-Ta/fa and WKY/DRT-fa rats	Hyperphagia, obesity, and insulin resistance similar to fa rats; more pronounced lipidemia and diabetes sexually dimorphic	Beta-cell hypertrophy and hyperplasio; abnormal response to secretogogues
■ CORPULENT GROUP OF SPECIES WITH MARKED PROPENSITY TO COMPLICATIONS (ALLELES OF fa, SUGGESTED DESIGNATION, fa^{cp} OR cp^{fa})		
SHR/N-cp rats	Corpulence, hyperinsulinemia, and resistance accentuated by sucrose diel; rise in counterregulatory hormones; hypertension; hyperlipidemia; nephropathy	Long-lasting secretion with hyperplasia persistion
SHHF/Mcc-cp rats	Obesity; hyperinsulinemia and resistance; hypertension; hyperlipidemia; sexual dimorphism; congestive heart failure and nephropathy	As above
Jcr:LA-cp rats	Obesity; hyperinsulinemia and resistance; no hypertension; hyperlipidemia; sexual dimorphism, marked cardiovascular atherosclerosis	As above

Species	Metabolic-Endocrine Abnormalities	Pancreatic Function
■ RODENTS WITH DIABETES ISOLATED BY SELECTIVE BREEDING		
GK rat	Nonobese, mildly hyperglycemic; insulin resistant, nonketoic; bred by selection pressure on glucose intolerance on regular diet	Islet deformation and secretion abnormality, gradual beta-cell loss
Cohen rats	Nonobese, hyperglycemic, transiently hyperinsulinemic then overtly diabetic; bred by selection pressure of glucose intolerance on high-sucrose diet	Defective first-phase and stimulated release, then beta-cell breakdown

Streptozotocin induced diabetes

In present study, Streptozotocin (STZ) was used to experimentally induce diabetes in rats. STZ [2-deoxy-2- (3-methyl-3-nitrosoureido-D-gluco pyranoside)] is a nitrosourea derivative isolated from the mould *Streptomyees grisevus* (Figure 3). The diabetogenic action of STZ destroys most islet beta cells. It is effective in different, species-specific doses, ranging from 25 to 200 mg/kg, in rats, dogs, mice, hamsters, monkeys,

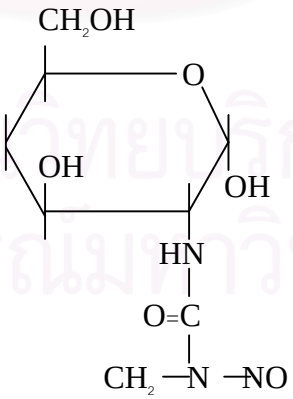


Figure 3 Molecular structures of streptozotocin, 2-deoxy-2-(3-methyl-nitrosoureido)-D-glucopyranose.
(From Porte D.,1997)

miniature pigs, pigs and rabbits (Porte D.,1997). It can induce severe insulin deficient diabetes in rats and other rodents, either when given as a single large dose (50-100 mg/kg in rats) or as multiple smaller doses; in the latter case, diabetes develops more gradually and appears to have an autoimmune, rather than a toxic, basis (Pickup JC.,1997). STZ-treated animals, though insulinopenic, retain some insulin-secretion capacity, are not ketotic, and do not usually require insulin support for survival. In fact, a mild diabetic state, resembling an insulin-poor form type 2 diabetes, may be induced in rats by a single low dose of about 35 mg/kg STZ. However, there is a tendency for spontaneous recovery in rats receiving doses below 35 mg/kg (Porte D.,1997).

STZ is unstable in solution even at acid pH, and should be injected promptly after dissolving in citrate buffer at pH 5.0. Its in vivo life span is less than 15 minutes (Porte D.,1997). After the intravneous administration of STZ, an early hyperglycaemic phase appears, followed by a hypoglycemic phase and then a perment diabetic phase occuring at appoximate 4,7 and 24 hours, respectively (Wong KK.,1996). The precise mechanism of STZ diabetogenicity has been described. STZ may act on both the membrane and the interior of the β cell (Figure 4). It damages β cell membrane and also induce fragmemtation of DNA (Pickup JC.,1997). STZ causes DNA strand breaks in pancreatic islet and stimulates nuclear poly (ADP-ribose) synthetase, and thus depletes the intracellular NAD and NADP levels. NAD depletion by STZ inhibits proinsulin synthesis and thus induces diabetes. The pathological and biochemical features of the model may be compatible to those of type I diabetes in humans (Ohkuwa T.,1995).

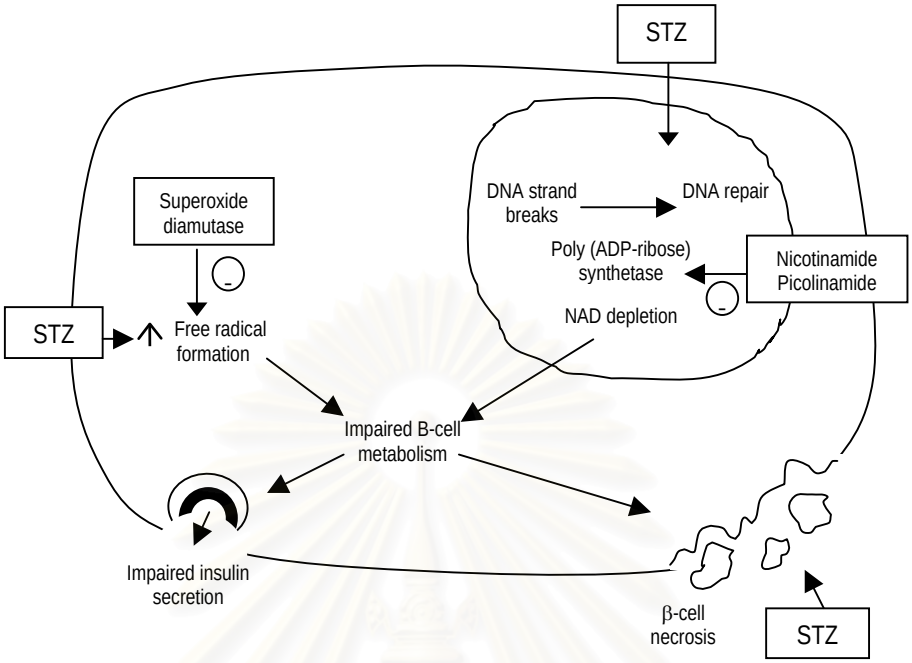


Figure 4 Suggested mechanisms of streptozotocin toxicity on the β cell.
(Modified from Pickup JC.,1997)

III. ENDOTHELIAL DYSFUNCTION IN DIABETES

Endothelial function

The endothelium, a continuous cellular monolayer lining the blood vessels, has an enormous range of important homeostatic roles. The endothelial cell (Ec) lining is ideally situated at the interface between the blood and the vessel wall to serve as a sensor and transducer of signals within the microenvironment (Gibbons GH.,1996). Endothelial cells orchestrate the homeostatic balance of the vessel through the production of the numerous vasoactive or platelet active agent (listed in Table 3) regulating vessel tone, coagulation state, cell growth and leukocyte trafficking (Vapaatalo H.,2001). We will highlight reviewes only on nitric oxide, a major vasoactive substance produced by endothelium.

Table 3 Endothelial factors which have vasoactive, hemostatic, growth modulating or inflammatory properties.

(From Vapaatalo H.,2001)

Vasodilators	**Vasoconstrictors**
Nitric oxide	Endothelin-1
Prostacyclin	Angiotensin II
Endothelium derived	Endoperoxide (PGH2)
Hyperpolarizing factor	Thromboxane A2
C-natriuretic peptide	
Antithrombotic	**Prothrombotic**
Tissue type plasminogen activator	Plasminogen activator inhibitor-1
Prostacyclin	Thromboxane A2
Nitric oxide	
Growth inhibitors	**Growth promotors**
Nitric oxide	Superoxide radicals
Prostacyclin	Endothelin
C-natriuretic peptide	Angiotensin II
Inflammation inhibitors	**Inflammation promotors**
Nitric oxide	Superoxide and other free radicals
	Tumor necrosis factor-alpha

Nitric oxide (NO)

Nitric oxide (NO) is an important messenger molecule that plays a critical role in a wide variety of physiological functions, including neuronal transmission, vascular relaxation, immune modulation and cytotoxicity (Kapur S.,1997). NO is a short-lived, free radical gas (Haendeler J.,1999). The discovery that NO is an unique diffusible molecular messenger in the vascular and immune system motivated searches for NO biosynthesis and action throughout the body. NO is produced catalytically by different NO-synthase (NOS) isoforms in a reaction scheme, involving the five electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO and stoichiometric amounts of citrulline. The reaction demands oxygen and NADPH as consubstrates, with numerous other redox cofactors including

enzyme bound heme, reduced thiols, FAD, FMN, and tetrahydrobiopterin (Brune B.,1998). In biological systems, NO is produced from the amino acid L-arginine by three different isoforms of nitric oxide synthase (NOS). Three isoforms of NOS have been identified, which are highly homologous in their primary structure (Figure 5) (Govers R.,2001). The neuronal NOS (nNOS), or type I NOS, was first cloned from rat cerebellum. Immunological or inducible NOS (iNOS), or type II NOS, was identified in macrophages, whereas the last isoform was the endothelial NOS (eNOS), or type III. iNOS, nNOS and eNOS are constitutively expressed, whereas iNOS expression requires stimulation with cytokines, microorganisms, or microbial products. The constitutively expressed isoforms

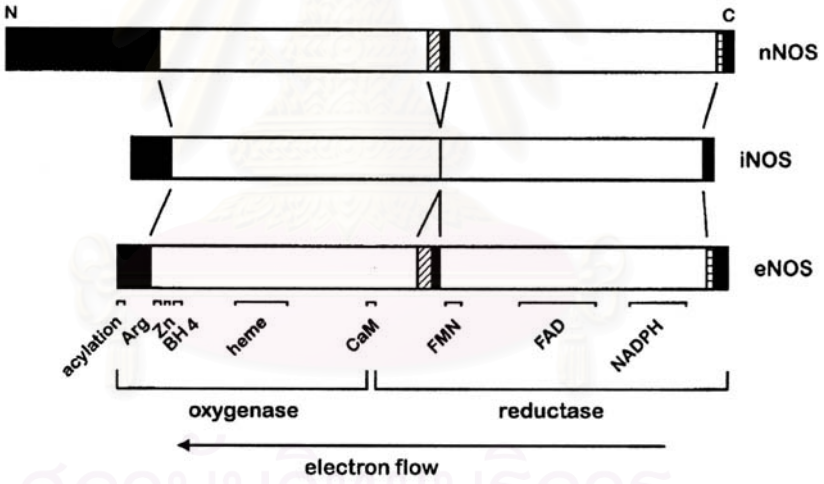


Figure 5 Structure of nitric oxide isoforms. NH2 (N) and COOH (C) termini are indicated. Homology in amino acid sequences are depicted: open boxes, homologous regions; hatched boxes, homologous in neuronal (nNOS) and endothelial nitric oxide synthase (eNOS); sold boxes, isoform-specific sequences. For eNOS, regions involved in acylation and in the binding of substrates and cofactors are indicated as well as the oxygenase and reductase domain and the direction of the intramolecular electron flow. Arg, arginine; BH4, tetrahydrobiopterin; CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

(From Govers R.,2001)

of NOS are calcium dependent and produce physiological concentration of NO in the picomolar range where as iNOS is calcium independent and releases larger amounts of NO in nanomolar concentrations (Brune B.,1998 , Haendeler J.,1999). Under physiological conditions, the endothelial isoform of NOS (eNOS) is the prevailing form in the vascular system. ***Therefore, this review will deal with the physiological role of endothelium-derived NO.***

Endothelium-derived nitric oxide

Physiological amounts of NO as produced by the eNOS play an important role as a mediator in the cardiovascular system. Endothelial-derived NO is a potent vasodilator, inhibits platelet aggregation and adhesion, and prevents smooth muscle cell proliferation and leukocyte activation. The best characterized effect of endothelium-derived nitric oxide is the activation of the guanylate cyclase, which leads to an increase in the concentration of cyclic GMP, mediates vascular relaxation (Figure 6). Nitric oxide from the vascular endothelium mediating vasodilation can be elicited by endothelium-dependent vasodilators such as acetylcholine, bradykinin, and calcium ionophores as well as fluid shear stress (Ranjan V.,1995). Endothelial-derived nitric oxide (NO) is synthesised from L-arginine by NO synthase (NOS). Nitric oxide diffuses across to vascular smooth muscle cell to cause vasorelaxation through cyclic GMP (cGMP) process (Chan NN.,2000). NO stimulates soluble guanylate cyclase in vascular smooth muscle cells, thereby increasing guanosine 3',5'-cyclic monophosphate. This second messenger reduces intracellular free calcium by several mechanisms and also reduces the calcium sensitivity of the contractile apparatus. In addition, high doses of NO can directly affect potassium channels, thereby inducing hyperpolarization and a subsequent reduction of calcium influx into the vascular smooth muscle cells. All

these events, alone or in combination, lead to a relaxation of vascular smooth muscle and, eventually, maximal vasodilation by application of physiological concentrations of endothelial stimuli (Pohl U.,1999).

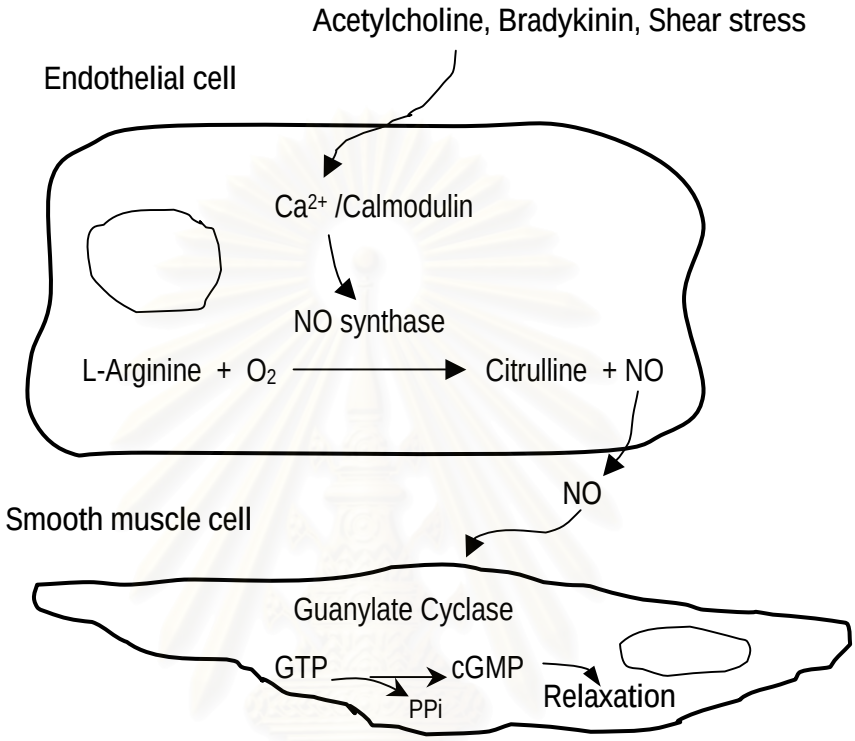


Figure 6 Nitric oxide diffuses across to vascular smooth muscle cell to cause vasorelaxation through cyclic GMP (cGMP) pathway.
(Modified from Chen NN.,2000)

Endothelial dysfunction

Endothelial dysfunction (ED) exists in many arterial diseases and is characterized by deterioration of endothelial vasodilator function. It can manifest either by decreased secretion of vasodilatory mediators, increased production of vasoconstrictors, increased sensitivity to vasoconstrictors and low resistance of vascular smooth muscle to endothelial vasodilators (Vapaatalo H.,2001). Moreover, ED is also characterized by vasospasm, inflammation, platelet aggregation, thrombosis, abnormal vascular proliferation and leukocyte adhesion result

in atheroselerosis and hypertension. It is important to realize the values of indicators for endothelial function tests to quantify the severity of disease in individual subjects. Indicators or markers which can be related in ED are listed in Table 4.

Table 4 Measurement of endothelial dysfunction

(From Vapaatalo H.,2001)

Nitric oxide metabolites (nitrite, nitrate) and second messenger of nitric oxide cyclic GMP in plasma/serum and urine
Functional tests of endothelium-dependent vasodilation
invasive coronary of forearm test
noninvasive coronary test (positron emission tomography)
noninvasive ultrasound method (flow mediated dilation)
Circulating markers of endothelial function
asymmetric dimethylarginine (ADMA)
endothelin-1
von Willebrand factor
tissue type plasminogen activator
plasminogen activator inhibitor-1
Adhesion molecules
intercellular adhesion molecule-1
vascular cell adhesion molecule-1
E-selectin
P-selectin

Diabetic endothelial dysfunction

Endothelial dysfunction, found in humans with both type I, insulin-dependent diabetes mellitus and type II, non-insulin dependent diabetes mellitus (Lund DD.,2000), has been suggested to be an early event in diabetic vascular disease (Giugliano D.,1996). The influence of diabetes on vascular function has been studied using a wide variety of human and animal models in a number of different vascular beds and different techniques (Michael T.,1993, Makino A.,1998, Lund DD.,2000, Richard

F.,2000). In 1998, Makino A. et al. examined the vasoconstriction induced by methoxamine in isloted mesenteric arterial beds from streptozotocin (STZ)-induced diabetic rats. **They suggested that the methoxamine-induced mesenteric vasoconstriction was attenuated in diabetic rats. Futhermore, impaired endothelium-dependent relaxation were demonstrated by Lund DD. (2000).** They found less relaxation of carotid artery in response to acetylcholine in diabetic than in normal rabbits was observed while relaxation to sodium nitroprusside, an endothelium-independent vasodilator, was similar in normal and diabetic animals. **Numerous studies suggest that the mechanism of impaired endothelium-dependent relaxation may involve inactivation of NO by oxygen-derived free radicals** (Pieper GM.,1996 , Plamer AM.,1998 , Higashi 1999 , Richard F.,2000).

The interaction between leukocytes and endothelium is one of the markers for evaluating endothelial function. Recruitment of leukocytes during inflammation is known to be a multistep process where initial rolling of leukocytes along the endothelium is a precondition for subsequent firm adhesion and extravasation (Salas A.,1998). Experimental evidence, using intravital microscopy, has demonstrated that diabetic state enhanced leukocyte adhesion and emigration in mesenteric postcapillary venules. **It was found that mesenteric venules of 3-wk diabetic animals had an increased baseline level of leukocyte rolling compared with controls** (Salas A.,1998). Moreover, it was demonstrated that exposure of the vascular endothelium to elevated glucose upregulates leukocyte-endothelium interaction in the rat mesenteric microcirculation. There is some evidence suggested that oxidative stress may be involved by quenching NO production (Booth G.,2001).

IV. FREE RADICALS

Free radicals may be defined broadly as molecules or ions that are capable of existing independently and containing one or more unpaired electrons in their outer orbitals. The unpaired electron causes the free radical to be highly reactive and unstable (Halliwell B.,1984, Sen CK.,1995). The conventional “radical dot” (•) designates the presence of one or more of the unpaired electrons (Arouma OI., 1994 , Asmus KD., 1994). To regain the stability, the free radicals will pull electrons from adjacent molecules. If free radical generation is intense and uncontrolled, the outcomes are general molecular chaos and loss of cell functions (Clark IA.,1986).

In general, the environment of the human cells is aerobic and consumes molecular oxygen in the process of producing energy. Oxygen free radical intermediates are yielded partly from the process of electron transfer in respiratory chain are harmful to aerobic cells (Ji LL.,1996). Normally, most of the oxygen consumed is used in the mitochondria for oxidative phosphorylation and is reduced to water. However, a small fraction of oxygen (approximately 2% to 5% v/v) is converted into several oxygen intermediates, i.e., superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Leninger AL.,1993, Ji LL.,1996, Goldfarb AH.,1994, Child RB.,1998) (Figure 7). Collectively, they are classified as “reactive oxygen species” (Viguie CA.,1993, Sen CK.,1995). These three reactive oxygen species (ROS) can cause a wide spectrum of cell damage including inactivation of enzymes, alteration in intracellular oxidation-reduction state and damage of DNA, protein and cell membrane (Halliwell B.,1985 , Slater TF.,1987)

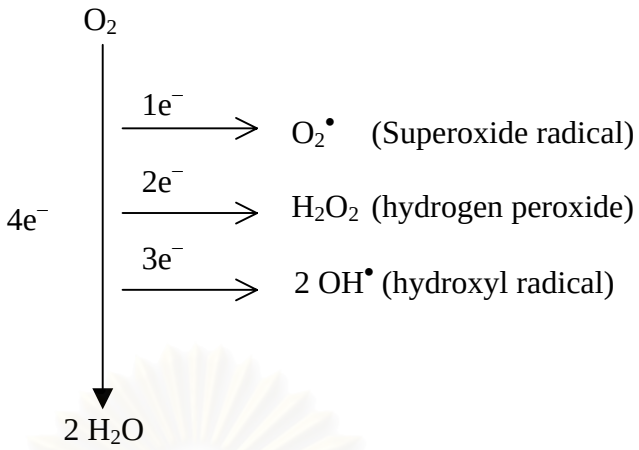


Figure 7 Univalent pathway for the reduction of molecular oxygen. By a series of single-electron transfers molecular oxygen is reduced first to the superoxide radical ($O_2^{\bullet}$) and from superoxide, with the addition of two electrons, to hydrogen peroxide (H_2O_2). Hydrogen peroxide is itself then univalently reduced, with the addition of another proton, to water and the hydroxyl radical ($OH^{\bullet}$). A final univalent reduction and the addition of another proton convert the hydroxyl radical to water. [Adapted from Bulkley Gb.].

(From Gregory B.,1983)

Free radicals induced cellular damage

Free radicals damage cell membranes by catalyzing amino acid oxidation, protein-protein cross-linking, protein strand scission, and attacking polyunsaturated fatty acid in cell membrane leading to the subsequent chain reaction process called lipid peroxidation (Halliwell B.,1984 , Gutteridge JM.,1990). Lipid peroxidation is a process by which any free radical ($R^{\bullet}$) in the presence of molecular oxygen (O_2), with sufficient energy, abstracts an hydrogen atom from a methylene group ($-CH_2$) of unsaturated fatty acid (LH) (Gutteridge JM.,1990 , Halliwell B.,1993) (Figure 8).

Normally, biomembranes contain relatively large amounts of polyunsaturated fatty acid (PUFA). The greater the number of double bonds in the fatty acid side chain the easier the removal of the hydrogen atom. So,

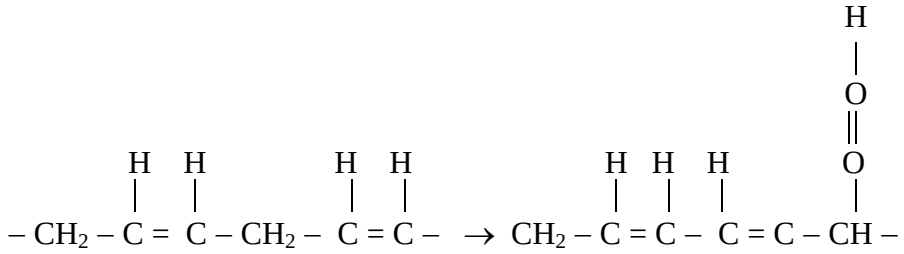


Figure 8 Change in the structure of lipid due to hydrogen abstraction by free radicals (From Asmus KD.,1994).

PUFAs are the major sites of lipid peroxidation (Asmus KD.,1994 , Sen CK.,1995). Oxidation of PUFA or lipid peroxidation leads to the disintegration of fatty acid and formation of hydrocarbon gasses (e.g., pentane) and aldehydic compounds, in particular the volatile low molecular weight aldehyde, malondiadehyde (MDA) (Wade CR.,1988 , Ji LL.,1996) (Figure 9). The deleterious health outcomes associated with accumulation of

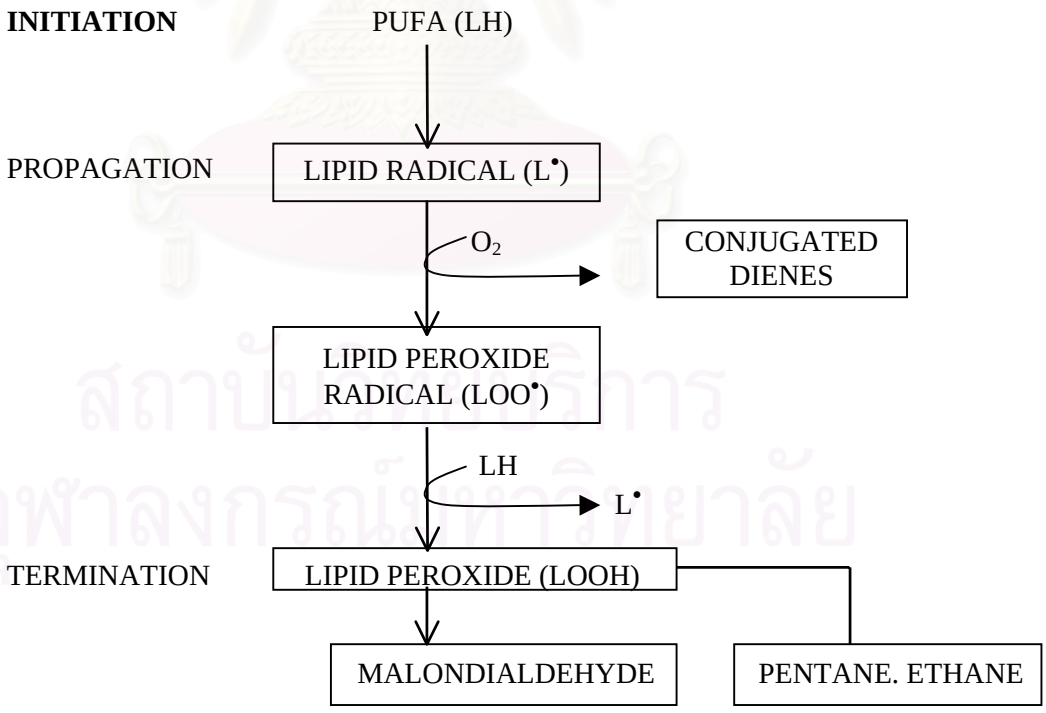


Figure 9 Three steps of lipid peroxidation: initiation, propagation and termination. (From Jenkins RR.,1993)

large amounts of lipid peroxidation byproducts such as MDA, include an increase in tumor frequency and incidence of atherosclerosis (Asmus KD.,1994).

Malondialdehyde (MDA)

MDA, a lipid peroxidation byproduct, is a toxic compound capable of reacting with sulfhydryl and amino groups of proteins. It is a bifunctional cross-linking agent since it can produce both intra- and intermolecular linkages which can lead to inactivation and polymerization of enzymes such as ribonuclease. Its reactivity toward amino groups can result in interactions with the endogenous base of DNA as well as inhibition of DNA, RNA and protein synthesis thereby affecting their biological functions (Tappel AL.,1973 , Lee DM.,1980 , Siu SM.,1982).

MDA can induce polymerization of membrane components and variety of cross-linking reactions such as lipid-lipid cross-linking and lipid-protein cross-linking (Gregory B.,1983) (Figure 10). These effects will result in deteriorative changes in cellular membranes such as loss of fluid properties and membrane flexibility due to the alteration in the fluid-mosaic bilayer, a decrease in membrane fluidity and an inability to maintain ionic gradients (Niess AM.,1996). Cellular swelling, a loss of cell integrity and cell inflammation will follow (Alessio HM.,1993). Free radicals may also disrupt subcellular membranes of important organelles including mitochondria, microsomes, and lysosomes (Gregory B.,1983 , Cotran RS.,1999). Lipid peroxidation of the mitochondrial membrane leads to swelling, lysis, and disintegration of the mitochondria. Microsomal membrane damage results in disaggregation of polyribosomes and inhibition of protein synthesis. For the lysosomes, since they contain hydrolytic enzymes, the lysosomal membrane damage will be followed by the enzymatic digestion of other cell components (Sen CK.,1995).

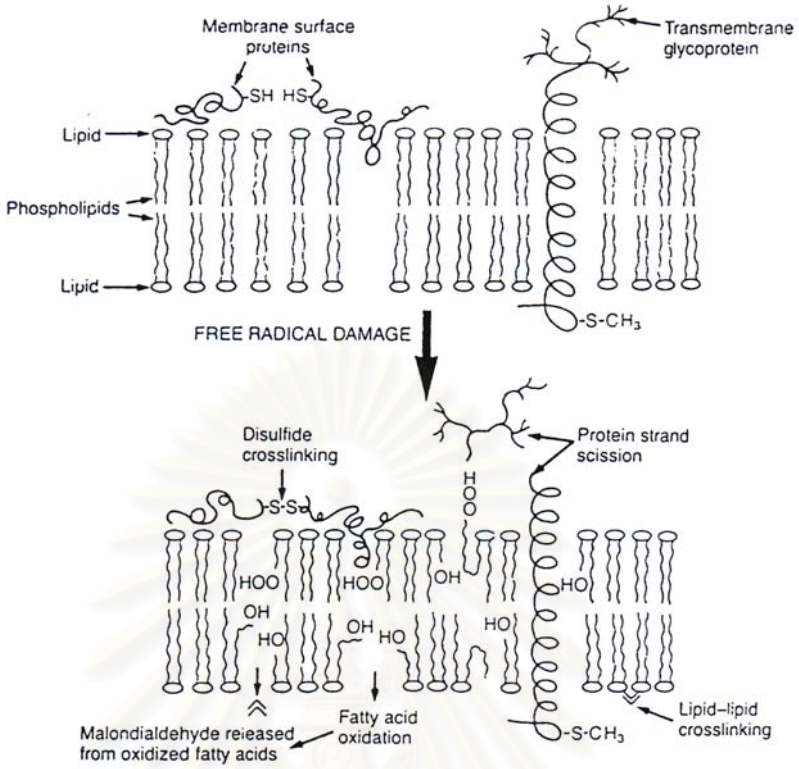


Figure 10 Free radical damage of membranes. Free radicals can affect lipids by initiating peroxidation, which leads to short chain fatty acyl derivatives and the byproduct malondialdehyde. A variety of cross-linking reactions can be mediated malondialdehyde reactions. Free radicals can also catalyze amino acid oxidation, protein-protein cross-linking, and protein strand scission.

(From Freeman BA.,1982)

V. ANTIOXIDANT SYSTEM

The human body is equipped with a sophisticated antioxidant system to deal with the production of reactive oxygen species (ROS). The system includes enzymatic antioxidants and non-enzymatic antioxidants. Antioxidant enzymes or “scavenging enzymes” provide the first line of defense against ROS by converting them to more reduced and more stable species. The three major scavenging enzymes are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Ji LL.,1996) (Figure 11). A second line of defense is provided by non-enzymatic or

exogenous antioxidants obtained primarily as nutrients or nutritional supplements such as, carotenoids, reduced glutathione, and vitamins E and C (Sen CK.,1995 , Margaritis I.,1997).

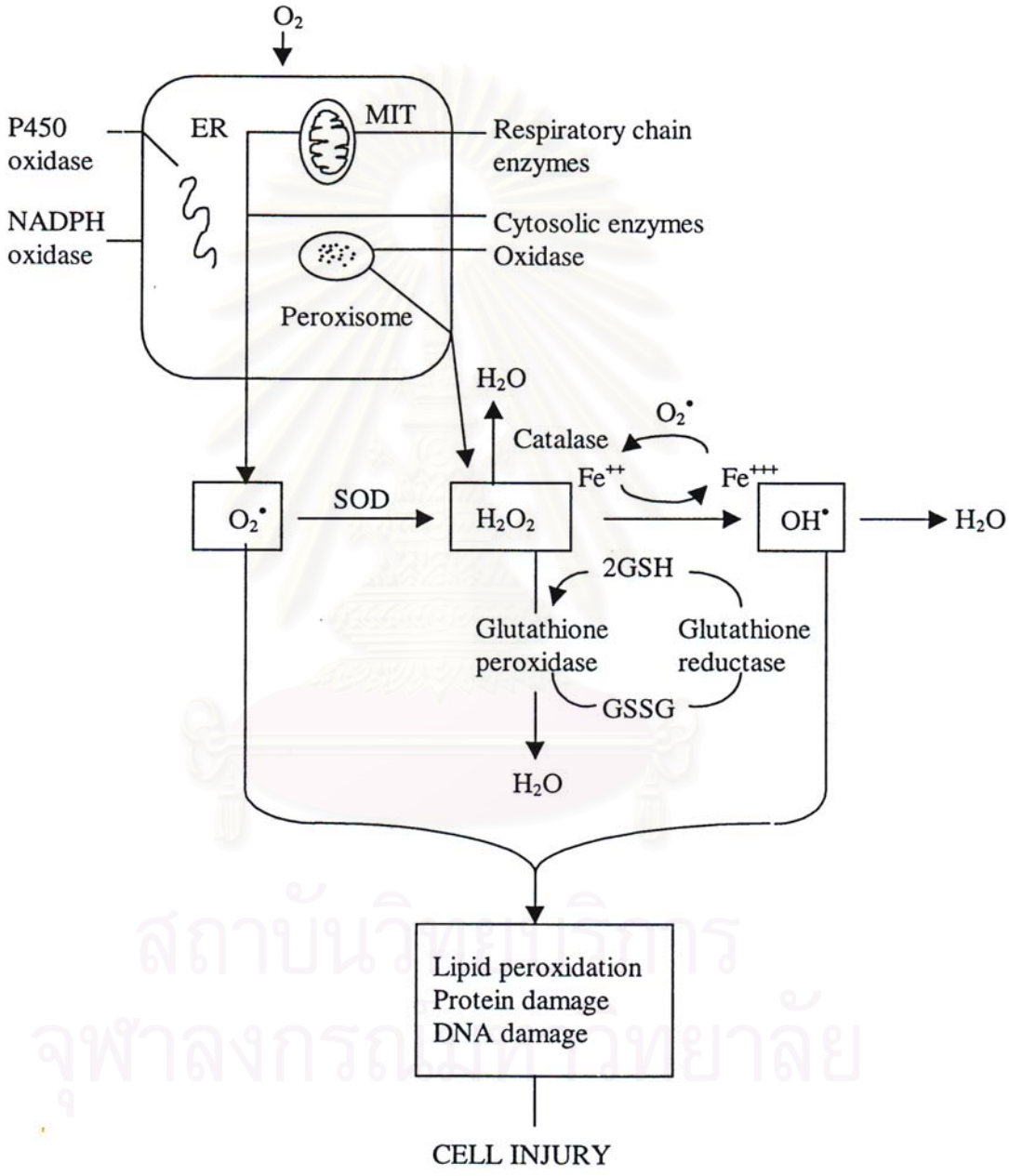


Figure 11 Antioxidant system to deal with the production of reactive oxygen species (ROS) (From Halliwell B.,1984)

The possible mechanisms by which antioxidants protect against oxygen toxicity are as follows: (Arouma OI.,1994 , Sen CK.,1995)

1. decreasing localized oxygen concentration and prevention at ROS formation via a fall in substrate
2. a chain-breaking mechanism whereby intermediate radicals (e.g., $O_2^{\bullet}$) are scavenged to prevent continuation of more deleterious forms (e.g., $OH^{\bullet}$, H_2O_2)
3. interception of ROS attack by scavenging the reactive metabolites and converting them to less reactive molecules and/or by enhancing the resistance of sensitive biological targets to ROS attack
4. facilitating the repair of damage caused by ROS and triggering the expression of genes that encode antioxidant proteins
5. providing a favorable environment for the effective functioning of other antioxidants (e.g., as a cofactor or by acting to maintain a suitable redox status)

Vitamin C

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

Structure

Vitamin C comprises essentially two compounds, L-ascorbic acid (mol. Wt 176), a strong reducing agent, and its oxidized derivative L-dehydroascorbic acid. Although most vitamin C in body fluids and tissues is in its reduced form, both ascorbic acid (AA) and dehydroascorbic acid (DHAA) have biological activity, and are interconvertible by an oxidation /reduction reaction (Figure 12). Some of the enzymes responsible for these interconversions are glutathione dehydrogenase and ascorbate oxidase (Tapan K. et. al., 1996).

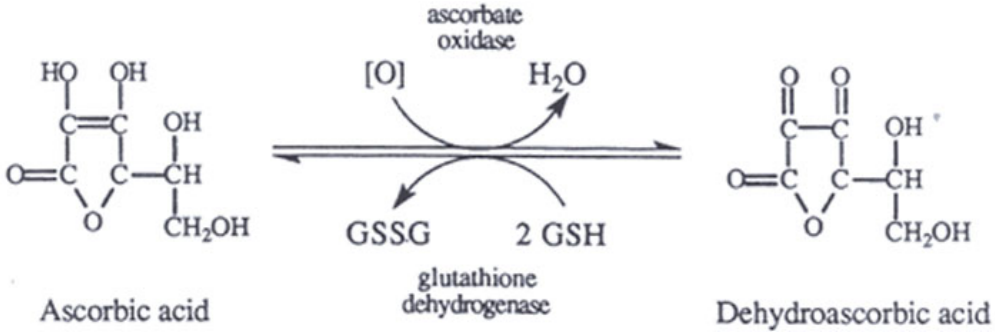


Figure 12 Interconvertibility of ascorbic acid by oxidation and reduction
(From Tapan K. Basu,1996)

Absorption, Transport and Storage

The absorption of vitamin C in humans occurs in the buccal mucosa, stomach, and small intestine. After absorption, vitamin C equilibrates rapidly 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 have high concentrators of vitamin C. Vitamin C exists in blood and tissues mainly in the reduced form; its oxidized form is generally constitutes less than 10% of the total (Tapan K. et. al.,1996).

Vitamin C is not stored in individual tissue deposits as is vitamin A. Rather it is distributed more generally 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 balance (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 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-1 and most is lost in the urine within 24 hours (Tapan K. et.al.,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. et. al.,1997).

Ascorbate may be involved in reducing damage to the cell from radicals. A simplified mechanism (Figure 13) shows the hydroxyl radical ($OH^{\bullet}$) reacting with a component in the cell, abstracting (pulling off) a hydrogen radical. The product is a radical, but it is one that is more stable than $OH^{\bullet}$. Ascorbate may donate a hydrogen radical ($H^{\bullet}$) to this product, thus repairing it before further deterioration can occur. Here, the ascorbate is converted to a semidehydroascorbate, a relatively stable radical, which

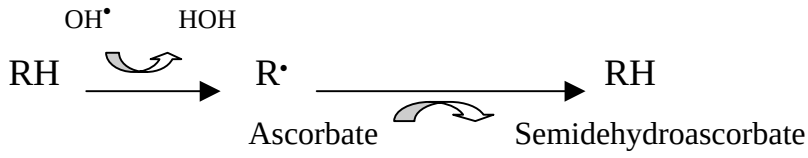


Figure 13 Possible use of ascorbate in reducing damage from radicals
(Modified from Brody T.,1994)

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).

The suggested antioxidant actions of vitamin C include the following (Manore M. et. al.,2000) :

- Stabilization of hydroxyl radical
- Quenching of singlet oxygen
- Reducion of oxidized form of vitamin E
- Reduction of nitrosamines to harmless species
- Protect of the lungs from ozone and cigarette smoke

Vitamin C and Diabetes

Abnormal endothelial function has been observed in patients with conditions predisposing them to the development of diabetes. While the mechanisms of endothelial dysfunction in diabetes individuals are not clear, there is strong evidence that inactivation of nitric oxide by increased oxygen-derives free radicals could be responsible (Lekakis JP.,2000). There is evidence to suggest that oxidative stress is increased in human with diabetes and in animal models of diabetes (Giuglano D.,1996). Moreover, evidence for oxidative stress in diabetes includes the 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 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 blunts the increased lipid peroxidation in alloxan diabetic rats by protecting antioxidants enzymes. Nazirogly M. et.

al. (1999) studied the effect of selenium, vitamins C and E on the lipid peroxidation, glutathione peroxidase (GSH-Px) and reduced glutathione (rGSH) activities in the lens of streptozotocin (STZ) rats. They suggested that vitamins 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.,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.,1995). **It was found that vitamin C concentrations of the brain, heart, lung, liver, kidney, and plasma of the diabetic rats were decreased significantly after 8 weeks of diabetes compared with those of the control group** (Sun F.,1999). As vitamin C deficiency has been implicated in some of the complications of diabetes such as angiopathy (Dai S.,1995, vitamin C supplementation may be beneficial. In 1997, Siman CM. et. al. 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.(2000), abnormal endothelial function in type 2 diabetes was restored by treatment with vitamin C supplementation. **Since diabetes mellitus in man and in experimental animals is associated with elevated plasma lipid levels, in particular triglycerides, it is of importance that Dai et al. showed that the production of hyperlipidemia in diabetes was prevented by vitamin C supplementation** (Dai S.,1997).

VI. EXERCISE

Physical activity is defined as any form of muscular activity. Therefore physical activity results in the expenditure of energy proportional to muscular work and is related to physical fitness. Exercise represents a subset of physical activity that is planned, with a goal of improving or maintaining fitness (Powers SK.,2001).

Exercise, or physical activity or work of a fairly vigorous nature, leads to a number of important bodily changes. The changes in function of the body are brought about by both single and repeated bouts of exercise. A single bout of exercise is sometimes called “acute exercise” whereas repeated bouts of exercise over several days or months is called “chronic exercise” or “exercise training” (Lamp DR.,1984).

The functional changes that occur when one exercises a single time are called responses to exercise. These functional changes are sudden, temporary and disappear shortly after the exercise period is finished. They are, for example, the increase in heart rate, the rise in blood pressure, the increase in breathing, the increase in blood flow to the working muscles and the decrease in blood flow to the stomach and the kidney. Each of these responses will persist no longer than a few minutes after the exercise is over (Lamp DR.,1984, Fox LE.,1993).

Training has the major objective of facilitating biologic adaptations that improve performance in specific tasks. An “adaptation” is a more or less persistent change in structure or function following training that apparently enables the body to respond more easily to subsequent exercise bouts. Ordinarily, adaptations are not seen until several weeks of training have passed, but some occur after only four or five days of training. The training is influenced by many factors such as intensity, duration, frequency and mode of exercise (Lamp DR.,1984, McArdle

WD.,1996). Each of these is important for physical adaptations. To achieve the training improvement, these major factors must be considered (Robergs RA.,1997).

Intensity

Intensity refers to the level of stress achieved during the exercise period. Intensity is determined best from measurements of oxygen consumption, but indirect methods are heart rate, respiration rate, or from the rating of perceived exertion (PPE). Exercise sessions can be low intensity or high intensity. Low intensity exercise would be equal or 50% to 60% of an individual's maximal heart rate, whereas 85% to 90% would relate to high intensity exercise. It is best to begin an exercise program at a low intensity and gradually increase the intensity over time.

Duration

Duration refers to the length of the training session. Duration and intensity are inversely related; that is, if the intensity of the exercise is high, the duration is generally low, and vice versa. The duration of the exercise session can be affected by environmental factors (e.g., heat, humidity, altitude). It can also be affected by the present fitness level or energy supply of an individual.

Frequency

Frequency refers to the number of training sessions per week. It is recommended that individuals try to exercise 4 or 5 days per week. The frequency of exercise depends on the type of exercise performed and the fitness status and goals of the individual.

Mode

Mode refers to the type of activity performed during the exercise session. Various modes of exercise can affect the components of fitness

differently. Choosing the correct mode of exercise is important because it has a direct effect on the outcome.

Physiology adaptations to training

The physiological adaptations that occur with chronic exposure to exercise improve both exercise capacity and efficiency. These adaptations are highly specific to the type of training (Wilmore JH.,1999).

The two contrasting types of training, anaerobic and aerobic, have difference consequences on these parameters anaerobic exercise (static or isometric) includes such activities as carrying a suitcase or weightlifting : muscle tension develops, but there is little or no displacement of the object worked against aerobic exercise (dynamic or isotonic) includes running, walking and related sports: regular muscular activity occurs, but against a light load (Opie H.,1998).

Adaptations to anaerobic (sprint) training

Anaerobic training is the training to improve the capacity to perform all-out exercise for brief periods of time (up to 60 seconds). Anaerobic exercise largely depends on ATP generated by the immediate and short-term anaerobic energy system. In training to enhance ATP-CP energy transfer capacity, the activities selected must engage the specific muscles at the movement speed and power output for which the experiment desires to improved anaerobic power. As the duration of all out effort extends beyond 10 second, dependence on anaerobic energy from the intramuscular phosphates decreases while the magnitude of anaerobic energy generated in glycolysis increases. To improve the capacity for energy transfer by the short-term lactic acid energy system, training must overload this aspect of energy metabolism (McArdle WD.,1996).

The anaerobic changes in skeletal muscle resuling from training involve increased capacities of (1) the phosphagen (ATP-PC) system; the capacity of the ATP-PC system is enhanced by two major biochemical changes: (a) increased levels of muscular stores of ATP and PC and (b) increased activities of key enzyme involved in the ATP-PC system [i.e., the enzymes myokinase (MK) and creatine kinase (CPK)] and (2) anaerobic glycolysis (i.e., lactic acid system; the glycolytic capacity is enhanced by increased the glycolytic enzyme activities i.e. phosphofructokinase (PFK) which is important in the early reactions of glycolysis (Fox E.,1993).

Adaptations to aerobic (endurance) training

The aerobic, or oxygen, system release energy for ATP production from the breakdown mainly of carbohydrate and fat, and sometime of protein (Fox E.,1993). The aerobic exercise is the activities to perform whole body activities for extended period of time (Prentice WE.,1999).

The effects of aerobic endurance training on the mechanisms of adaptation have been reviewed previously throughly with respect to the cardio-respiratory system, and skeletal muscle. (Zuluagn M.,1995).

1. Cardio- respiratory system adaptations

Adaptations within the cardiovascular system with training may be considered as either central or peripheral in origin. Central adaptations include changes in cardiac output, blood volume and arterial oxygen-carrying capacity. Peripheral adaptations include skeletal muscle blood flow and capillarization. The functional significance of these changes may be assessed by examining their respective roles in the improvements in VO_2max and endurance performance after endurance training. Endurance training program that increase VO_2max involve a large muscle was in dynamic exercise (e.g., running, cycling or swimming) for twenty to sixty

minutes per session three to five times per week at an intensity of about 50% to 85% VO_2max (American Collage of Sports Medicine,1998).

Maximal aerobic power, VO_2max, is a value expressed quantitatively of a person's capacity for aerobic resynthesis of ATP. It provides important information on the capacity of the long term energy system (Heyward VH.,1997). Since VO_2max is equal to the product of systemic blood flow (cardiac output) and systemic oxygen extraction (arteriovenous oxygen difference), changes in VO_2max would have to be due to changes in one or more of those variables (Figure 14). Endurance

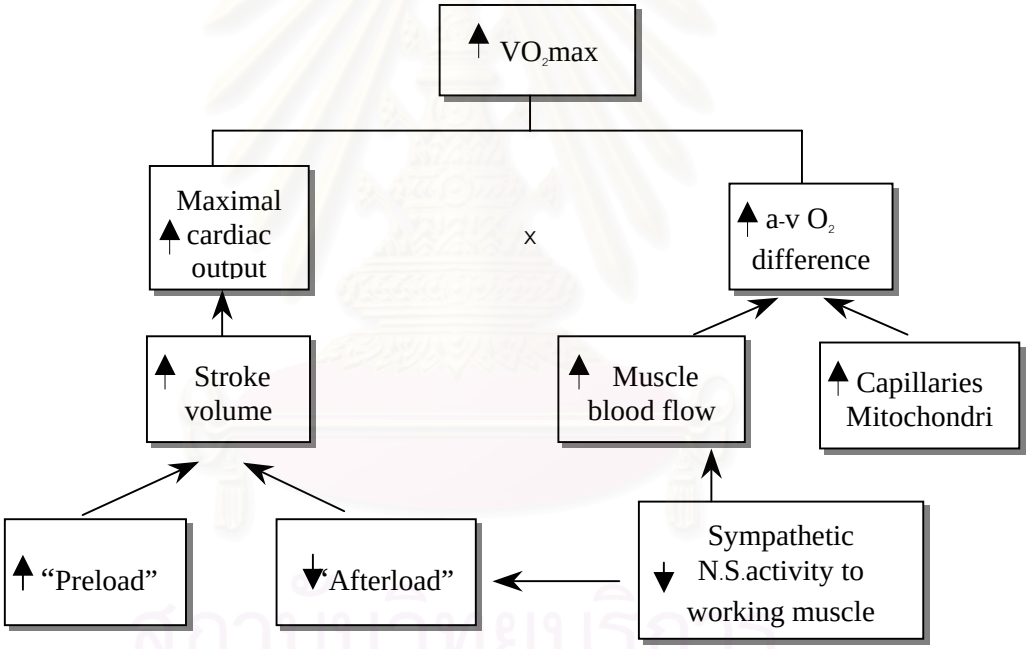


Figure 14 Summary of factors causing an increase in VO_2max with endurance training (From Powers SK.,2001)

training induced increases in maximal cardiac output are due to an increase in stroke volume by both an increase in preload and a decrease in afterload. The increased in the arteriovenous O_2 difference could be due to an elevation of the arterial oxygen content (higher hemoglobin or PO_2) or a decrease in the mixed venous oxygen content. The increase capacity

of the muscle to extract O_2 following endurance training is believed to be due to the increase in capillary density and mitochondria number. The increase in capillary density in trained muscle accommodates the increase in muscle blood flow during exercise, decrease the diffusion distance to mitochondria and slows the rate of blood flow to allow time for diffusion to take place. The increases in mitochondria following endurance training favor O_2 transport from the capillary and contribute to the expanded a - v O_2 differences (Powers SK.,2001).

Changes in heart size and heart rate are the cardiovascular adaptations which occur in response to training. In response to increased work demand, the heart's weight and volume and thus the left ventricle's wall thickness and chamber size all increase as a result of a normal adaptation to chronic endurance training (Wilmore JH.,1999). A reduction in heart rate is an adaptation to training for a submaximal exercise load that nearly always follows several weeks of training. This reduction in exercise heart rate seems to enable the heart to pump the same amount of blood to the body's tissues at a lower energy cost for the heart (McArdle WD.,1996). The actual mechanisms responsible for this decrease are not entirely known, but training appears to increase parasympathetic activity in the heart while decreasing sympathetic activity (Wilmore JH.,1999).

2. Skeletal muscle adaptations

The aerobic adaptations occur in skeletal muscle mainly as a result of endurance-training programs (Fox E.,1993). After endurance training, exercise at a given power output is characterized by a reduced decline in muscle phosphagen (ATP and CP) and glycogen stores and a smaller accumulation of lactate in both muscle and blood. These changes were suggested originally to result from the increased mitochondrial volume and capillarization evident with endurance training (Power SK.,2001).

One of the most striking skeletal muscle adaptations with endurance training is the marked increase in mitochondrial enzyme activities, reflecting an increase mitochondrial volume in skeletal muscle. Enzymes involved in the Krebs Cycle, electron transport chain and beta oxidation (fat metabolism) are all increase with endurance training. These changes are reversible, demonstrating a sharp decline after only 1 week of detraining. The molecular mechanisms underlying mitochodrial upregulation with training remain obscure, but may be associated with increases in 3'5' cyclic adenosine monophosphate (cAMP), or changes in CP stores in skeletal muscle with contractile activity (Zuluagn M.,1995).

Exercise training and Diabetes Mellitus

Exercise training is known to have beneficial effects on diabetes. One of those is an increase in glucose uptake and metabolism (Enevoldse LH.,2000). Glucose transport has been shown to be defective in diabetic humans and streptozotocin-induced diabetic animals (Garvey WT.,1993). Streptozotocin-induced diabetes results in decrease in myocardial sarcolemmal GLUT-4 protein, responsible for insulin-stimulated glucose uptake, total membrane GLUT-4 protein extract, and GLUT-4 mRNA (Garvey WT.,1993). Hall JL. demonstrated that exercise training has been shown to enhance myocardial glucose metabolism in streptozotocin-induced diabetic rats and young control rats. **They found that moderate treadmill exercise training in streptozotocin-induced diabetic rats increased myocardial GLUT-4 levels by 40%** (Hall JL.,1995).

Moreover, as exercise continues, fat rather than carbohydrate becomes the predominant fuel that is burned. **In both lean and obese diabetic subjects exercise training causes significant reductions in the VLDL, triglyceride concentration and, to a lesser extent, in LDL**

cholesterol. HDL cholesterol also has been shown to increase. These beneficial effects on lipids are observed in both NIDDM and IDDM individuals (Defronzo RA.,1998). Dose-response relationships between exercise training volume and blood lipid changes suggest that exercise can favourably alter blood lipids at low training volumes, although the effects may not be observable until certain exercise thesholds are met (Durstine JL.,2001).

In addition, physical training might reflect the improvement in endothelial function in diabetes (Rigla M.,2001). Endothelial cells produce several substances such as von Willebrand factor (vWF) and thrombomodulin (TM), which have been proposed as markers of endothelial damage (Seigneur M.,1993). These markers have been found to be increased in patients with Type I (insulin-dependent) diabetes mellitus (Iwashima Y.,1990) and in patients with Type II (non-insulin-dependent) diabetes mellitus (Knobl P.,1993 , Kario K.,1995), especially in those with microvascular complications. **There is evidence that after 3 months of a supervised training programm, TM decreased in both groups and became similar to the concentrations observed in their respective control subjects** (Rigla M.,2001).

Recent works support the hypothesis that physical training improves endothelial function, particularly in patients with high cardiovascular risk including diabetes (Higashi Y.,1999). The mechanisms responsible for these beneficial effects have been studied in experimental models. There is growing understanding that as a consequence of the increased vascular shear stress derived from repetitive exercise, endothelial cells respond by increasing their production of nitric oxide and other vasodilatory products (Rigla M.,2001).

In regard to the literature review from above, it might be summarized the idea as that the generation of reactive oxygen species (oxidative stress) may play an important role in the etiology of diabetic complications. Therefore, this present study was desired to evaluate whether or not the adverse effects of high glucose on endothelial functions, such as reduced endothelial dependent relaxation and increased leukocyte-endothelial cell interactions can be reversed by antioxidant, vitamin C, and/or regular exercise training.

CHAPTER III

MATERIALS AND METHODS

In present study, the effects of vitamin C supplementation and exercise training on microvascular function in streptozotocin-induced diabetic rats was studied. The experimental protocol of this study was divided into 3 parts. First, physiological characteristics in all animals groups were examined. Second, microcirculation study including leukocyte-endothelium interaction and vasodilation responses of mesenteric arterioles to endothelium-dependent and -independent vasodilators were investigated using intravital fluorescense microscopic techniques. And third, chemical analyses including determination of MDA levels, SOD activity, and eNOS protein level were performed. All protocols and procedures employed in this study were reviewed and approved by the Committee on Human Rights Related to Animal Experimentation, Faculty of Medicine, Chulalongkorn University, Bangkok.

Chemicals

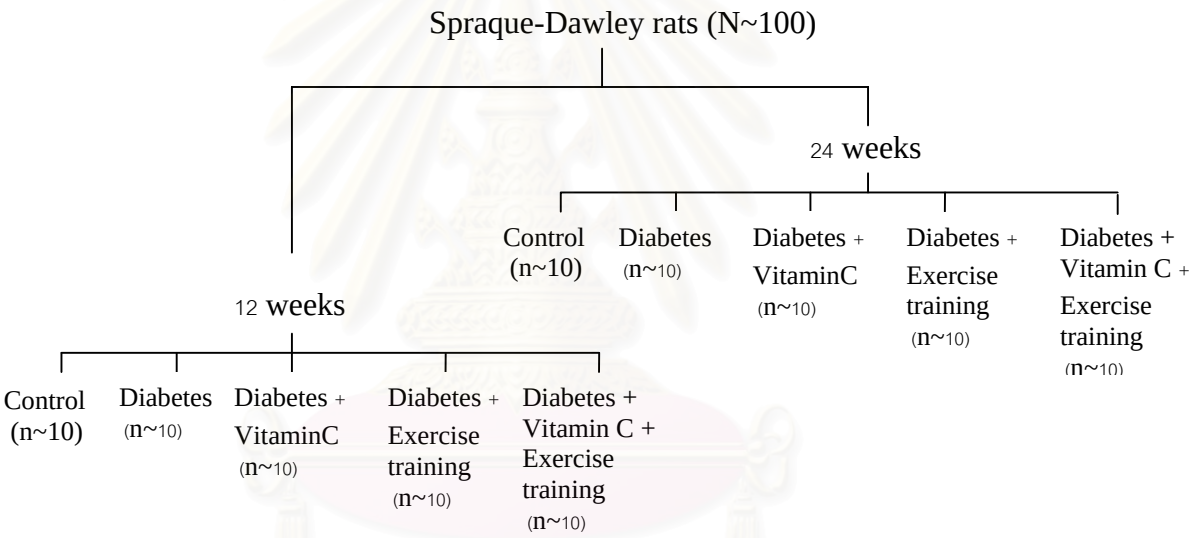
All chemicals and reagents used throughout the investigation were analytical grade. L-ascorbic acid (vitamin C), fluorescense dyes (FITC-dextran and Acridine orange), and reagents for determination of MDA, SOD, and eNOS protein were purchased from Sigma Chemical Company (St. Louis, USA). Diethyl ether and acetic acid (100%) were purchased from Merck (Darmstadt, Germany).

Animal preparation

Male Spraque-Dawley rats weighing 200-250 g were purchased from the National Animal Center at Salaya, Mahidol University, Thailand.

They were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University before being used in the experiment. The animals were kept in a room where temperature was 25 ± 3°C. Four rats were housed per stainless steel cage and fed ad libitum with regular dry rat chow and water.

All rats were divided randomly into two groups: at the 12 weeks and 24 weeks after injection with citrate buffer alone or streptozotocin (STZ). Each group consisted of five subgroups as follows:



The reasons that we have performed the experiment at 12 and 24 weeks are based on our previous results. Since our colleaque have be able to approve that the diabetic induced endothelial dysfunction was significantly demonstrated at 12 wk after STZ injection (Jariyapongskul A.,2000). Therefore, by using these experimental periods, the effects of vitamin C supplementation and/or exercise training on diabetic induced endothelial dysfunction would be able to evaluate. Besides, the data of our colleaque have also demonstrated the significantly decrease in plasma vitamin C in 12 wk STZ-induced diabetic rats (Jariyapongskul A.,2000).

Diabetic Induction

To induce diabetes mellitus, streptozotocin (STZ) (Sigma Chemical Co.) was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co.) and injected immediately into the tail vein of fasted rats, at a dose of 50 mg/kg body weight (Midaoui AE.,1996). Hyperglycemia (glucose concentration > 200 mg/dl) was confirmed by measurement of glucose concentration in blood samples obtained from the tail vein two days after the STZ-injection. Blood glucose was determined by using glucometer (Advantage Glucometer, Boehringes Mannheim, Germany). Samples were analyzed by applying a drop of blood to a control strip inserted into the monitor. Streptozotocin treated rats that failed to exhibit an elevation of blood glucose level at 48 hours greater than 200 mg/dl were excluded from the study (Panes J., 1996).

Vitamin C Supplementation

Supplementation of rats with vitamin C (L-ascorbic acid, 99%, Sigma USA) started 48 hours after administration of streptozotocin. Vitamin C was prepared daily by dissolving in drinking tap water at a concentration of 1 g/L; the experimental rats had free access to this vitamin C drinking water (Dai S. et al., 1995).

Exercise training protocol

Low-intensity exercise training protocol modified from the studies of De Angelis et al. (1997) and Wang JS. et al. (2000) was performed. This protocol sufficient to improve myocardial dysfunction in diabetic rats and reduced oxidized LDL in diabetic rats. The 12- and 24- wk exercise training protocol consisted of running on a motorized treadmill (SPORTSART 1190) five times per week with an incremental increase in treadmill velocity and run duration during the first 6 wk of training to accommodate increased fitness (Figure 15).

Figure 15 The rats employed exercise training by running on a motorized treadmill.

Initial exercise intensity was 13 m/min, 0° grade for 10 min. Exercise intensity was increased to 15 m/min, 0° grade and the running time was extended until a running time of 30 min/day was reached and intensity was then maintained for the duration of the training regimen. The highest level of activity (15 m/min, 0° grade for 30 min) included 5-min warm up period at 13 m/min, 0° grade and the final 5 min consisted of a warm down at 13 m/min, 0° grade. The training was executed after 5 p.m. to ensure that the exercise took place in their daily active cycle.

Experimental protocol

The experiments were performed at 12 and 24 week (wk) after the injection of citrate buffer or STZ. On the day of experiment, rats were

anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kgBW) and a tracheostomy was performed. A catheter was inserted into a carotid artery for measuring arterial blood pressure and collecting blood. A jugular vein was canulated with a catheter where fluorescence tracer was supplied as needed.

Three experimental protocols were separately performed as follow :

Protocol 1: Physiological characteristics

In the present study, the physiological characteristics were determined including: body weight (BW), heart weight (HW), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR). Arterial pressure and heart rate were monitored via a canular inserted into the carotid artery by using Polygraph system (NIHON KOHDEN, Japan). Arterial pressure was reported in terms of mean arterial blood pressure (MAP) calculated by MAP = 1/3 (SP + 2DP).

Besides, blood glucose (BG), plasma vitamin C, glycosylated hemoglobin (HbA_{1C}) and lipid profile (cholesterol, triglyceride, HDL and LDL, were also determined. All these parameters were determined at the end of experiment in arterial blood withdrawn from the carotid artery via catheter. Blood glucose was determined by using glucometer (Advance Glucometer, Boehringer Mannheim, Germany). HbA_{1C} and Lipid profile were analyzed from whole blood using colourimetric method by Bangkok RIA lab CO, LTD. Plasma vitamin C level was measured using enzyme-assisted spectrophotometric method and analyzed by Research center, Ramathibodi Hospital, Mahidol University.

Protocol 2: **Study of mesenteric microcirculation**

In this study, the mesenteric microcirculation was use to observe the endothelial dysfunction in diabetic rats due to this tissue model is available and easy to observe the function of microvessel in vivo in particular using fluorescence microscope. Although blood flow in the splanchnic area is not increased during physical effort (Shepherd JT.,1987). However, the functional changes in this mesentery could reflect the more generalized vascular adaptations induced by exercise as the other organs. Previous study reported that the endothelium-dependent relaxation in mesenteric arteries was enhanced by exercise training same as those in carotid arteries of the obese rats (Arrola P.,1999).

On the day of experiment, after anesthesia with sodium pentobarbital (60 mg/kgBW.ip.), rats were prepared for intravital microscopy according to a technique described previously (Booth G. et. al.,2001, Schaffler A. et. al.,1998). The abdominal cavity was opened via a midline laparotomy, and a loop of mesentery was exteriorized through the midline incision and placed on a Plexiglas chamber for microscopic observation (Figure 16). The mesentery was then pulled and fixed with a 37°C -Krebs Ringer Solution (pH 7.4) –soaked gauze. The exteriorized mesentery was superfused continuously with 37^{o}C Krebs Ringer Solution (pH 7.4) to avoid dehydration throughout the experiments. The study of mesenteric microcirculation consisted of two aspects; leukocyte-EC interaction and vascular responses to vasodilators.

Leukocyte-EC interaction

The animal was placed on the stage under fluorescence microscope (Figure 16). The acridine orange at the concentration of 25 mg/ml was i.v. injected as a bolus (0.5 ml) through the canulated jugular vein. The epi-fluorescent image of acrydine orange labeled leukocytes in

mesenteric microcirculation was then observed by using a set of intravital fluorescent microscopic equipment. The equipment set is consisted of video microscope (Nikon, Tokyo, Japan) with a 40x objective lens (Nikon, Japan) an 10x eyepieces (Nikon, Japan), video camera (MTI SIT68), video recorder (Sony GUM-1411QM, Japan) and video timer (Sony, Japan).

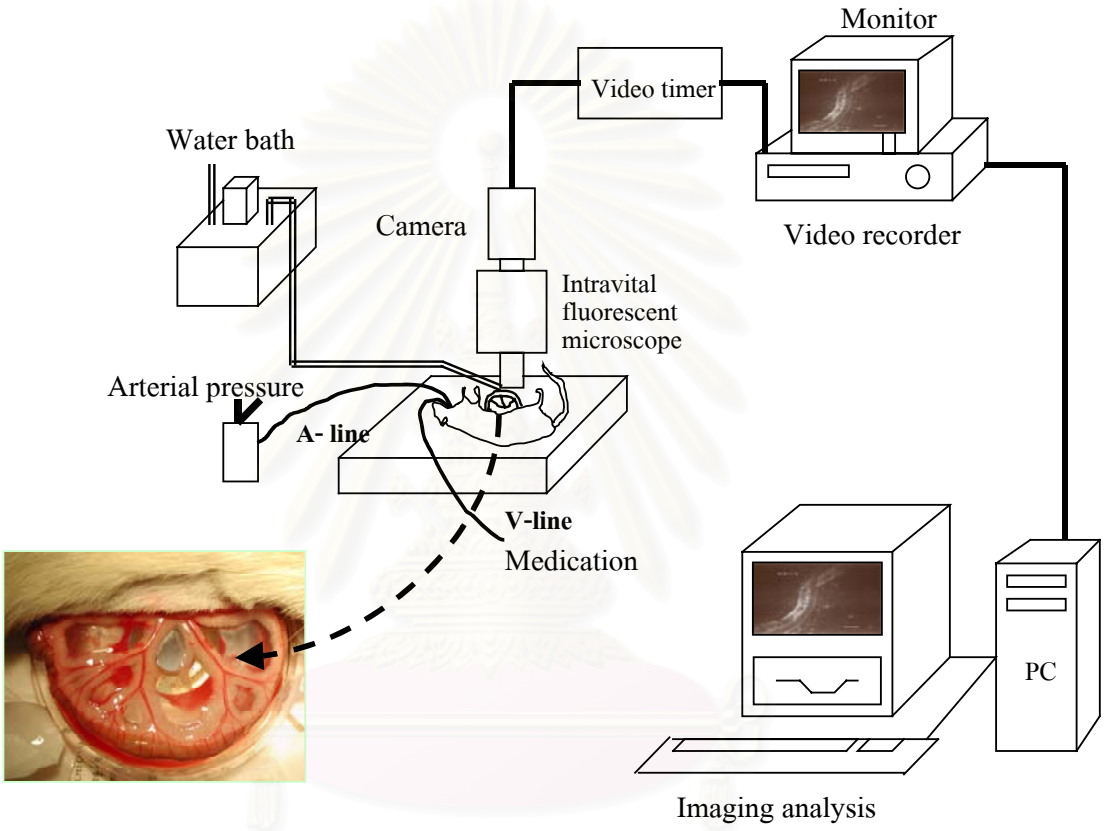


Figure 16 Schematic of a setup for intravital fluorescent microscopy of the mesenteric microvasculature in the rat.

By using this set of intravital fluorescent set, the real time video image of mesenteric microcirculation was able to record using video recorder (Sony SVT-124P). During the experiment, the video image of studied area was also observed on monitor (Sony SLV x311) and printed by videoprinter (Sony videographic printer UP-890CE). The videotape of each expriment was then analyzed off-line using digital image processing software called Global Lab II.

The rats were allowed to stabilize for 20-30 min following surgery. After stabilization, a single unbranched mesenteric venule (20 to 45-μm diameter, >150 μm length) was chosen for observation (Kubes P.,1991, Salas A.,1998). Video recordings were made at 20 min after initiation of acridine orange injection for quantification of leukocyte adherence. The numbers of leukocytes adherence were determined off-line during video-playback. A leukocyte was considered to be adherent to postcapillary venular (d=20 to 45-μm) if it remained stationary for 30 seconds. Leukocyte adherence was expressed as the number per 100-μm length of the venule (Lefer DJ.,1999 , Sanz MJ.,1999).

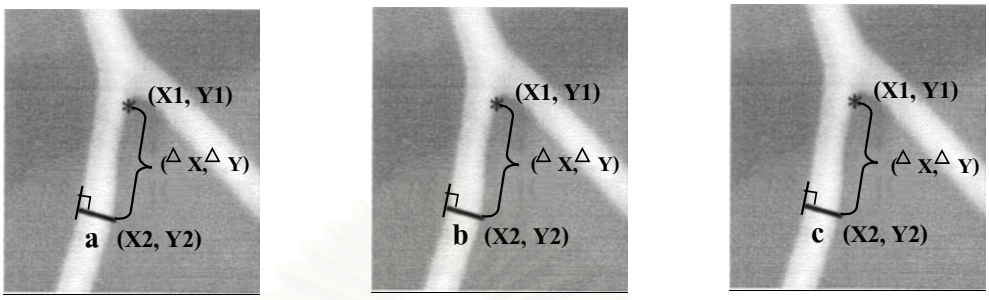
Vascular responses to vasodilators

To visualize the vascular reactivity to vasodilators, a fluorescent marker FITC-dextran-150 (FITC-D150 Sigma, USA, MW.=150,000 dalton) was administered intravenously. An intravital video microscope with a x 10 eyepiece lens and a x 20 objective lens was used to observe mesenteric arterioles of 15-45 μm diameter.

Mesenteric arterioles were studied for their vascular reactivity to two kinds of vasodilators. The vessels were preconstricted with norepinephrine (NE, 10^{-5}M ; 0.1 ml) superfusion before adding acetylcholine (Ach, 10^{-5}M ;10 ml/5min). After that, we allowed vessels to return to baseline by rinsing with phosphate buffer solution. Finally, sodium nitroprusside (SNP,10^{-5}M ;10ml/5min) was superfused after preconstriction with NE.

Based on FITC-D150 images, mesenteric arteriolar diameter was measured with a Global Lab image device. The arteriolar diameter in micrometer (μm) was calculated as the mean of triple measurements from three video frames by using the same reference point as a marker for measuring at the same position in each frame as shown in figure 17. Vasodilation responses were expressed as the percentage of maximal

relaxation after preconstriction with NE and the calculation method was shown in figure 18.

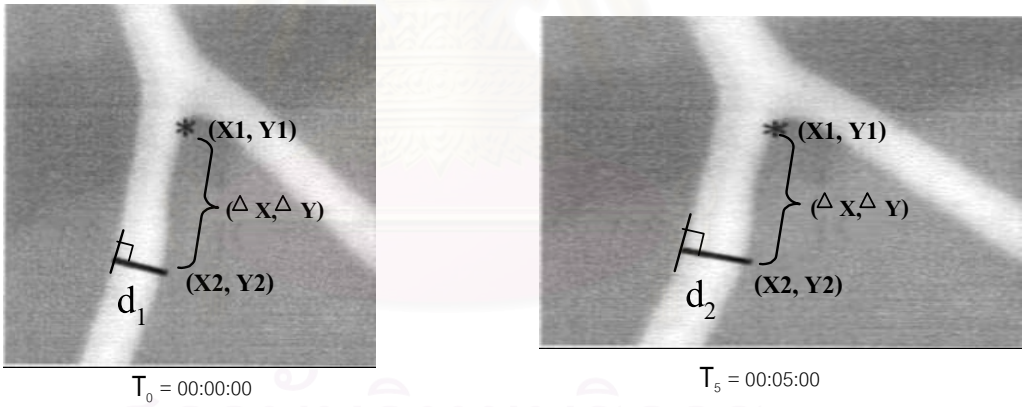


Frame 1: t = 00:00:01 Frame 2: t = 00:00:02 Frame 3: t = 00:00:03

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

x_1 , y_1 = reference point (*) of the branching

Figure 17 Method for measurement of arteriolar diameter.
(Modifiled from Jariyapongakul A.,2000)



T_0 = 00:00:00 T_5 = 00:05:00

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

x_1 , y_1 = reference point (*) of the branching

Figure 18 Method for calculation the percentage of arteriolar diameter change.
(Modifiled from Jariyapongakul A.,2000)

Protocol 3: **Chemical analysis**

After the study of microcirculation was finished, the abdominal wall was opened, then three organs: liver, gastrocnemius muscle, and heart, were excised. All organs were immersed in an ice-cold 0.9% NaCl then fat and fibrous tissue were removed before the organ was then weighed. The liver and muscle were collected to measure malondialdehyde (MDA) level and superoxide dismutase (SOD) activity. The hearts were collected to measure eNOS protein level. Those parameters were assay by technique as mention following .

1. Malondialdehyde (MDA) level was determined using thiobarbituric acid reaction as described by Ohgawa et.al., 1979 (Appendix II)

2. Superoxide dismutase (SOD) activity was determined by the modified method of Winterbourn, 1975 (Appendix III).

3. eNOS protein levels were determined by Western blot analysis as described previously in Resta TR.,2001 (Appendix IV).

4. Total protein was determined for eNOS protein analysis by using Lowry's method,1951 (Appendix V).

Although, in each parameter, sources of tissue were different (Due to amount of tissue was not enough for all parameters and saving the animal life was consider.), we assumed that they all presented the same direction of level in each parameter.

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 statistical significant.

CHAPTER IV

RESULTS

This chapter of results composed of three major parts which were served to examine the effects of vitamin C supplementation and exercise training on endothelial function in diabetic rats. These three major parts were listed in followings :-

Part 1 Physiological characteristics

: Body weight (BW)

: Heart weight (HW)

: Heart rate (HR)

: Systolic blood pressure (SBP), Diastolic blood pressure (SBP) and Mean arterial blood pressure (MAP)

: Blood glucose (BG)

: Glycosylated hemoglobin (HbA_{1c})

: Plasma vitamin C

: Lipid profile cholesterol (chol), Triglyceride (Trig), High density lipoprotein (HDL) and Low density lipoprotein (LDL).

Part 2 Study of mesenteric microcirculation

: Leukocyte-Ec interaction

: Vascular responses to vasodilators (Ach, SNP)

Part 3 Chemical analysis

: Malondialdehyde (MDA) level in liver and muscle homogenates

: Superoxide dismutase (SOD) activity in liver and muscle homogenates

: eNOS protein level in heart homogenate

1. Physiological characteristies

1.1 Body weight

Values for the body weight of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) are shown in Table 5 and Figure 19.

The body weight of those rats at 12 wk were 426±4, 183±8, 228±15, 214±8 and 191±15 gram, respectively. All four groups of diabetic rats had significantly lower in the body weight when compared with control rats. At the 24 wk the body weights were 490±11 , 295±8 , 267±11 , 284±19 and 289±14 gram in Con, DM, DM+Vit.C, DM+Ex, and DM+Vit.C+Ex groups, respectively. It was also shown significatly lower ($p<0.001$) in still different from that of the control rats at the 12 wk.

1.2 Heart weight

Heart weight of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) are shown in Table 6 and Figure 20.

At both 12 and 24 wk, the heart weight of DM (4.71±0.14 and 4.07±0.09 g/kgBW) and DM+Vit.C groups (4.30±0.30 and 4.45±0.25 g/kgBW) was found to be significantly greater (0.001) than age-match Con group (3.22±0.08 and 2.91±0.08 g/kgBW), wherease the heart weight of both exercise trained diabetic rats, DM+Ex (3.74±0.14 and 3.47±0.10 g/kgBW) and DM+Ex+Vit.C (3.95±0.10 and 3.47±0.10 g/kgBW), were

not significantly different from Con group. Moreover, it was found that DM+Vit.C+Ex group had also significantly lower in heart weight than DM group at 12 weeks after STZ injection. Furthermore, heart weight of DM+Ex group at both monitered time point were also significantly lower than age-match DM rats.

1.3 Arterial blood pressure

Table 7 and Figure 21, 22 show systolic blood pressure (SBP) and diastolic blood pressure (DBP) of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk).

At both 12 and 24 wk, the SBP of DM (134.38±5.13 and 139.50±7.58 mmHg) were significantly higher ($p<0.05$ and $p<0.01$) than age-match Con group (115.00±7.50 and 108.89±4.70 mmHg). In the same as SBP, the DBP of DM (101.88±2.30 and 110.00±6.58 mmHg) were also showed significantly higher ($p<0.05$ and $p<0.001$) than Con group (88.33±6.29 and 78.89±2.61 mmHg). However, both SBP and DBP of DM+Vit.C, DM+Ex and DM+Vit.C+Ex at 12 wk (110.00±7.68 / 83.75±6.03 mmHg, 100.71±4.81 / 76.43±4.19 mmHg and 102.22±2.65 / 75.00±4.17 mmHg, respectively), and 24 wk (111.25±6.93 / 85.00±5.26 mmHg, 100.25±4.06 / 82.50±4.01 mmHg and 95.71±6.85 / 74.29±4.93 mmHg, respectively) were significantly lower than the age match DM group.

The data of mean arterial blood pressure (MAP) were also shown in Table 8 and Figure 23. At 12 wk, MAP was 90.71±6.60 mmHg in Con, 113.54±3.16 mmHg in DM, 92.70±6.47 mmHg in DM+Vit.C, 84.52±4.36 mmHg in DM+Ex and 84.07±3.52 mmHg in DM+Vit.C+Ex groups. At

24 wk, MAP were 88.33±3.18 mmHg, 119.82±6.71 mmHg, 93.75±5.79 mmHg, 88.20±3.90 mmHg and 81.42±5.56 mmHg, in those groups of animals respectively. It was found that at both 12 and 24 wk after injection, the DM group had significantly higher MAP than the Con group. However, MAP of DM+Vit.C, DM+Ex and DM+Vit.C+Ex groups were significantly lower than the age-match DM group at both 12 and 24 weeks after STZ injection.

1.4 Heart rate

Table 9 and Figure 24 show heart rate of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values for heart rate of rats in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex group at 12 weeks were 332.14±15.2, 292.86±7.14, 294.44±17.07, 210.34±13.90 and 247.88±40.59 beats per minute (bpm) and at 24 weeks were 308.33±8.33, 290.63±14.89, 296.88±13.72, 237.71±16.54 and 238.31±19.60 bpm, respectively. Among all group of rats, both diabetes with exercise trained groups (DM+Ex and DM+Ex+Vit C) had significantly lower in heart rate than Con groups at both 12 ($p<0.001$ and $p<0.01$) and 24 wk ($p<0.05$ and $p<0.05$), respectively.

1.5 Blood glucose

The level of blood glucose of rats at 12 and 24 weeks after treatment are shown in Table 10 and Figure 25. The blood glucose concentration of rats in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex groups at 12 wk were 93.13±70 , 418.33±17.24 , 380.13±18.61 , 328.00±26.03 and 351.11±21.21 mg/dl, respectively. At 24 wk after injection, there were 98.00±5.10 , 327..67±20.82 ,

287.80±6.47 , 311.88±23.17 and 361.00±12.6 mg/dl in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex groups, respectively. Blood glucose levels of all diabetic groups were significantly higher than that of age-match Con group ($p<0.001$) at both 12 and 24 wk after injection with STZ and citrate buffer. It was found that only DM+Ex trained group had significantly lower ($p<0.05$) in blood glucose when compared with diabetic rats at 12 wk.

1.6 Glycosylated hemoglobin

Table 11 and Figure 26 show glycosylated hemoglobin (HbA_1C) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or STZ.

At 12 wk after injection, they were 3.68±0.05, 10.86±0.24, 9.81±0.30, 9.81±0.45 and 9.70±0.30% in Con, DM, DM+Vit.C, DM+Ex, DM+Ex+Vit.C groups while at 24 wk they were 3.41±0.09, 11.20±0.38, 9.80±0.30, 10.12±0.25 and 9.75±0.44%, respectively. All four groups of diabetic rats had significantly higher in HbA_1C than that of control rats at both time points ($p<0.001$). However, at 24 wk, there were significantly lower in HbA_1C of DM+Ex ($p<0.05$) and DM+Vit.C+Ex ($p<0.05$) groups when compared with DM group.

1.7 Plasma vitamin C

Table 12 and Figure 27 show plasma vitamin C levels of rats at 12 and 24 weeks (wk) after injection with citrate buffer or STZ. The concentrations of plasma vitamin C at 12 and 24 weeks in control (Con) group were 1.30±0.15 and 1.17±0.11 mg/dl, in Diabetes (DM) group were 0.62±0.02 and 0.51±0.04 mg/dl, in diabetes with vitamin C supplemented

rats (DM+Vit.C) group were 0.96±0.11 and 1.58±0.14 mg/dl, in diabetes with exercise trained (DM+Ex) group were 0.77±0.18 and 0.67±0.06 mg/dl and in diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) group were 1.00±0.20 and 1.12±0.06 mg/dl.

DM group had plasma vitamin C levels significantly lower than Con group at both 12 and 24 wk after injection ($p<0.05$). Although there were no significant difference in plasma vitamin C level among all group of diabetes at 12 wk but it was found significantly higher in those of DM+Vit.C ($p<0.05$) and DM+Vit.C+Ex group ($p<0.05$) when compared with diabetic group at 24 wk after STZ injection.

1.8 Lipid profile

Cholesterol

Table 13 and Figure 28 show plasma cholesterol of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

At 12 wk, plasma cholesterol of rats in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex group were 93.11±2.95, 89.13±6.23, 91.80±5.66, 105.88±2.84 and 75.88±6.86 mg/dl, respectively. Whereas plasma cholesterol of those groups at 24 wk was 86.71±4.44, 91.33±8.45, 96.44±6.03, 81.50±2.19 and 97.14±9.97 mg/dl, respectively. There were no significant difference in plasma cholesterol levels among all group of rats at either 12 or 24 wk.

Triglyceride

Table 14 and Figure 29 show plasma triglyceride levels of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

The concentration of plasma triglyceride in DM group at 12 and 24 wk (159.13±23.75 and 150.83±17.49 mg/dl, respectively) were significantly higher ($p<0.05$ and $p<0.001$) than the age-match Con group(80.38±7.31 and 64.71±9.91 mg/dl, respectively). The only significant difference among all treated diabetic rats when compared with control rats was the, DM+Vit.C group at 24 weeks which had significantly higher in plasma triglyceride (122.25±11.35 mg/dl) than age-match Con group ($p<0.05$). However, at 12 wk, plasma triglyceride was significantly lower in DM+Vit.C+Ex group (90.57±7.62 mg/dl) than age-match diabetic rats. Moreover, at 24 wk, DM+Ex and DM+Vit.C+Ex group had also significantly lower ($p<0.05$) plasma triglyceride (91.50±10.5 and 93.57±14.1 mg/dl) than the age-match diabetic rats ($p<0.05$).

High density lipoprotein

Table 15 and Figure 30 show the high density lipoprotein data of (HDL) of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) rat groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) at 12 wk and 24 wk after the injection of citrate buffer or STZ.

At 12 wk, HDL were 59.11±2.07 mg/dl in Con, 61.38±4.46 mg/dl in DM, 54.80±3.45 mg/dl in DM+Vit.C, 65.50±2.45 mg/dl in DM+Ex and 62.13±2.81 mg/dl in DM+Vit.C+Ex groups. At 24 wk, HDL was 55.29±2.89 mg/dl in Con, 58.17±4.92 mg/dl in DM, 58.11±3.56 mg/dl in DM+Vit.C, 52.88±2.22 mg/dl in DM+Ex and 64.00±7.41 mg/dl in DM+Vit.C+Ex groups, respectively. There were no significant differences in this parameter among all groups of diabetes relative to control.

Low density lipoprotein

Table 16 and Figure 31 show the Low density lipoprotein (LDL) levels of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

At 12 wk, plasma LDL of rats in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex group was 17.11±1.11, 12.88±1.44, 15.00±1.25, 19.50±0.65 and 13.88±1.25 mg/dl, respectively. At 24 wk plasma LDL of those groups at 24 wk was 11.86±1.20, 13.17±0.95, 15.56±1.46, 10.50±1.30 and 18.00±3.12 mg/dl, respectively. There were no significant difference in plasma LDL level among all group of rats at either 12 or 24 wk.

2. Study of mesenteric microcirculation

2.1 Leukocyte / Endothelial Interactions

The number of leukocyte adherence was determined in mesenteric venules of control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at

12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) and these data are summarized in Table 17 and Figure 32, 33 and 34.

At 12 wk, the number of leukocytes adhering to venules of Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex rats were 2.00±0.50, 9.17±1.45, 4.71±0.47, 3.14±0.70 and 3.78±0.62 cell/100μm, respectively. At 24 wk, the number of leukocyte adhering to venules of those groups of rats was 5.57±0.97, 11.86±0.86, 3.14±1.06, 5.13±1.13 and 5.14±0.67 cell/100μm.

The data demonstrate that the number of leukocyte adherence per 100 μm of vessel length was significantly greater in DM group compared with the age-match Con group at both 12 and 24 wk ($p<0.001$ and $p<0.01$). Interestingly, all treatments groups had significantly lower in the number of leukocyte adhering when compared with age-matched diabetic rats at both monitored time point.

2.2 Endothelial / Smooth Muscle Function ; Arteriolar tone

The presence of functional endothelium was assessed by the dilation of arterioles in response to Ach and SNP and the data were shown in Table 18, 19 and Figure 35, 36. In the present experiment, arterioles with in the same range of diameter values (d=15-45 μm) were sampled. The average arteriolar diameter value of rat was no significantly different among the groups.

At 12 and 24 wk, the percentage of relaxation responses to Ach in Con group were 14.08±1.89 and 12.59±1.45% ; in DM group they were 4.72±1.52 and 3.24±0.51% ; in DM+Vit.C they were 14.4±1.53 and 10.85% ; in DM+Ex they were 7.64±1.13% and 6.71±1.08% and in the DM+Vit C+Ex groups were 9.16±1.76 and 9.62±1.66%.

The arteriolar response to Ach was significantly attenuated in DM group compared with Con group ($p<0.001$ and $p<0.01$, at 12 and 24 wk respectively). However, in DM+Vit.C and DM+Vit C+Ex group the magnitude of the dilatation at both 12 and 24 weeks to Ach was significantly higher than in DM group. There was no significant different in the percentage of relaxation responses to Ach between DM+Ex and Con rats.

When the endothelial independent vasodilater, SNP, was used. The arteriolar tone response did not show the same trend as the response to Ach. While there were no significant differences among all group of rats in the percentage of relaxation to SNP at both monitered time point, there was a tendency to for a decrease in relaxation to SNP when compared to the age-matched control group.

3. Chemical analysis

3.1 Malondialdehyde

ROS generation in this study was determined using Malondialdehyde (MDA) as an indicator. The influence of Vitamin C supplementation and/or exercise training on this indicator is shown in Table 20 and 21 and Figure 37 and 38.

Diabetic rats had an MDA level of liver homogenate at 12 and 24 wk of 6141±366 and 5646±232 nmole/g wet wt , respectively and the MDA level of muscle homogenate at 12 and 24 wk of 2843±286 and 2994±148 nmole/g wet wt respectively which was significantly higher than the MDA level of liver and muscle homogenates of in age-matched Con groups (12 wk : 3766±232 and 1643±101 nmole/g wet wt , 24 wk : 3632±441 and 2505±254 nmole/g wet wt).

At 12 wk, the MDA level of liver homogenate in DM+Vit.C group (4355±508 nmole/g wet wt), DM+Ex (4388±305 nmole/g wet wt) and DM+Vit.C+Ex (4188±194 nmole/g wet wt) groups were significantly lower ($p<0.001$) than those of age-match diabetic rats. For muscle homogenate, MDA levels were found to be lower ($p<0.05$) in only the DM+Vit C+Ex group (1947±144 nmole/g wet wt) compared with age-match diabetic groups. At 24 wk, the MDA level of liver homogenate in DM+Ex (4135±131 nmole/g wet wt) and DM+Vit C+Ex (4121±303 nmole/g wet wt) groups was significantly lower ($p<0.01$) than in age-match diabetic rats. In muscle homogenate by comparison, the MDA level of DM+Vit.C (2539±156 nmole/g wet wt), DM+Ex (2449±201 nmole/g wet wt) and DM+Vit.C+Ex (2299±161 nmole/g wet wt) groups was significantly lower ($p<0.01$) than the MDA level of age-matched diabetic rats.

3.2 Superoxide dismutase

Superoxide dismutase (SOD) levels (Units/g wet weight) in liver and muscle homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) are shown in Table 22, 23 and Figure 39, 40.

At 12 wk, SOD activity of liver and muscle homogenate in Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex were 5641±656 and 1782±308 , 4612±625 and 1527±285 , 3396±498 and 1929±579 , 3995±388 and 1618±297 and 5266±593 and 1426±302 , respectively. No significant difference in SOD activity of either tissue homogenate was

found among any of the groups of rats. At 24 wk, SOD activity of liver homogenate was significantly lower ($p<0.01$) in DM (5312±195 Units/g wet wt) and DM+Ex (5140±442) groups when compared with Con group (7740±403). Moreover, in muscle homogenate, SOD activity was also significantly lower in DM group (1012±214) compared with the age-matched Con group (2679±590).

3.3 eNOS protein level

eNos protein level in heart homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ) were shown in Table 24 and Figure 41.

For comparisons for the data at 12 wk, it was found that DM group had significantly lower ($p<0.05$) eNOS protein levels (1.10±0.70 μg/5μg Protein) than Con group (3.85±0.19 μg/5μg Protein). Significant increases of eNOS protein level ($p<0.05$) were observed in DM+Vit C (4.18±0.21 μg/5μg Protein) and DM+Vit C+Ex (4.56±1.17 μg/5μg Protein) groups when compared with diabetic group. At 24 wk of the experiment, eNOS protein level of homogenate heart of Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex were 3.79±0.49, 2.45±0.66, 4.77±0.55, 1.57±0.81 and 4.35±0.40 μg/5μg Protein. In DM+Vit.C and DM+Vit.C+Ex groups, eNOS protein of heart homogenate were significantly higher than age-mactch group. There are trends to decrease in eNOS protein of DM and DM+Ex groups compared to age-match Con group.

Table 5 Body weight (g) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Body weight (g)	
	12 wk	24 wk
Con	425.66 ± 4.46 (n = 9)	489.82 ± 10.98 (n = 11)
DM	182.70 ± 7.87*** (n = 10)	294.65 ± 8.07*** (n = 10)
DM+Vit.C	228.00 ± 15.18***, ## (n = 9)	266.50 ± 11.17*** (n = 9)
DM+Ex	213.83 ± 7.85*** (n = 9)	283.72 ± 19.12*** (n = 9)
DM+Vit.C+Ex	190.85 ± 15.18*** (n = 10)	289.38 ± 14.14*** (n = 8)

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

##$p < 0.01$: significant difference from diabetic group.

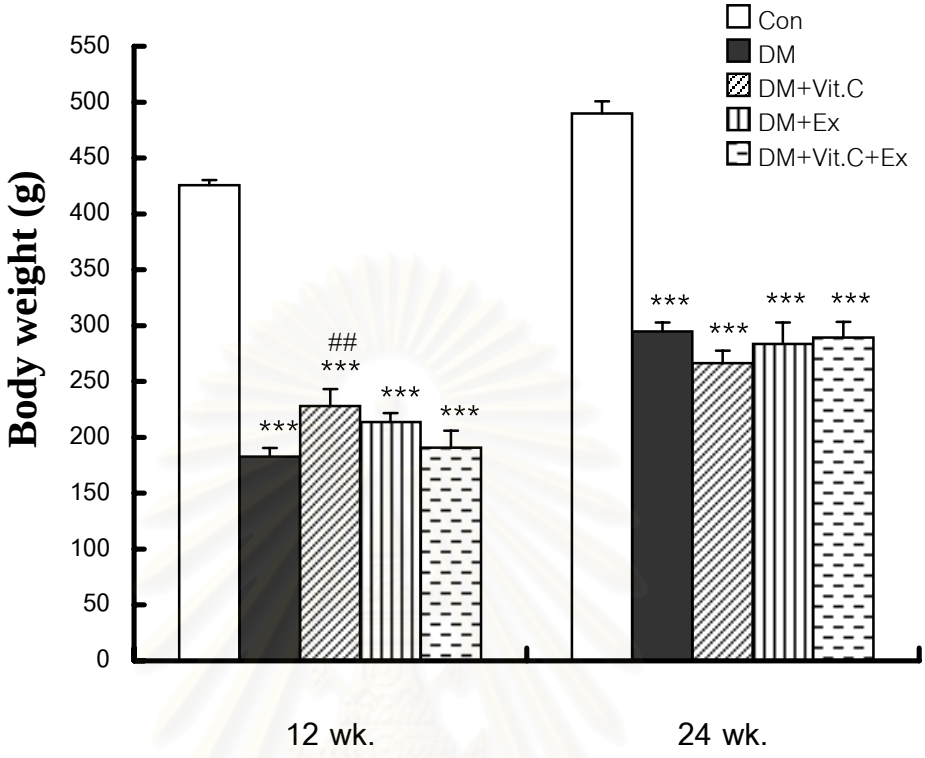


Figure 19 Body weight (g) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

$^{\#\#}p < 0.01$: significant difference from diabetic group.

Table 6 Heart weight (g/kg bw) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Heart weight (g/kg bw)	
	12 wk	24 wk
Con	3.22 ± 0.08 (n = 9)	2.91 ± 0.08 (n = 10)
DM	4.71 ± 0.14*** (n = 9)	4.07 ± 0.09*** (n = 8)
DM+Vit.C	4.30 ± 0.30*** (n = 8)	4.45 ± 0.25*** (n = 9)
DM+Ex	3.74 ± 0.14## (n = 10)	3.47 ± 0.1# (n = 10)
DM+Vit.C+Ex	3.95 ± 0.1# (n = 10)	3.47 ± 0.10 (n = 7)

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

#$p < 0.05$, ##$p < 0.01$: significant differences from diabetic group.

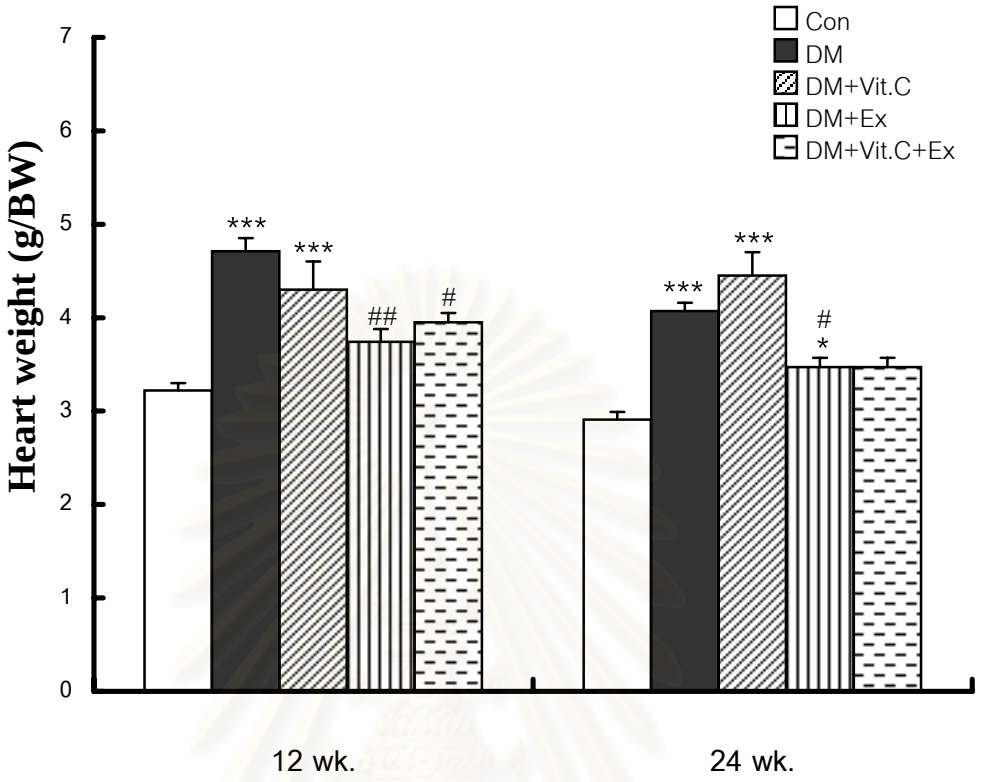


Figure 20 Heart weight (g/kgBW) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

**$p < 0.05$, ***$p < 0.001$: significant differences from control group

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$: significant differences from diabetic group.

Table 7 Systolic and Diastolic blood pressure (mmHg) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Arterial blood pressure (mmHg)			
	12 wk		24 wk	
	Systolic	Diastolic	Systolic	Diastolic
Con	115.00 ± 7.50 (n = 9)	88.33 ± 6.29 (n = 9)	108.89 ± 4.70 (n = 9)	78.89 ± 2.61 (n = 9)
DM	134.38 ± 5.13* (n = 8)	101.88 ± 2.30* (n = 8)	139.50 ± 7.58** (n = 10)	110.00 ± 6.58*** (n = 10)
DM+Vit.C	110.00 ± 7.68$^{\#}$ (n = 8)	83.75 ± 6.03$^{\#}$ (n = 8)	111.25 ± 6.93$^{\#}$ (n = 8)	85.00 ± 5.26$^{\#\#}$ (n = 8)
DM+Ex	100.71 ± 4.81$^{\#\#}$ (n = 7)	76.43 ± 4.19$^{\#}$ (n = 7)	100.25 ± 4.06$^{\#\#}$ (n = 8)	82.50 ± 4.01$^{\#\#}$ (n = 8)
DM+Vit.C +Ex	102.22 ± 2.65$^{\#\#}$ (n = 9)	75.00 ± 4.17$^{\#\#}$ (n = 9)	95.71 ± 6.85$^{\#\#\#}$ (n = 7)	74.29 ± 4.93$^{\#\#\#}$ (n = 7)

Values are means ± SEM.

*p < 0.05 , **p < 0.01 , ***p < 0.001 : significant differences from control group.

$^{\#}$p < 0.05 , $^{\#\#}$p < 0.01 $^{\#\#\#}$p < 0.001 : significant difference from diabetic group.

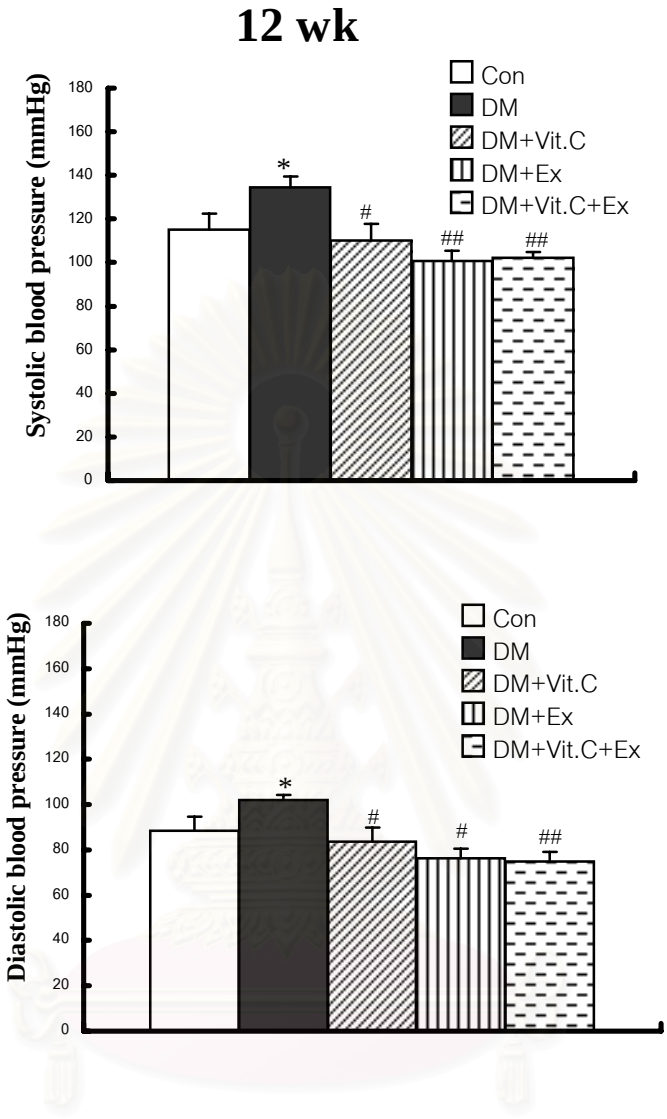


Figure 21 Systolic and Diastolic blood pressure (mmHg) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$: significant differences from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$: significant differences from diabetic group.

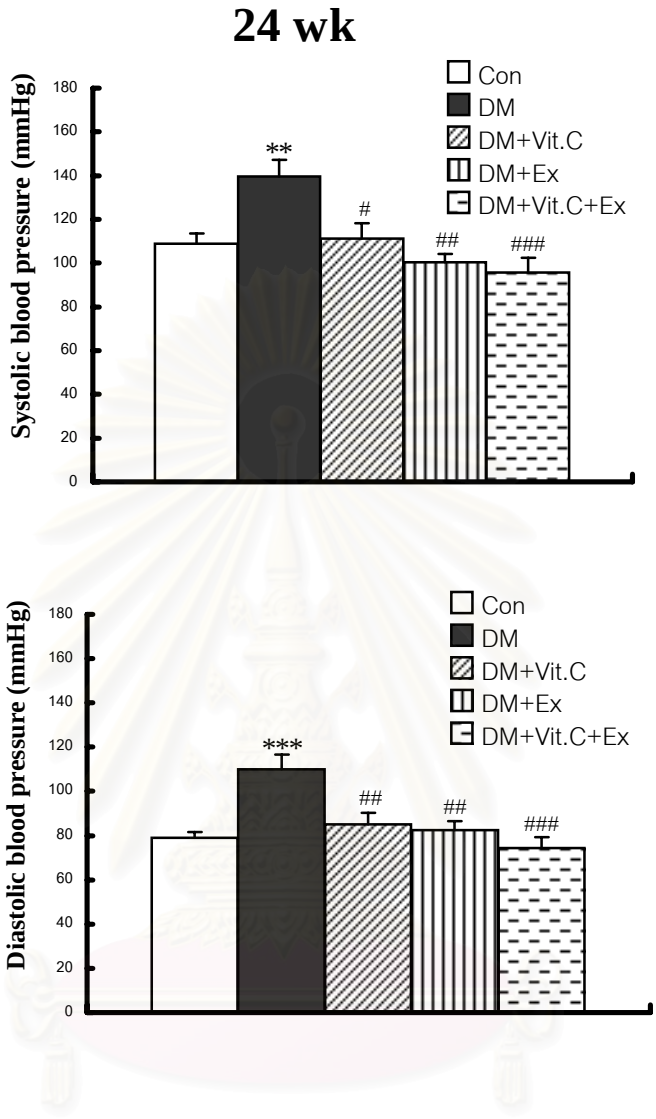


Figure 22 Systolic and Diastolic blood pressure (mmHg) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

**p < 0.01, ***p < 0.001 : significant differences from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: significant differences from diabetic group.

Table 8 Mean arterial blood pressure (mmHg) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Mean arterial blood pressure (mmHg)	
	12 wk	24 wk
Con	90.71 ± 6.60 (n = 7)	88.33 ± 3.18 (n = 8)
DM	113.54 ± 3.16* (n = 8)	119.82 ± 6.71** (n = 9)
DM+Vit.C	92.70 ± 6.47$^{\#}$ (n = 8)	93.75 ± 5.79$^{\#\#}$ (n = 10)
DM+Ex	84.52 ± 4.36$^{\#\#}$ (n = 7)	88.20 ± 3.9$^{\#\#}$ (n = 8)
DM+Vit.C+Ex	84.07 ± 3.52$^{\#\#}$ (n = 9)	81.42 ± 5.56$^{\#\#\#}$ (n = 8)

Values are means ± SEM.

*$p < 0.05$, **$p < 0.01$: significant difference from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: significant differences from diabetic group.

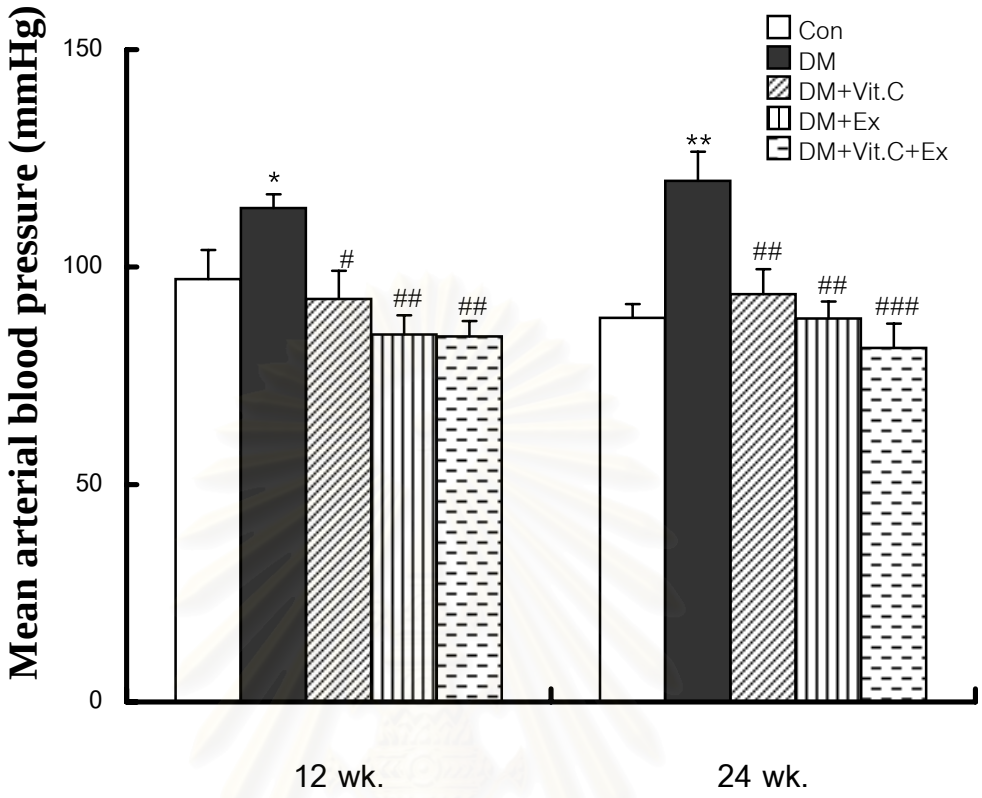


Figure 23 Mean arterial blood pressure (mmHg) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$, **$p < 0.01$: significant difference from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: significant differences from diabetic group.

Table 9 Heart rate (bpm) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Heart rate (bpm)	
	12 wk	24 wk
Con	332.14 ± 15.2 (n = 7)	308.33 ± 8.33 (n = 9)
DM	292.86 ± 7.14 (n = 7)	290.63 ± 14.89 (n = 8)
DM+Vit.C	294.44 ± 17.07 (n = 9)	296.88 ± 13.72 (n = 8)
DM+Ex	210.34 ± 13.9***,## (n = 8)	237.71 ± 16.54* (n = 7)
DM+Vit.C+Ex	247.88 ± 40.59** (n = 9)	238.31 ± 19.63* (n = 7)

Values are means ± SEM.

*p < 0.05 , **p < 0.01 , ***p < 0.001 : significant difference from control group.

##p < 0.01 : significant differences from diabetic group.

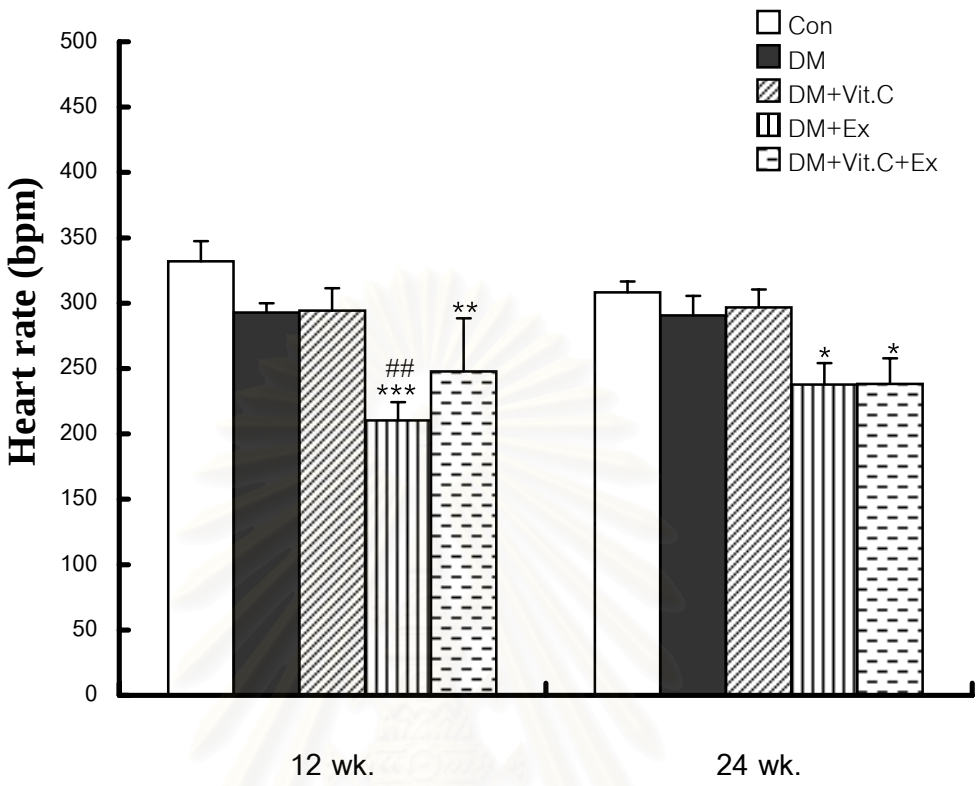


Figure 24 Heart rate (bpm) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*p < 0.05 , **p < 0.01 , ***p < 0.001 : significant difference from control group.

##p < 0.01 : significant differences from diabetic group.

Table 10 Blood glucose (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Blood glucose (mg/dl)	
	12 wk	24 wk
Con	93.13 ± 7.70 (n = 8)	98.00 ± 5.10 (n = 8)
DM	418.33 ± 17.24*** (n = 9)	327.67 ± 20.82*** (n = 9)
DM+Vit.C	380.13 ± 18.61*** (n = 8)	287.80 ± 6.47*** (n = 10)
DM+Ex	328.00 ± 26.03***,# (n = 9)	311.88 ± 17*** (n = 8)
DM+Vit.C+Ex	351.11 ± 21.21*** (n = 9)	361.00 ± 12.55*** (n = 8)

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

#$p < 0.05$: significant difference from diabetic group.

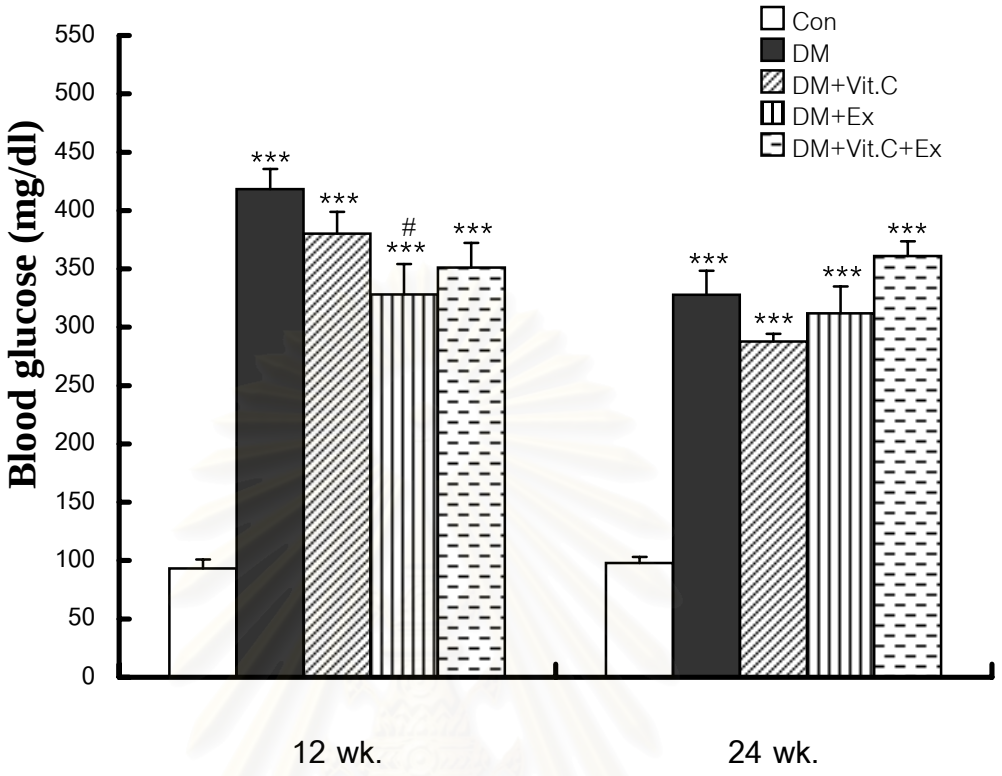


Figure 25 Blood glucose (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

$^{\#}p < 0.05$: significant difference from diabetic group.

Table 11 Glycosylated hemoglobin (%) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Glycosylated hemoglobin (%)	
	12 wk	24 wk
Con	3.68 ± 0.05 (n = 6)	3.41 ± 0.09 (n = 8)
DM	10.86 ± 0.24*** (n = 7)	11.20 ± 0.38*** (n = 8)
DM+Vit.C	9.81 ± 0.30*** (n = 8)	9.80 ± 0.30***,# (n = 8)
DM+Ex	9.81 ± 0.45*** (n = 7)	10.12 ± 0.25*** (n = 9)
DM+Vit.C+Ex	9.70 ± 0.30*** (n = 7)	9.75 ± 0.44***,# (n = 8)

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

#$p < 0.05$: significant difference from diabetic group.

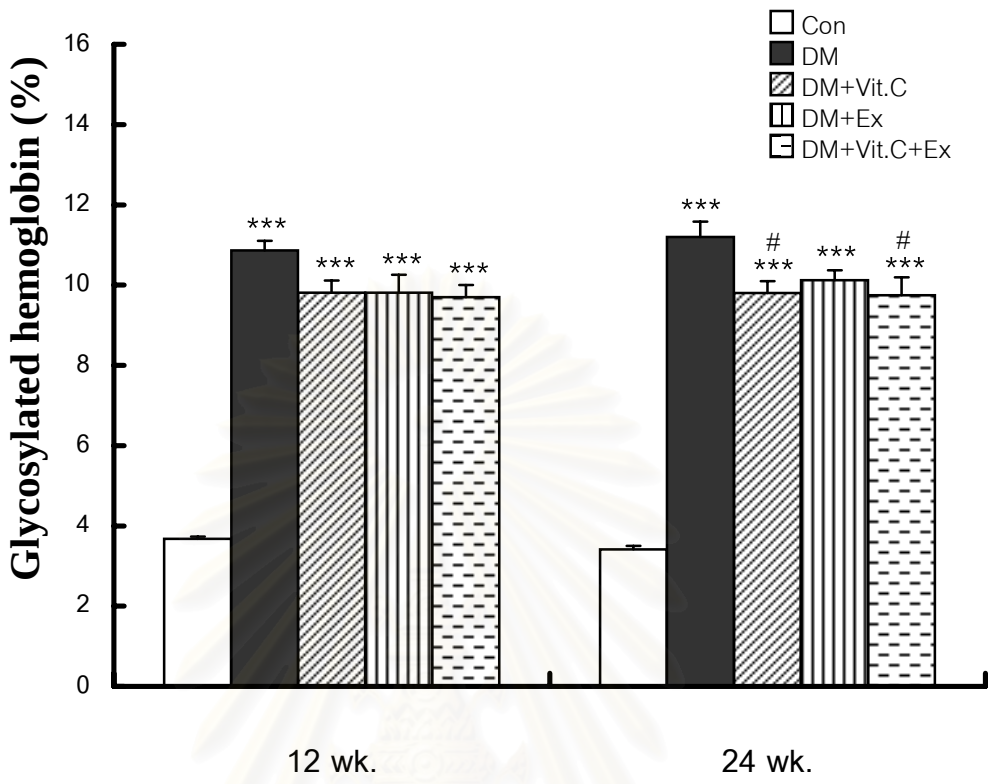


Figure 26 Glycosylated hemoglobin (%) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

$^{\#}p < 0.05$: significant difference from diabetic group.

Table 12 Plasma vitamin C (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Plasma vitamin C (mg/dl)	
	12 wk	24 wk
Con	1.30 ± 0.15 (n = 6)	1.17 ± 0.11 (n = 7)
DM	0.62 ± 0.02* (n = 7)	0.51 ± 0.04* (n = 7)
DM+Vit.C	0.96 ± 0.11 (n = 6)	1.58 ± 0.14# (n = 8)
DM+Ex	0.77 ± 0.18 (n = 7)	0.67 ± 0.06 (n = 7)
DM+Vit.C+Ex	1.00 ± 0.20 (n = 8)	1.12 ± 0.06# (n = 8)

Values are means ± SEM.

*$p < 0.05$: significant difference from control group.

#$p < 0.05$: significant difference from diabetic group.

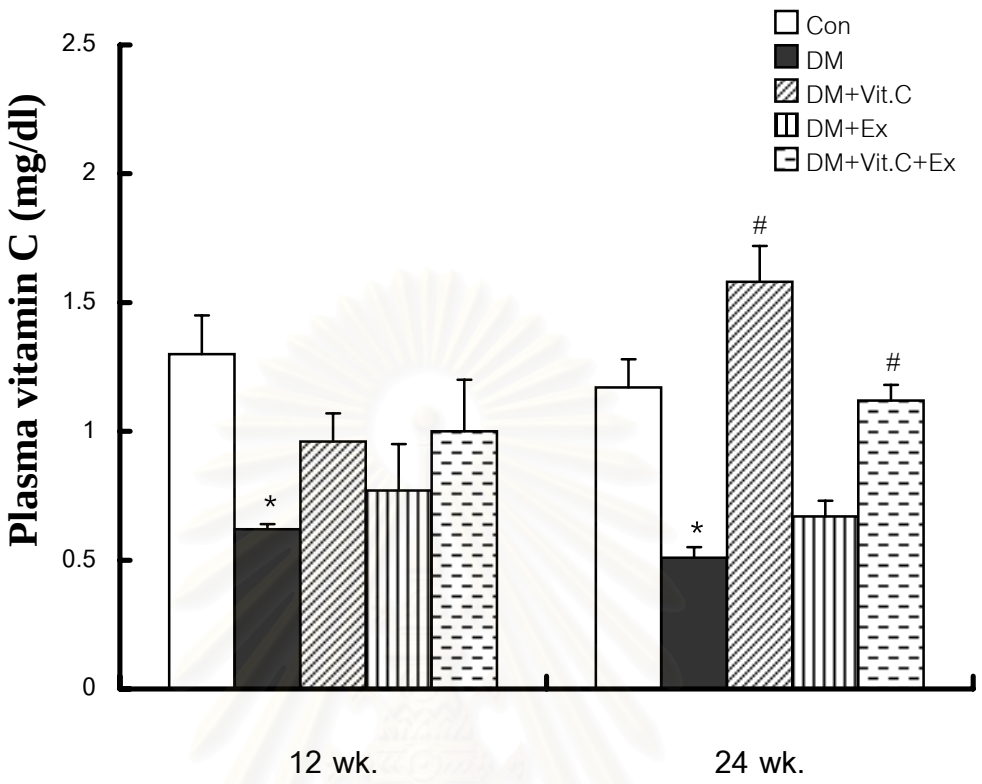


Figure 27 Plasma vitamin C (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$: significant difference from control group.

#$p < 0.05$: significant difference from diabetic group.

Table 13 Plasma Cholesterol (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Plasma Cholesterol (mg/dl)	
	12 wk	24 wk
Con	93.11 ± 2.95 (n = 9)	86.71 ± 4.44 (n = 7)
DM	89.13 ± 6.23 (n = 8)	94.33 ± 8.45 (n = 6)
DM+Vit.C	91.80 ± 5.66 (n = 10)	96.44 ± 6.03 (n = 9)
DM+Ex	105.88 ± 2.84 (n = 8)	81.50 ± 2.19 (n = 8)
DM+Vit.C+Ex	75.88 ± 6.86 (n = 8)	97.14 ± 9.97 (n = 7)

Values are means ± SEM.

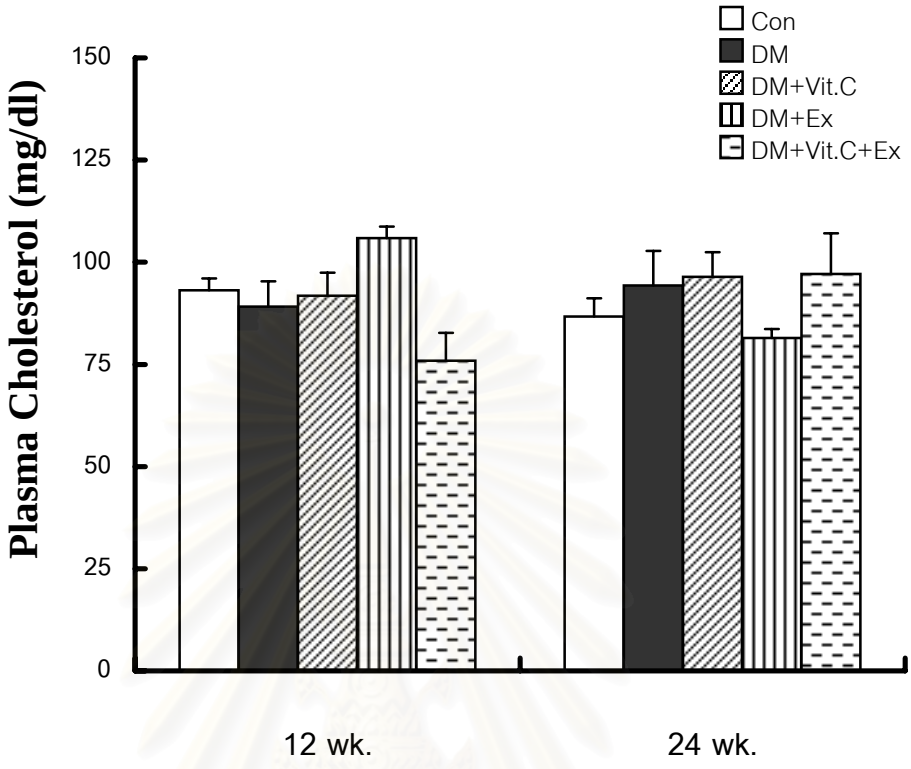


Figure 28 Plasma Cholesterol (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

Table 14 Plasma Triglyceride (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Plasma Triglyceride (mg/dl)	
	12 wk	24 wk
Con	80.38 ± 7.31 (n = 8)	64.71 ± 9.71 (n = 7)
DM	159.13 ± 23.75* (n = 8)	150.83 ± 17.71*** (n = 6)
DM+Vit.C	121.00 ± 16.43 (n = 9)	122.25 ± 11.35* (n = 8)
DM+Ex	145.71 ± 17.10 (n = 7)	91.50 ± 10.5# (n = 8)
DM+Vit.C+Ex	90.57 ± 7.62# (n = 7)	93.57 ± 14.05# (n = 7)

Values are means ± SEM.

*$p < 0.05$, ***$p < 0.001$: significant differences from control group.

#$p < 0.05$: significant difference from diabetic group.

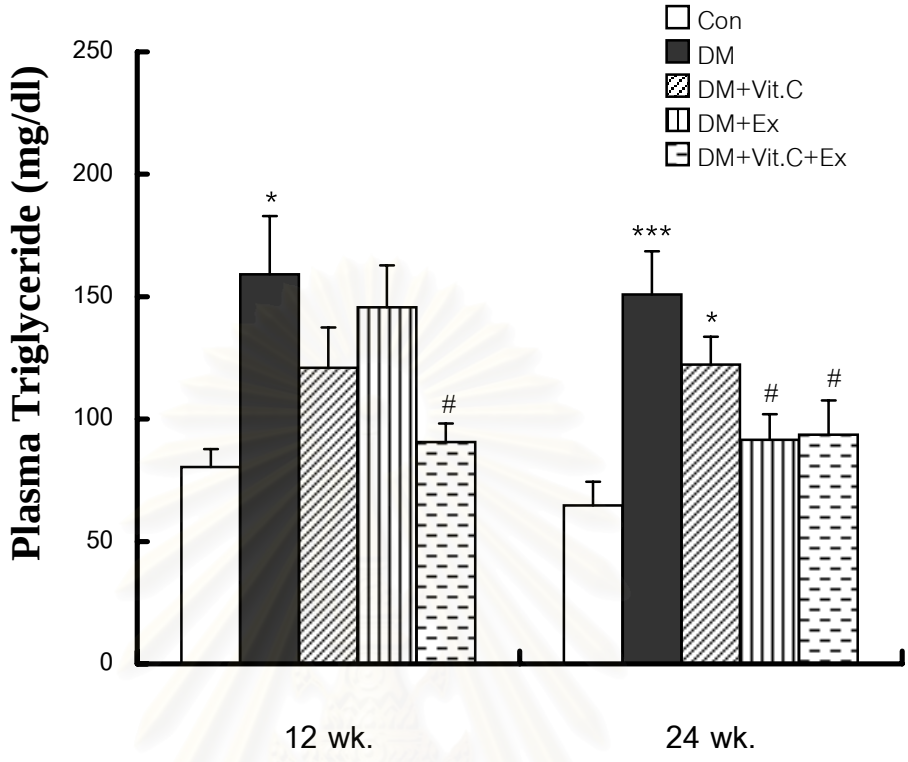


Figure 29 Plasma Triglyceride (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

* $p < 0.05$, ***$p < 0.001$: significant differences from control group.

$^{\#}p < 0.05$: significant difference from diabetic group.

Table 15 High density lipoprotein (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	High density lipoprotein (mg/dl)	
	12 wk	24 wk
Con	59.11 ± 2.07 (n = 9)	55.29 ± 2.89 (n = 7)
DM	61.38 ± 4.46 (n = 8)	58.17 ± 4.92 (n = 6)
DM+Vit.C	54.80 ± 3.45 (n = 10)	58.11 ± 3.56 (n = 9)
DM+Ex	65.50 ± 2.45 (n = 8)	52.88 ± 2.22 (n = 8)
DM+Vit.C+Ex	62.13 ± 2.81 (n = 8)	64.00 ± 7.41 (n = 8)

Values are means ± SEM.

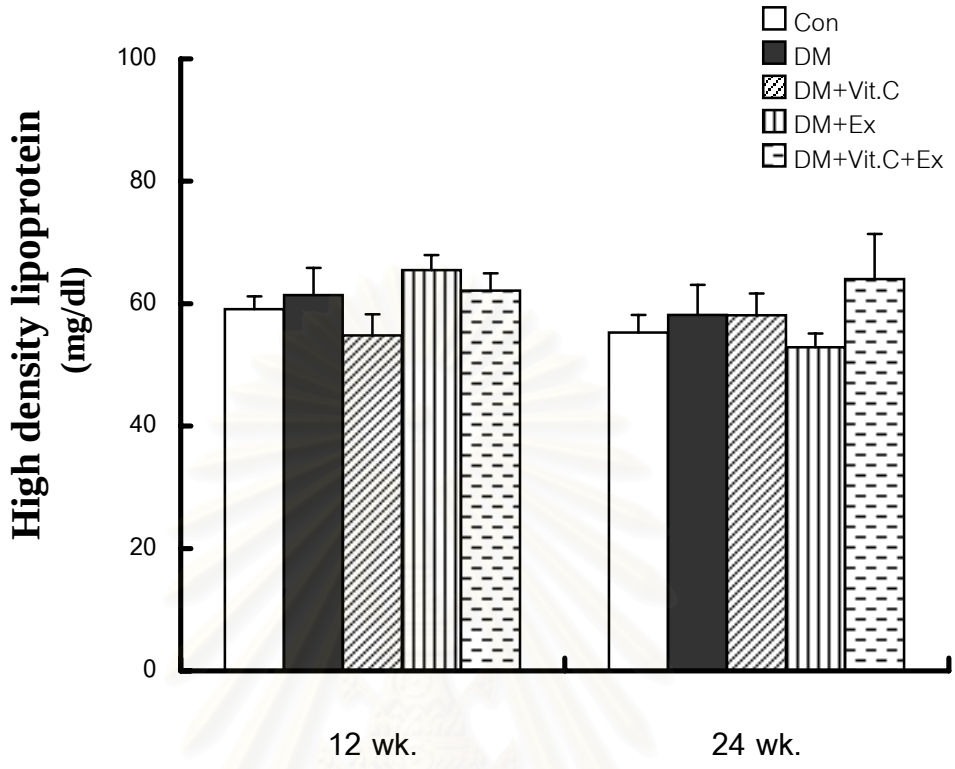


Figure 30 High density lipoprotein (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

Table 16 Low density lipoprotein (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	Low density lipoprotein (mg/dl)	
	12 wk	24 wk
Con	17.11 ± 1.11 (n = 9)	11.86 ± 1.20 (n = 7)
DM	12.88 ± 1.44 (n = 8)	13.17 ± 0.95 (n = 6)
DM+Vit.C	15.00 ± 1.25 (n = 8)	15.56 ± 1.46 (n = 9)
DM+Ex	19.50 ± 0.65 (n = 8)	10.50 ± 1.38 (n = 8)
DM+Vit.C+Ex	13.88 ± 1.25 (n = 8)	18.00 ± 3.12 (n = 7)

Values are means ± SEM.

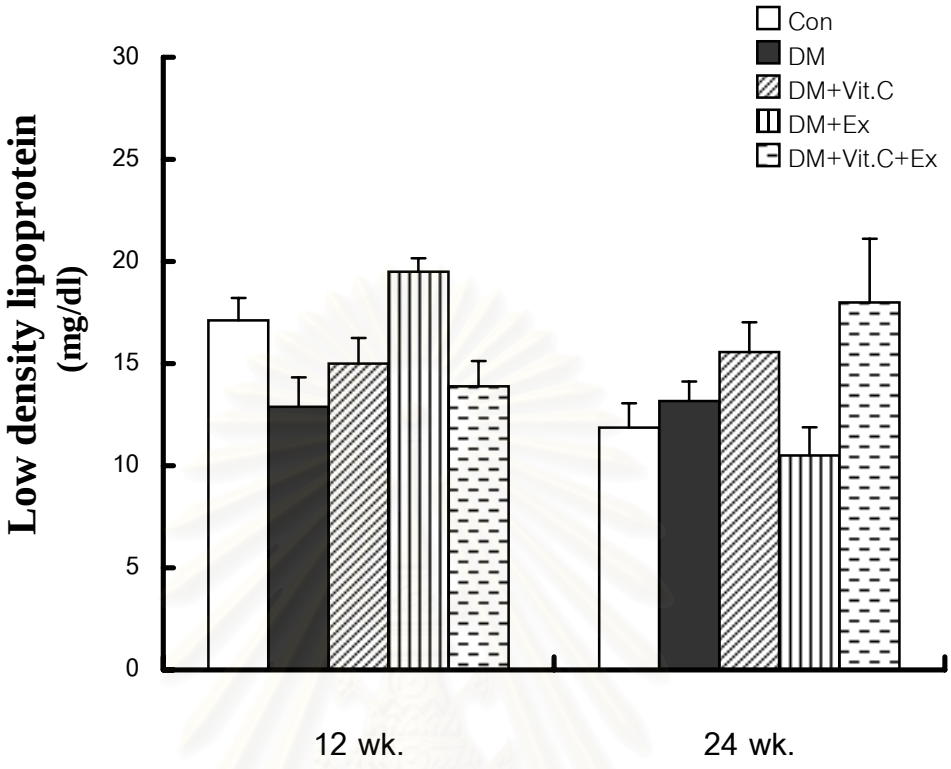


Figure 31 Low density lipoprotein (mg/dl) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

Table 17 The number of leukocyte adherence in mesenteric venules (cells/100μm)of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	The number of leukocyte adherence (cells/100μm)	
	12 wk	24 wk
Con	2.00 ± 0.50 (n = 8)	5.57 ± 0.97 (n = 7)
DM	9.17 ± 1.45*** (n = 6)	11.86 ± 0.86** (n = 7)
DM+Vit.C	4.71 ± 0.47$^{\#\#}$ (n = 7)	3.14 ± 1.06$^{\#\#\#}$ (n = 7)
DM+Ex	3.14 ± 0.70$^{\#\#\#}$ (n = 7)	5.13 ± 1.13$^{\#\#\#}$ (n = 8)
DM+Vit.C+Ex	3.78 ± 0.62$^{\#\#\#}$ (n = 9)	5.14 ± 0.67$^{\#\#\#}$ (n = 7)

Values are means ± SEM.

**p < 0.01 , ***p < 0.001: significant differences from control group.

$^{\#\#}$p < 0.01 , $^{\#\#\#}$p < 0.001 : significant differences from diabetic group.

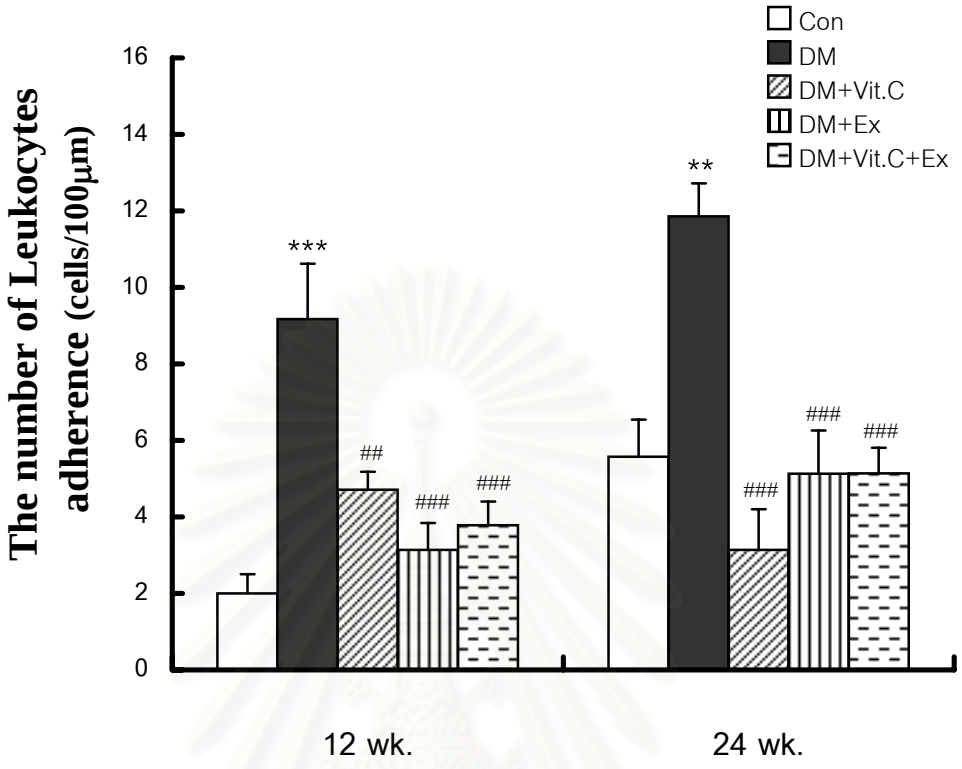


Figure 32 The number of leukocyte adherence in mesenteric venules (cells/100μm) of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

**p < 0.01, ***p < 0.001 : significant differences from control grou

##p < 0.01, ###p < 0.001 : significant differences from diabetic group.

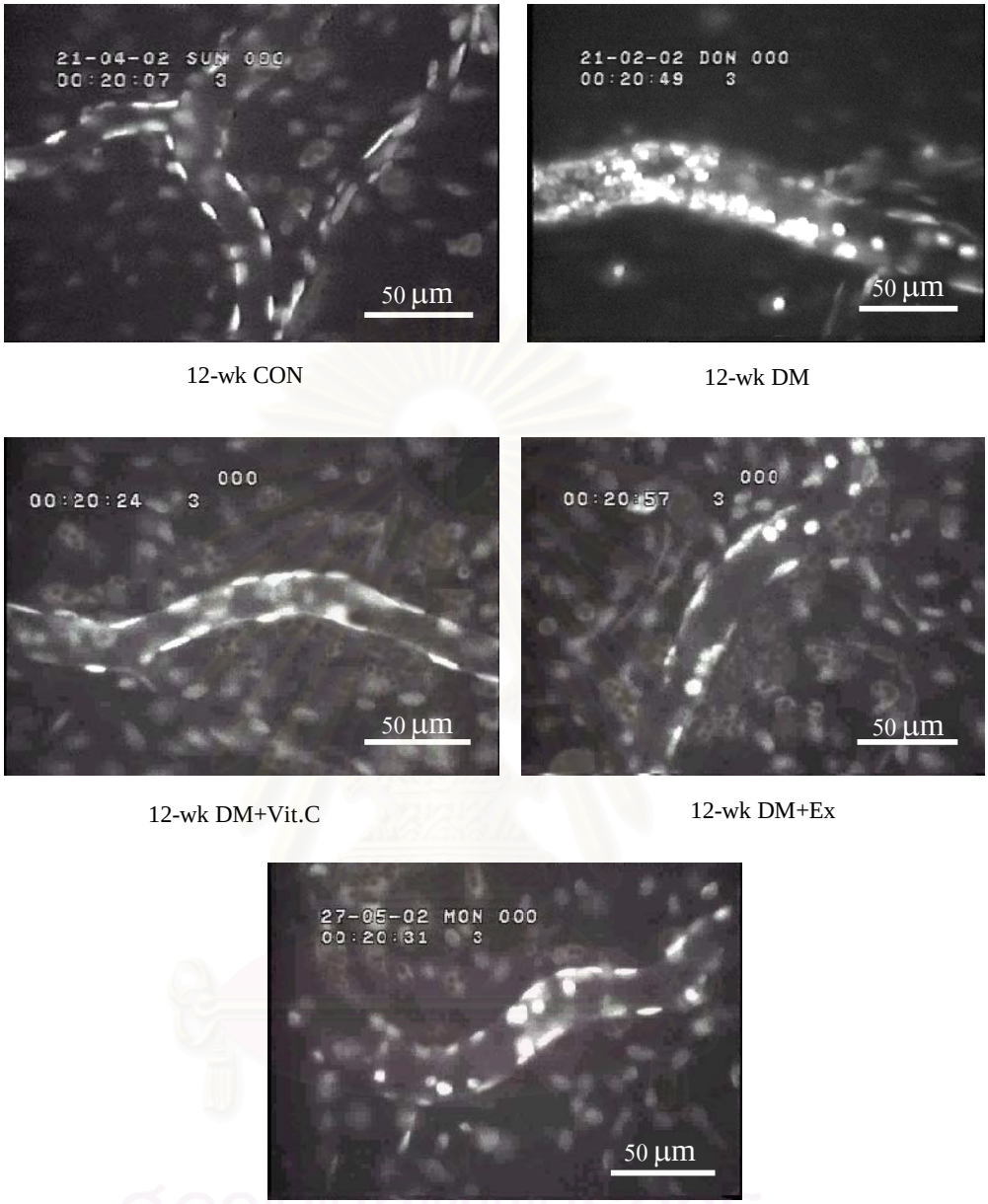


Figure 33 Videoimages of mesenteric postcapillary venules (d=15-45 µm) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ). Round white dots represent the Leukocytes labeled by acridine orange.

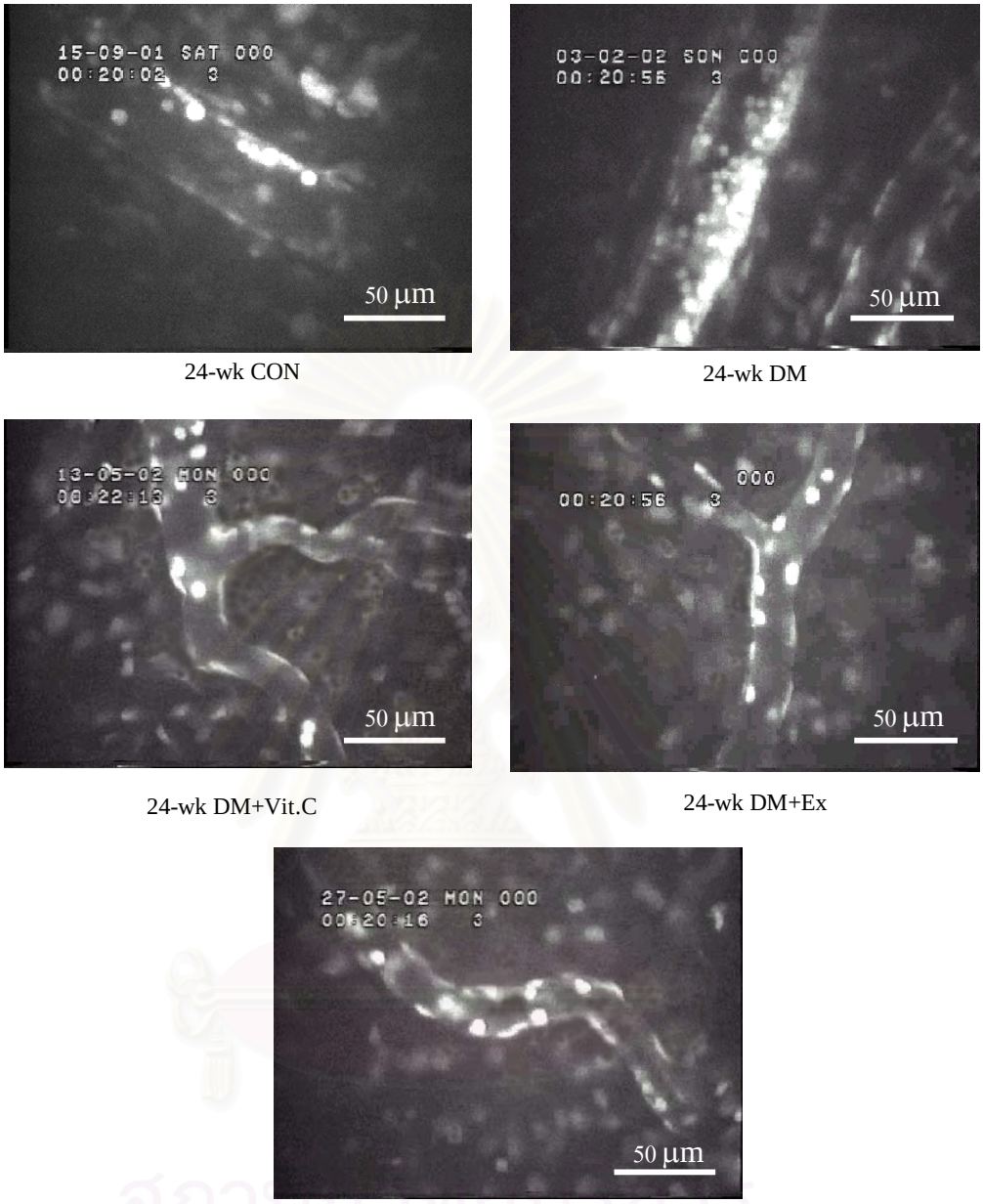


Figure 34 Videoimages of mesenteric postcapillary venules (d=15-45 µm) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ). Round white dots represent the Leukocytes labeled by acridine orange.

Table 18 % Relaxation of mesenteric arterioles (10^{-5}M) to the applications of the vasodilators., Ach (10^{-5}M) and SNP (10^{-5}M) after the precontraction with NE (10^{-5}M) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	% Relaxation (12 wk)	
	Ach	SNP
Con	14.08 ± 1.89 (n = 8)	15.19 ± 0.90 (n = 6)
DM	4.72 ± 1.52*** (n = 7)	9.42 ± 1.61 (n = 6)
DM+Vit.C	14.14 ± 1.53## (n = 7)	12.66 ± 2.02 (n = 7)
DM+Ex	7.64 ± 1.13* (n = 8)	9.94 ± 0.90 (n = 8)
DM+Vit.C+Ex	9.16 ± 1.76# (n = 8)	10.08 ± 1.00 (n = 8)

Values are means ± SEM.

*p < 0.05 , ***p < 0.001 : significant differences from control group.

#p < 0.05 , ##p < 0.01 : significant difference from diabetic group.

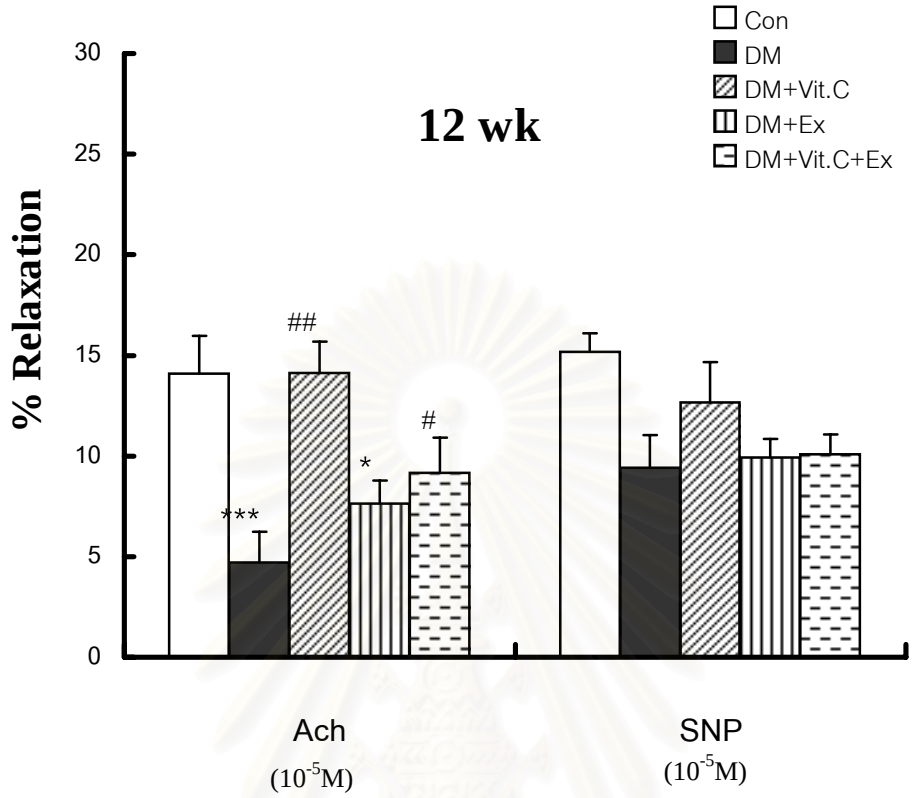


Figure 35 % Relaxation of mesenteric arterioles (10^{-5}M) to the applications of the vasodilators, Ach (10^{-5}M) and SNP (10^{-5}M) after the precontraction with NE (10^{-5}M) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$, ***$p < 0.001$: significant differences from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$: significant difference from diabetic group.

Table 19 % Relaxation of mesenteric arterioles (10^{-5}M) to the applications of the vasodilators, Ach (10^{-5}M) and SNP (10^{-5}M) after the precontraction with NE (10^{-5}M) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	% Relaxation (24 wk)	
	Ach	SNP
Con	12.59 ± 1.45 (n = 7)	16.61 ± 2.46 (n = 7)
DM	3.24 ± 0.51** (n = 8)	9.78 ± 1.13 (n = 9)
DM+Vit.C	10.85 ± 2.59$^{\#}$ (n = 7)	12.60 ± 1.55 (n = 8)
DM+Ex	6.71 ± 1.08 (n = 8)	11.23 ± 1.35 (n = 8)
DM+Vit.C+Ex	9.62 ± 1.66$^{\#}$ (n = 8)	14.94 ± 2.22 (n = 9)

Values are means ± SEM.

**$p < 0.01$: significant difference from control group.

$^{\#}p < 0.05$: significant difference from diabetic group.

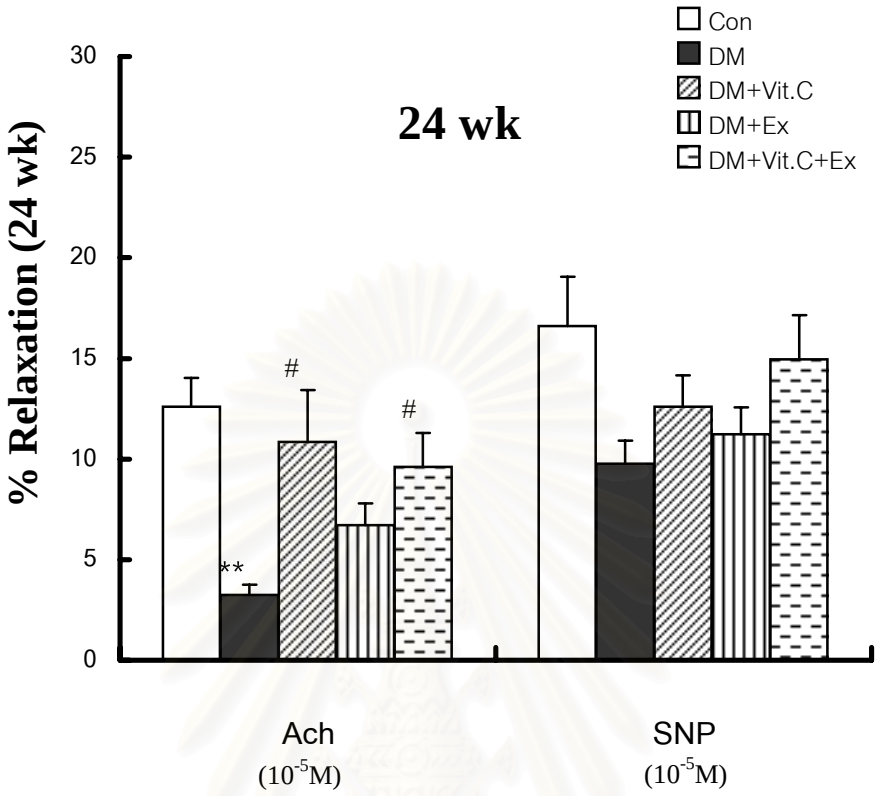


Figure 36 % Relaxation of mesenteric arterioles (10^{-5}M) to the applications of the vasodilators, Ach (10^{-5}M) and SNP (10^{-5}M) after the precontraction with NE (10^{-5}M) in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

**$p < 0.01$: significant difference from control group.

$^{\#}p < 0.05$: significant difference from diabetic group.

Table 20 Malondialdehyde (MDA) levels (nmole/g wet wt.) in liver homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	MDA levels of Liver (nmole/g wet wt.)	
	12 wk	24 wk
Con	3766.37 ± 232.45 (n = 10)	3632.14 ± 441.61 (n = 7)
DM	6141.22 ± 366.09*** (n = 10)	5646.56 ± 232.52*** (n = 10)
DM+Vit.C	4355.83 ± 508.27$^{###}$ (n = 9)	5480.95 ± 215.67 (n = 10)
DM+Ex	4388.96 ± 305.54$^{###}$ (n = 10)	4135.98 ± 131.53$^{##}$ (n = 8)
DM+Vit.C+Ex	4188.90 ± 193.57$^{###}$ (n = 10)	4121.52 ± 303.17$^{##}$ (n = 10)

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

$^{##}p < 0.01$, $^{###}p < 0.001$: significant differences from diabetic group.

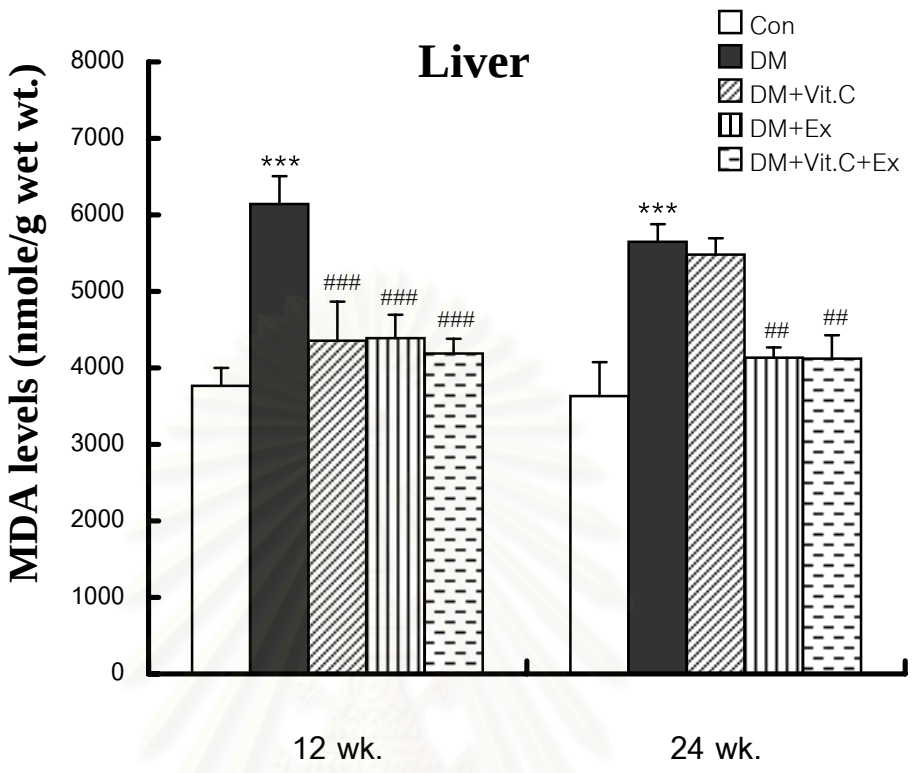


Figure 37 Malondialdehyde (MDA) levels (nmole/g wet wt.) in tissue liver homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

***$p < 0.001$: significant difference from control group.

$^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: significant differences from diabetic group

Table 21 Malondialdehyde (MDA) levels (nmole/g wet wt.) in muscle homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	MDA levels of Muscle (nmole/g wet wt.)	
	12 wk	24 wk
Con	1643.87 ± 101.15 (n = 10)	2505.62 ± 254.74 (n = 7)
DM	2843.83 ± 286.42*** (n = 10)	2994.39 ± 148.41** (n = 9)
DM+Vit.C	2260.69 ± 243.04$^{\#}$ (n = 9)	2539.67 ± 155.68$^{\#\#}$ (n = 9)
DM+Ex	2437.43 ± 251.55* (n = 9)	2449.67 ± 200.95$^{\#\#}$ (n = 10)
DM+Vit.C+Ex	1947.38 ± 143.57$^{\#}$ (n = 12)	2299.62 ± 161.41$^{\#\#}$ (n = 10)

Values are means ± SEM.

*p < 0.05 , **p < 0.01 , ***p < 0.001 : significant differences from control group.

$^{\#}$p < 0.05 , $^{\#\#}$p < 0.01 : significant differences from diabetic group.

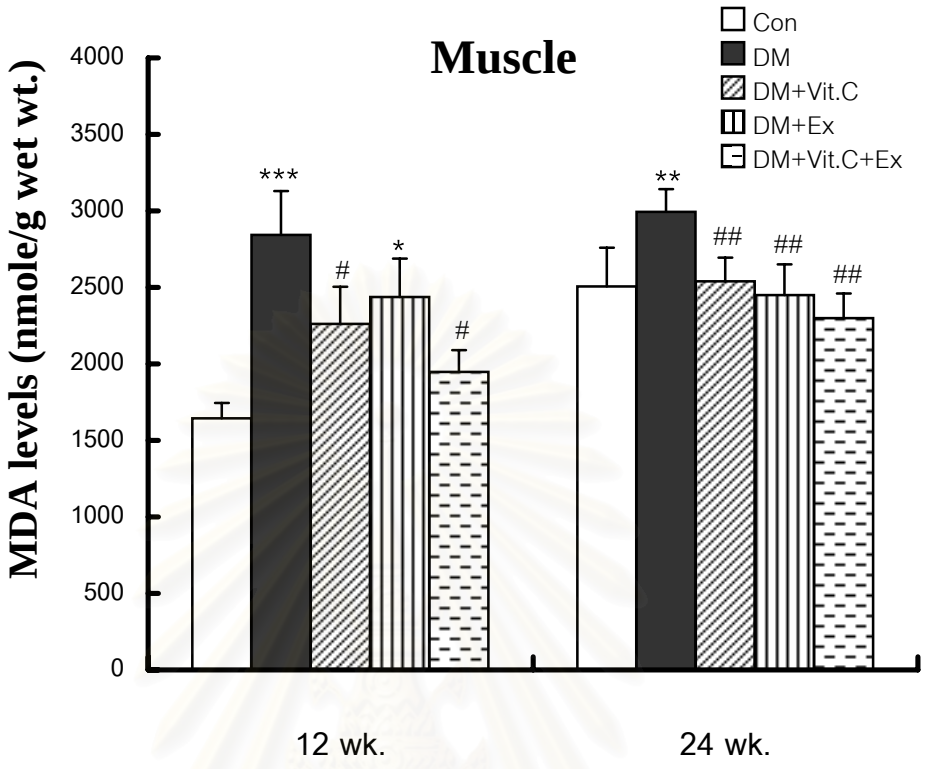


Figure 38 Malondialdehyde (MDA) levels (nmole/g wet wt.) in tissue muscle homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*p < 0.05 , **p < 0.01 , ***p < 0.001 : significant differences from control group.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$: significant difference from diabetic group.

Table 22 Superoxide dismutase (SOD) activity (Units/g wet wt.) in liver homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	SOD activity of Liver (Units/g wet wt.)	
	12 wk	24 wk
Con	5640.66 ± 656.02 (n = 6)	7740.09 ± 403.18 (n = 6)
DM	4611.93 ± 624.73 (n = 6)	5311.73 ± 195.31** (n = 6)
DM+Vit.C	3396.32 ± 498.26 (n = 6)	6132.88 ± 435.58 (n = 6)
DM+Ex	3994.94 ± 387.98 (n = 6)	5140.45 ± 441.08** (n = 6)
DM+Vit.C+Ex	5266.33 ± 593.26 (n = 6)	6511.97 ± 565.61 (n = 6)

Values are means ± SEM.

**$p < 0.01$: significant difference from control group.

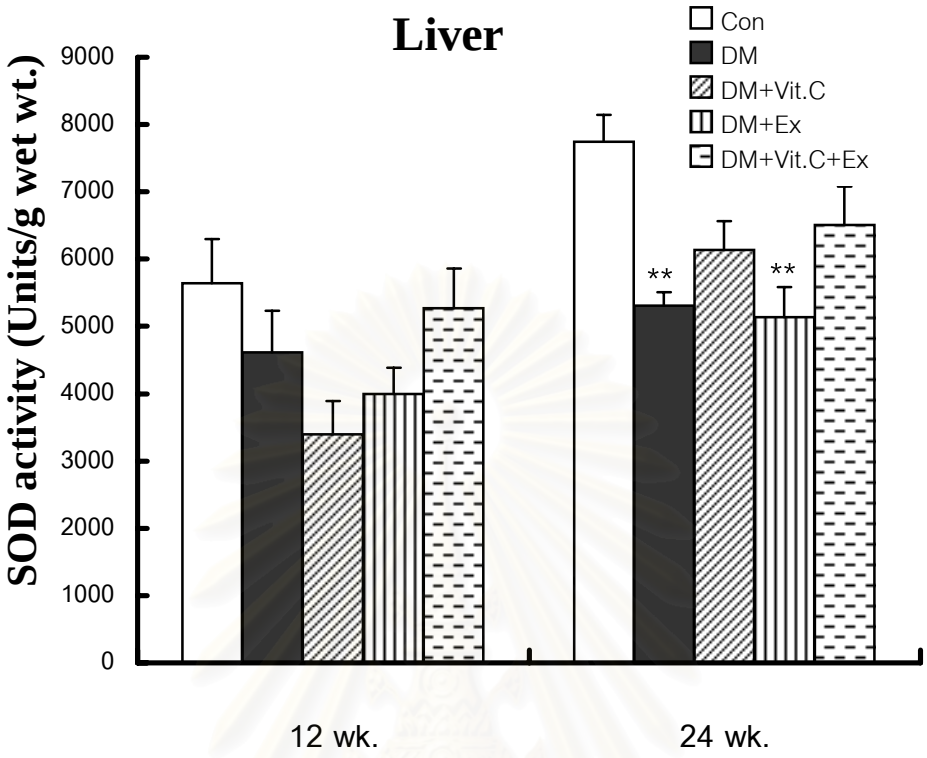


Figure 39 Superoxide dismutase (SOD) activity (Units/g wet wt.) in tissue liver homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

**$p < 0.01$: significant difference from control group.

Table 23 Superoxide dismutase (SOD) activity (Units/g wet wt.) in muscle homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	SOD activity of Muscle (Units/g wet wt.)	
	12 wk	24 wk
Con	1781.79 ± 307.67 (n = 6)	2678.71 ± 589.71 (n = 6)
DM	1527.19 ± 284.87 (n = 6)	1011.97 ± 213.55* (n = 6)
DM+Vit.C	1929.36 ± 578.85 (n = 6)	1492.43 ± 71.29 (n = 6)
DM+Ex	1617.83 ± 297.13 (n = 6)	2032.90 ± 344.31 (n = 6)
DM+Vit.C+Ex	1426.39 ± 302.24 (n = 6)	1499.90 ± 366.93 (n = 6)

Values are means ± SEM.

*$p < 0.1$: significant difference from control group.

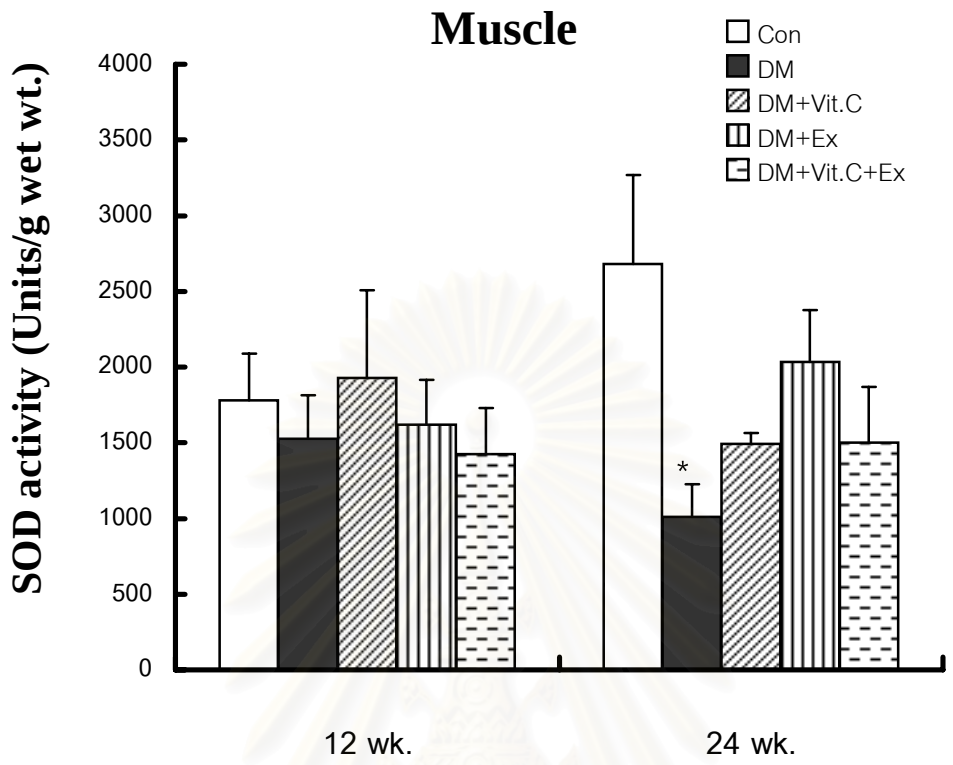


Figure 40 Superoxide dismutase (SOD) activity (Units/g wet wt.) in tissue liver homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$: significant difference from control group.

Table 24 eNOS protein (μg/5μg Protein) in tissue heart homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Group	eNOS protein of Heart (μg/5μg Protein)	
	12 wk	24 wk
Con	3.85 ± 0.19 (n = 6)	3.79 ± 0.49 (n = 6)
DM	1.10 ± 0.70* (n = 6)	2.45 ± 0.66 (n = 6)
DM+Vit.C	4.18 ± 0.21# (n = 6)	4.77 ± 0.55# (n = 6)
DM+Ex	1.85 ± 0.85 (n = 5)	1.57 ± 0.81 (n = 5)
DM+Vit.C+Ex	4.56 ± 1.17# (n = 5)	4.35 ± 0.40# (n = 5)

Values are means ± SEM.

*$p < 0.1$: significant difference from control group.

#$p < 0.1$: significant difference from diabetic group.

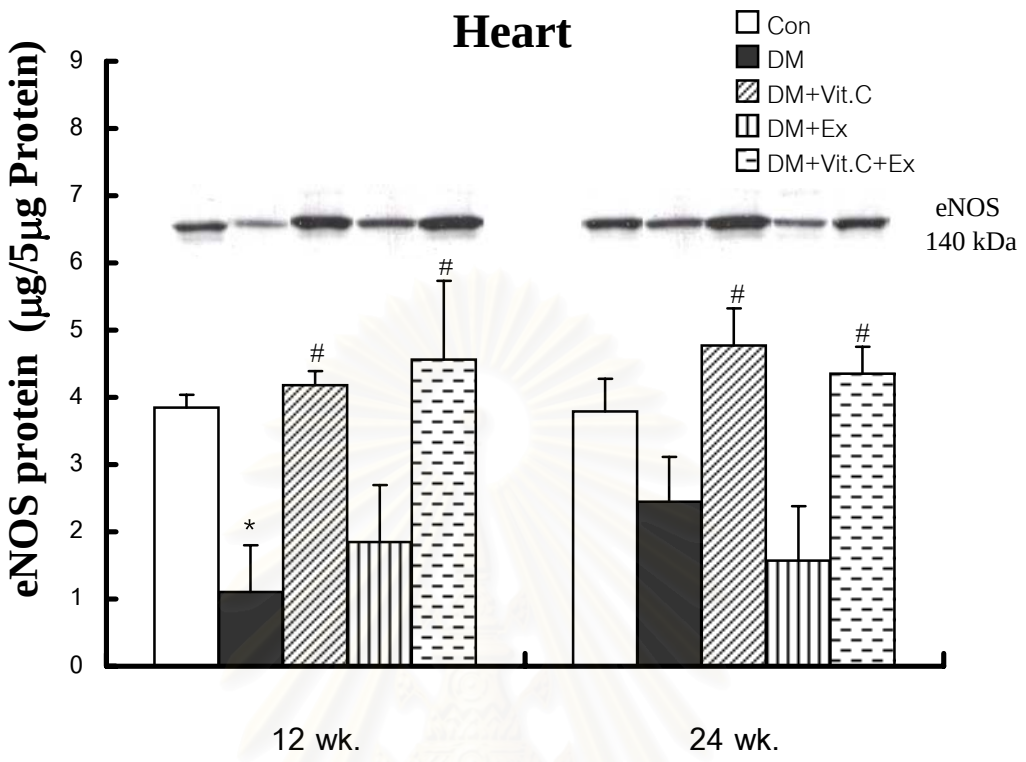


Figure 41 eNOS protein (µg/5µg Protein) in tissue heart homogenate of rats in control (Con), diabetes (DM), diabetes with vitamin C supplemented (DM+Vit.C), diabetes with exercise trained (DM+Ex) and diabetes with vitamin C supplemented plus exercise trained (DM+Vit.C+Ex) groups at 12 and 24 weeks (wk) after the injections of citrate buffer or streptozotocin (STZ).

Values are means ± SEM.

*$p < 0.05$: significant difference from control group.

#$p < 0.05$: significant difference from diabetic group.

CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to investigate the effects of vitamin C supplementation and/or exercise training on diabetic endothelial dysfunction. From the results, STZ-induced diabetic rats developed hyperglycemia, hypertension, hypertriglyceridemia, increased leukocyte endothelial cells interaction, impaired endothelium dependent vasodilation, imbalance of oxidants/antioxidants as well as decreasing eNOS protein production. Vitamin C supplementation and exercise training play a role in preventing these deliterious effects by means of different mechanisms. Discussion is expressed in relation to the heading topics as follow.

1. Effects of diabetes mellitus

1.1 Physiological characteristics of diabetic rats

Streptozotocin (STZ) had highly specific cytotoxic action on the beta cells of the Islets of Langerhans. This drug has been widely used, especially in rats, to induce the experimental model imitated type 1 diabetes mellitus. Numerous studies are performed with this model, providing evidence of its usefulness in studying diabetic complications (Crijns FR.,1999). In the present study, hyperglycemia occurs 48 hr after 50 mg/kg STZ intravenous administration and persist throughout the experiment. We found that diabetic symptoms appear abruptly at around 6-8 weeks of age including marked hyperglycemia, polyphagia, polydipsia, polyuria and weight loss. These symptoms can be explained as follow.

Severe hyperglycemia leads to glycosuria and induces an osmotic diuresis and thus polyuria, causing a profound loss of water and electrolytes. The renal water loss combined with the hyperosmolarity resulting from the increased levels of glucose in the blood trends to deplete intracellular water, triggering the osmoreceptors on the thirst centers of the brain (polydipsia). With a deficiency of insulin, the scales swing from insulin-promoted anabolism to catabolism of proteins and fats which tends to induce a negative energy balance, and which, in turn, leads to increasing appetite (polyphagia). Despite the increased appetite, catabolic effects prevail, resulting in weight loss and muscle weakness (Cotran RS.,1999).

Blood glucose (BG), Glycosylated hemoglobin (HbA_{1C}) and Lipid profile

Our measurements of plasma blood glucose levels indicated a greater than 3-fold elevation in diabetic rats compared to those in controls. Because a single blood glucose determination is often an insensitive indicator of metabolic control, we also measured HbA_{1C} concentrations. **HbA_{1C} levels were elevated more than 2-fold in the diabetic rats compared to those in control**. The increased HbA_{1C} levels in the diabetic rats reflect long-term exposure to high blood glucose levels. Based upon these data along with the marked differences in both blood glucose and HbA_{1C} between groups, we concluded that STZ injection induced a state of severe diabetes in this study. Besides, we found the abnormal lipid profile in **STZ-induced diabetic rats which expressed as the significantly higher in plasma triglyceride, but not plasma cholesterol and LDL** (Table 13, 14, 15 and 16). This study consistent with the study of Ting HH. et al. (1996). They reported that the total cholesterol, HDL and LDL were similar in diabetic and nondiabetic

subjects, but diabetic subjects had significantly higher in triglyceride levels, the reasons for the increase in serum triglycerides were increased production and decreased clearance of them. Triglyceride turnover is increased in both insulin-deficient and hyperinsulinaemic diabetics, but there is contradictory evidence concerning triglyceride clearance form the circulation, which depends on the activity of the enzyme lipoprotein lipase (Keen H.,1999). Importantly, it has been reported that the **elevated triglyceride may effect endothelial function** (Palmer AM.,1998). Since an abundance of lipids provides the substrate for free radical attack that undergo lipid peroxidation (Radak Z.,2000) result in altering membrane fluidity and permeability of endothelial cells. *According to this higher triglyceride level and its possibility to damage EC, therefore, we will discuss about this matter again in the following session of endothelial dysfunction in diabetes.*

Arterial blood pressure

Hypertension is more prevalent both among persons with IDDM and those with NIDDM than in the general population (Kahn CR.,1994). **Our results demonstrated the significant elevation of systolic, diastolic and mean arterial blood pressure in DM-rats at both 12 and 24 weeks after the STZ injection.** For the increased of blood pressure (BP), changes in vascular structure and function, and thereby in total peripheral resistance (TPR) could be involved. The vascular endothelium plays an important role in regulation of vasomotor tone and, subsequently, blood pressure (Rankinen T.,2000). The major endothelium-derived relaxing factor is NO (Taddei S.,1998). **From the results of this study, we suggested that the elevated BP in diabetic rats are mediated through the increase in TPR as well. Since in our experiment on endothelial functions we have found the abnormality**

of arteriolar responses. As such results, it will be described in the following session as well. Based on several cardiovascular diseases, the inhibition of NO synthesis has been mostly referred for its attribution to the vasoconstriction effects and impaired endothelial vasomotion function. These would be increased TPR and then resulted in increase arterial BP. However, BP is also regulated by compensatory system as well. Therefore, diabetic hypertension might be explained by multiple factors. For instance, the raise of intracellular sodium and calcium concentrations which could increase contractility of vascular smooth muscle and so peripheral vascular resistance and blood pressure as well. Furthermore, the increase of the renin-angiotensin-aldosterone system in type 1 diabetes has been also described for causing diabetic hypertension (Pickup JC.,1997).

Plasma vitamin C

Vitamin C or L-ascorbic acid (AA) is a naturally occurring major antioxidant, essential for the scavenging of toxic free radicals, both in the plasma and tissues (Kashiba M.,2002). Vitamin C concentrations in plasma, serum, red blood cells, and white blood cells can be determined by several techniques, such as: colorimetric, fluorometric, chromatographic, and electrochemical techniques, high-performance liquid chromatography (HPLC) methods. Plasma concentrations of vitamin C are generally believed to reflect dietary intake. Leukocyte AA concentrations, though difficult to measure, are thought to reflect both AA tissue content and body pool concentrations. DHAA assays are particularly difficult to perform, and few studies have used rigorous assay techniques (Will JC.,1996).

In this study, we have evaluated the plasma vitamin C in diabetic rats by using the colorimetric method. The data showed that **plasma**

vitamin C concentration of diabetic rats was significantly lower than control rats at both 12 and 24 wk after STZ-injection (Table 12 and Figure 27). This finding supports the previous studies that found the decrease of vitamin C concentrations of the brain, heart, lung, liver, kidney and plasma in the 8 wk diabetic rats compared with those of the control group (Sun F.,2001). Moreover, the recent study demonstrated that AA concentrations in plasma and tissues was decreased in STZ-induced diabetic rats (Kashiba M.,2002). The mechanisms for this evidence has not been fully explored yet. It might be due to competitively inhibits DHAA uptake or further downregulates the V_{max} of the uptake mechanism by chronic exposure of cell to high glucose levels. Transport of vitamin C through biological membrane is facilitated by glucose transporters, especially GLUT-1 (Siman CM.,1997). Therefore, chronic hyperglycemia may impose an intracellular deficit of AA through **competitive inhibition of membrane transport of AA by the elevated plasma glucose** (Dai S.,1995). Moreover, that plasma and tissue AA levels in STZ diabetes are decreased can be explained by other several factors, including the impaired hepatic and renal regeneration, increased urinary excretion as well as impaired hepatic biosynthesis of AA in diabetic rats (Kashiba M.,2002).

1.2 Oxidants/antioxidants imbalance and eNOS production in diabetes

Malondialdehyde (MDA)

There is currently great interest in the potential contribution of increased oxidative stress to the development of various disease. For practical reasons, neither the steady-state levels of ROS nor the rate of oxidant production are easily measured directly ex vivo in biological systems. Thus, steady-state levels, of the extent of accumulation of

oxidation products in tissues and plasma or the changes in antioxidant status are commonly used as measures of increased oxidative stress (Van DPS.,1995 , Cominacini L.,1996). In fact, the measurement of parameters of lipid peroxidation, such as lipid peroxides, conjugated dienes, and malondiadehyde (MDA), can be considered the cornerstone of the assessment of oxidative damage in vivo (Van DPS.,1995).

In current study, the degree of ROS occurred can be estimated by the assessment of its main product, malondialdehyde or MDA by using Thiobarbituric acid reactive substances (TBARS) technique (Halliwell B.,1993 , Margaritis I.,1997). This TBARS technique has been shown to be sensitive to MDA, a good general index of oxidative stress in biological systems and widely used to measure lipid peroxidation in cell membranes and fatty acids (Ohkawa H.,1979). Even though the reliability of using MDA as an indication for oxidative stress has been questioned, it remains a useful marker for gross oxidative damage if a multifaceted approach is adopted and the limitations are recognized (Ji LL.,1996). The control level of MDA found in this study was in a range comparable with that found by other investigators using the same TBARS method (Ohkawa H.,1979).

In present study, it is seen from the result that MDA levels in liver and muscle homogenates were significantly higher in both 12 and 24 wk diabetic rats (Table 20 and 21). We speculated that the significance of increased HbA_{1C} levels in DM rats, could be represented for poor metabolic control. As such the poor control of blood sugar could cause the generation of primary initiation oxygen radicals, superoxide anion ($O^{\bullet}_2$) within the peripheral circulation in DM rats, and then transferred to MDA (Davison GW.,2002). Strong evidences from the literature supporting our results both in humans (Aydin A.,2001 , Santini SA.,1997) and animals

(Ahmed I.,2001) models have been demonstrated. Hyperglycemia has been shown to stimulate superoxide generation by increasing the activity of the enzyme NADPH cytochome P-450 oxidoreductase, more commonly termed NADPH oxidase. It was found that vascular superoxide production in the setting of diabetes was reduced by preventing PKC-mediated activation of NADPH oxidase (Hink U.,2001). Besides, there are many suggestions regarding the origins of oxidative stress in diabetes, including free radical reactions related to glycation of proteins, consumption of NADPH through the polyol pathway, glucose autoxidation and hyperglycemia-induced pseudohypoxia (Vanderjagt DJ.,2001).

The high level of MDA is important in the late complications of diabetes. The endothelial inter-molecular cross-linking of protein and MDA not only reduced its optimal functioning, but reduces its already low turnover. Consequently it allows further glycation by glucose and its own oxidation products to initiate further oxidation of fatty acids. More MDA was produced and thereby propagating a feedback cycle of protein and fatty acid damage in endothelium (Davison GW.,2002).

Superoxide dismutase (SOD)

Our results show that after 24 weeks of diabetic induction, DM rats had significantly lower SOD activity as compared with those of control rats. Whereas at 12 weeks, DM rats had trended to decrease in SOD activity, but did not reach statistical significance. This findings is consistent with observations regarding SOD activity in bovine erythrocytes in diabetic patients (Inukai I.,2002) and with studies performed in experimental animals (Matkovics B. et al.,1997 , Power RW.,1996).

Superoxide dismutase (SOD) is an important scavenger of one of ROS, superoxide anion ($O^{\bullet}_2$). The first step of scarvenging ROS is the reduction of $O^{\bullet}_2$ to hydrogen peroxide (H_2O_2) by SOD (Halliwell

B.,1999). However, SOD enzyme activity has been reported to be inactivated by high concentration of H_2O_2 (Bray RC.,1974). Since, ROS was found to be increased in diabetes in our study. Thus the production of H_2O_2 may be increased in diabetes, and these elevated concentrations of H_2O_2 can reduce SOD activity. We suggested that a reduction in basal SOD activity lead to further ROS leftover and then consequently damaged cell compositions in diabetic rats.

However, some reports indicated an increase in both SOD gene expression and SOD activity in brain of STZ-induced diabetic rats (Huang WC.,1999) and in embryos of a diabetic rat strain displaying major malformations in vivo (Kakkar R.,1997). Disagreement among findings may be explained by different species and organ types used.

1.3 Endothelial dysfunction in diabetes

Endothelial nitric oxide synthase (eNOS)

In our study, eNOS protein of heart homogenate from DM rats was significantly decreased in comparison with the control heart. This finding was consistent with one reported by Zhao G. et al. (2000). They reported that the eNOS protein levels in aortic endothelium from diabetic dogs were decreased by 66% compared with those from normals. In addition, 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.,2000).

The reason why hyperglycemia could effect on eNOS protein has been described in a variety aspects. The increase in ROS, which is properly demonstrated by MDA level, has proposed as a potential mechanism. Synthesis of NO by eNOS is crucial for normal vascular homeostasis. In normal endothelium, eNOS activity is regulated by increase in intracellular Ca^{2+} concentration. Both agonists and hemodynamic could

mediated NO production (Ranjan V.,1995). Recently, localization of eNOS to specialized plasma membrane invaginations termed caveolae has been proposed to be required for maximal eNOS activity (Garcia-Cardena G.,1996). Moreover, a decrease in eNOS protein synthesis in diabetes is therefore, possibly explained through the ROS damaged protein-lipid interactions. As such it results in decreasing eNOS and caveolin interaction or called downregulation of both eNOS localization and activity (Peterson TE.,1999). In addition, ROS may inhibited posttranslational modifications of eNOS by a result of oxidation of critical amino acid residues and inhibition of necessary enzymes (Shaul PW.,1996).

However, the effect of hyperglycemia on activity of the NO system is somewhat controversial, since different studies have provided for: increase (Cosentino F.,1997 , Graier WF.,1996) , decrease (Balon TW.,1997 , Pieper GM.,1998) and even no change (Schmettenor L.,1997 , Smits P.,1993) in NO activity and its content. The tissue sources might be account for as such discrepancy.

Leukocyte-endothelium interaction

Our results demonstrated that the number of leukocyte adhering per 100 μm of vessel length was significantly increased at both 12 and 24 wk diabetic rats compared with control rats (Table 17). Schaffler A. et al. (1998) have reported that in STZ-induced diabetes, adhesion and emigration were significantly enhanced while shear rate were severely reduced. According to the study of Booth G. et al. (2001), they found the increased leukocyte rolling, adherence and transmigration after exposure of the mesentery to D-glucose for 12 hr. However, Cruz JWMC. et al. (2000) have observed that in alloxan-induced diabetes, the number of leukocytes rolling along the venular endothelium were reduced by about 70% and in postcapillary venules by 60%. The different result may be due

to the different strain and/or different method, since the studies of Schaffler A. et al. and Booth G. et al. as well as this present study, we used male Spraque-Dawley rats and STZ, whereas male Wistar rats and alloxan have been used by Cruz JWMC. et al.

Glucose-induced leukocyte adhesion is dependent upon the upregulation of adhesion molecules includings, E-selectin, ICAM-1, and VCAM-1 (Morigi M.,1998). It was found that sera from diabetic patients with elevated glycemia and abnormal HbA_{1C} induced a significant adhesive response as compared with sera from abnormal subjects (Morigi M.,1998). The increased leukocyte-endothelial interaction could be explained by loss of NO in chronic diabetes (Huvers FC.,1999). As demonstrated in experimental result, eNOS protein was significantly reduced in diabetic rats. These are the most likely cause of the over production of ROS in diabetes as we have described previously. The importance of NO is a regulator of vascular homeostasis. As such, NO also acts as a potent inhibitor of leukocyte adhesion to the microvascular endothelium (Kubes P.,1991). It was found that mice deficient in eNOS and nNOS exhibit enhanced leukocyte adhesion to vascular endothelial cell in the mesentery (Lefer DJ.,1999). Therefore, quenching NO may be associated with elevated surface expression of adhesion molecules within the microcirculation. Decrease in NO would result in activated PKC which could cause an upregulation of P-selection expression, respectively (Murohana T.,1996). Therefore, at this point we would like to suggest from our findings that high blood glucose levels could mediate the adhesive molecules expressed on the endothelial surface. **Hyperglycemic oxidative stress which results in reducing NO availability could be the major cause and lead to enhance leukocyte-endothelium interaction.**

Endothelium-mediated relaxation

To observe the function of endothelium, acetylcholine (Ach) which is attributed to increase nitric oxide (NO) synthesis or release from the endothelium for obtaining muscle relaxation (Tribe RM.,1998) was commonly used. A blunted Ach-induced relaxation has been generally use as an indicator for a global defect in the endothelium (Palmer AM.,1998).

Impaired endothelium-dependent vasodilatation has been reported in aorta (Keegan A.,1995), mesenteric (Palmer AM.,1998) and femeral (Taylor PD.,1995) arteries of the streptozotocin (STZ) rats and in diabetic patients (Huvers FC.,1999). In agree with previous reports, the present results showed that both 12- and 24-wk diabetic rats demonstrated a significant reduction in the percentage of maximum relaxation to Ach as compared to their age-matched controls (Table 18 and 19).

Acetylcholine binds to its receptor on vascular endothelial cells, resulting in a complex chain of events that results in calcium influx, stimulation of eNOS, and increased production of NO. NO, in turn, diffuses across the basement membrane to cause relaxation of adjacent vascular smooth muscle cells (Wang SP.,1998). In current study, the normal response of diabetic mesenteric vessels to nitroprusside, which acts as a NO donor, suggested that the smooth muscle cell function remains intact. Thus the impaired of vascular response to Ach appeared in this study, suggested that only the endothelial cells were defect.

1.4 ROS causes endothelial dysfunction in diabetes

In this study, there is substantial evidence to indicate that diabetic endothelial dysfunction is mediated by ROS. Since from our results, we found the markedly increased MDA level in STZ-induced diabetic animals. Hyperglycemia increase the protein glycation and auto-oxidation

as demonstrated by the significantly elevation of HbA_{1C} and dyslipidemia. In addition, we have showed the decreased antioxidant enzyme, SOD, simultaneously with the increased MDA level. Moreover, the plasma vitamin C level was also found to be decrease in diabetes. Since, those antioxidant substances were used to scavenge the harm of ROS. Therefore, the more decrease in these scavengers, the more increase in the deleterious effects of ROS. We therefore, believed that the over production of ROS in diabetes is a primary mechanism of development of endothelial dysfunction.

This finding demonstrated that the increased production of ROS after exposed to hyperglycemia accounts for a significant proportion of endothelial dysfunction in diabetes demonstrated by increased leukocytes adhesion to endothelium and impaired vascular reactivity to acetylcholine. Moreover, the significant decrease in eNOS protein expression in the heart of both 12 and 24 weeks DM rats was also presented in this study. It ,therefore, confirms that endothelial function is impaired in diabetes. Oxidative stress may damage endothelial function through several mechanisms. ROS, especially hydroxyl radicals may injure the modify proteins and DNA as well as irreversible modify proteins in endothelial cells. Diminished capacity of eNOS which results in to decrease of NO production could be the mechanisms for further development of endothelial dysfunction.

Considerable to leukocyte adhering, the harm of these phenomenon is that contact of circulating leukocytes with the vascular endothelium promotes a cascade of events leads to further leukocyte activation. Once activated, leukocytes are able to release oxygen-derived free radicals, proteolytic enzymes, and cytokines. Superoxide radicals released form leukocytes have been shown to inactivate NO, induce vasoconstriction,

and disrupt cellular membranes through lipid peroxidation (Booth G.,2001). All these processes lead to further leukocyte activation and aggravate vascular endothelial dysfunction. In particular, leukocyte stasis as well as infiltration of circulating, can have detrimental effects in diabetes, as leukocytes contribute to endothelial cell injury leading to diabetic microangiopathy (Lutty GA.,1997). (*As the conclusion of this moment, our hypothesis of hyperglycemic oxidative stress induced endothelial dysfunction was partly demonstrated within the diagram showed in Figure 43.)*

2. Effects of vitamin C supplementation on diabetic endothelial dysfunction

In the present study, the level of plasma vitamin C in DM rats was much smaller as compared to control rats. This deficit has been able to prevent by supplementation of exogenous vitamin C. Within our experimental period, vitamin C supplementation has showed no effect on hyperglycemia but did lower glycosylated hemoglobin (HbA_{1C}), reduced MDA level, prevent the decrement of eNOS protein expression and then prevent endothelial dysfunction.

Currently, endothelial dysfunction has been widely referred to its contribution to pathogenesis of hypertension especially, in diabetes. Therefore, this might be the fact to explain why high blood pressure in DM-rat was significantly prevented by vitamin C supplemented in this study. Furthermore, in present study, we observed the beneficial effects of vitamin C supplementation on mesenteric vascular function both venules and arterioles site in diabetic rats using intravital fluorescent microscopy. At the venular site, the present results have shown that the long term supplementation of vitamin C reduced the enhanced leukocyte adhesion

to mesenteric venular endothelium in STZ-induced diabetic rats. Jariyapongskul A. et al. (2002) who studied the cerebral microcirculation observed directly through a cranial window in STZ-induced diabetic rats, have also demonstrated the preventive effect of long-term supplemented vitamin C as well. Besides, Lehr et al. (1997) have demonstrated an important role of ascorbate in inhibiting leukocyte-endothelial cell interactions induced by cigarette smoke likely by antioxidant mechanisms. At the arteriolar site, this current investigation showed that vitamin C prevented the attenuated endothelium-dependent vasodilation observed in both 12-and 24 wk diabetic rats. Further, chronic administration of the antioxidant, vitamin C, significantly prevented mesenteric arterioles endothelium-dependent vasodilation to Ach, whereas the endothelium-independent vasodilator response to the exogenous nitric oxide donor SNP, was not significantly altered by administration of vitamin C. Similar to our study, numerous clinical studies have consistently demonstrated beneficial effects of vitamin C treatment on endothelium-dependent vasodilation in chronic heart failure (Horning B.,1998), hypertension (Taddei S.,1998) and coronary artery disease (Heitzer T.,2001).

One potential mechanism leading to diabetic endothelial dysfunction observed in this experiment was believed on the roles of hyperglycemia induced ROS, since we found the high levels of its product, MDA. Protein glycation is one of the main sources of those ROS in diabetes (Vallejo S.,2000). Recently study both in vitro and vivo described a high correlation between HbA_{1C} values and endothelial dysfunction in rats with STZ-induced diabetes (Angulo J.,1998 , Rodriguez-Manas L.,1998). Although there was no significant difference in plasma glucose concentration after vitamin C supplementation.

However, we cannot say that the improvement of diabetic endothelial function in this study is not due to a correction of the diabetic state, regarded to HbA_{1C} in diabetic rats was found to be decreased in this study. However, another sources of ROS that have been proposed in this study, lipid abnormality, was unchanged by administration of vitamin C. This finding is consistent with the study of Taddei S. et al. (1998) and Horning B. (1998). They found that administration with vitamin C did not change abnormal lipid patient. In contrast to our study, Muralikrishman G. demonstrated a beneficial effect of ascorbic acid treatment on dyslipidemia in albino female Wistar rats. The discrepant results may be explained by differences in the studied model and treatment regimen.

Endothelium is an important modulator of vascular tone, regulation of endothelial cell permeability and adhesivity as well as inhibition of platelet activation through the synthesis and release of endothelium-derived nitric oxide (EDNO) (Stamler JS.,1994). EDNO is synthesized from L-arginine through the action of constitutive and inducible isoforms of the NADPH-dependent enzyme NO synthase. The enzyme requires a number of cofactors, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) and possibly thiols. Inactivation of EDNO by reactive oxygen species such as superoxide anion ($O_2^{\bullet}$) may contribute to endothelial dysfunction (Saram K.,2002). We have demonstrated that endothelial dysfunction in diabetic rats was manifested as increased leukocyte-endothelial interaction and impaired vasoactivity to vasodilator. Interestingly, these abnormalities were prevented when vitamin C was supplemented. Since, vitamin C effectively scavenges superoxide and other reactive oxygen species and protects lipid against peroxidation (Heller R.,1999). It can be implied that ROS is a key factor causing abnormal endothelial function founded in

hyperglycemia. We present evidence that continued vitamin C supplementation in diabetic rats resulted in a highly significant reduction in MDA levels. This finding is supported by Ashton J. (1999), they demonstrated the reduction of lipid hydroperoxide and MDA by supplementation with vitamin C. Moreover, we found that DM rats with vitamin C supplementation had a significantly higher in eNOS protein expression as revealed by western blot analysis. We hypothesized that increased in eNOS protein may be results in the increase of NO production. In agreement with our findings are those of Heller R. et al. (1999), who demonstrated that L-ascorbic acid potentiates agonist-induced NO formation in cultured endothelial cells in a dose-dependent fashion. Thus, we indicated that dietary antioxidants, vitamin C, can protect against the development and progression of endothelial dysfunction in diabetes via prevented NO inactivation by degenerate ROS formation.

Ascorbic acid has improved endothelium-dependent vasodilation by augments NO bioavailability as showed in Figure 42 (Taddei S.,1998, Carr AC.,2000, Sherman DL.,2000). The potential mechanisms underlying the salubrious effects of vitamin C on NO generation are first, vitamin C may be decreasing the levels of superoxide radicals and oxidized LDL, both of which react with and inactivate NO (Carr AC.,2000). Vitamin C may act extracellularly as a superoxide anion scavenger and intracellularly by affecting the redox state (Beckman JA.,2001). Second, vitamin C can directly reduce critical thiol moieties on the enzyme and thereby up-regulate eNOS activity (Smith AR.,2002). Third, vitamin C might act through effects on the availability of NADPH, FAD, FMN and calcium/calmodulin which are cofactors of endothelial NO formation (Heller R.,1999). Finally, physiological concentrations of ascorbate

increase the synthesis and biological activity of NO by increasing intracellular tetrahydrobiopterin (Huang A.,2000, Mullan BA.,2002)

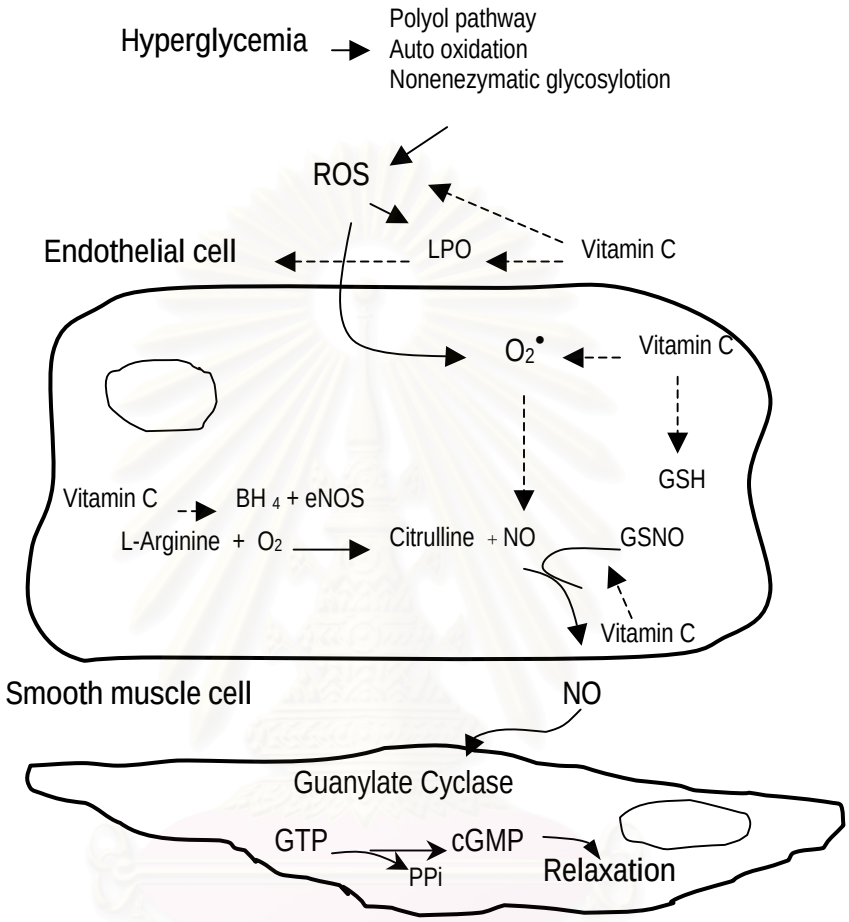


Figure 42 Potential mechanisms by which vitamin C enhances the preserves of endothelial function. Vitamin C scavenges superoxide radicals ($O_2^{\bullet}$) and prevents plasma membrane lipid peroxidation (LPO), which otherwise would decrease NO levels either by direct reaction of $O_2^{\bullet}$ with NO or by interruption of agonist-induced NO synthesis. Which can decrease the synthesis and biological activity of NO. Vitamin C has a number of other functions, as follows: sparing intracellular glutathione (GSH), which in turn can stabilize NO through the formation of biologically active S-nitrosoglutathione (GSNO); release of NO from S-nitrosothiols; and preservation of cofactors of endothelial NOS (eNOS), in particular tetrahydrobiopterin (BH_4). (Modified from Carr AC.,2000)

In the present study, the untreated DM group exhibited a significant elevation of arterial blood pressure. We have suggested that high blood

glucose levels might alter the properties of the endothelium by increased oxidative stress resulted in reducing NO availability. Therefore, it leads to endothelial dysfunction in diabetes. Administration of vitamin C resulted in a significantly amelioration of hypertension. We also demonstrated that vitamin C supplementation decreased HbA_{1C} in our study and we find a decrease of MDA level concomitant with an increased in eNOS protein expression indiabetic rats with supplemented vitamin C. We indicated that the beneficial effects of vitamin C supplementation in the attenuation of those diabetic endothelial dysfunction in this study because of 1) Decreased HbA_{1C}, 2) reduced ROS formation 3) increased eNOS protein expression and 4) maintained antioxidant capacity.

3. Effects of exercise training on diabetic endothelial dysfunction

In present study, the exercise training protocol consisted of running on a motorized treadmill five times per week (with an incremental increase in treadmill velocity and run duration) for 12 and 24 wk periods. The maximum level of exercising activity was 15 m/min, 0^{o}grade for 30 min. Although the training protocol used in this study were relatively lower intensity as compared with the other studies in diabetic model (Hall JL.,1995 , Midaoui A.,1996 , Osborn BA.,1997 , Angelis KLD.,2000). However, this training program is adequate to induce physical adaptations to training as which the decrease in resting heart rate, heart weight and arterial blood pressure were indicated in diabetic rats. This study was supported by the previous study of Gava NS. et al. (1995). They demonstrated that resting bradycardia and attenuation of tachycardia during dynamic exercise are significantly more pronounced after low-intensity than after high intensity exercise training. The mechanisms of resting bradycardia resulting from exercise training have been discussed

elsewhere (Foss ML.,1998 , Wilmore JH.,1999). It most likely involves a decrease sensitivity of cardiac tissue to the cathecholamines and an increase in parasympathetic (vagal) predominance on the cardiac pacemaker rate as a result of a decrease in sympathetic activity caused by exercise training.

Our results showed a significant reduction in heart weight in DM+Ex rats at both 12 and 24 wk of treadmill running compared with DM rats and a tendency increasing of those compared with Con rats. These data supported the study of Paulson DJ. et al. (1987). They found that heart from diabetic rats had significantly elevated heart weight-to body weight ratios but those of trained diabetic rats was not significantly affected relative to controls. Cardiac hypertrophy induced by diabetes is the enlargement of the heart reflecting a pathological state. Evidence from animal and human studies indicates that diabetes can adversely affect myocardial function. At cellular level, defects in calcium transport and fatty-acid metabolism have been identified, and are postulated to lead to hypertrophy of the myocytes and perhaps to myocardial fibrosis (Pickup JC.,1997). The defects in the myocardium of STZ-diabetic rats have been suggested to reduce myocardial contractility by an increase in the activity of the muscarinic acetylcholine receptor signaling system (Fu L-X et al.,1994) and a decrease in the activity of the calcium pump in the sarcoplasmic reticulum of the myocyte (Zarain Herzberg A. et al.,1994). While cardiac muscle undergoes hypertrophy with exercise training, this is recognized as a normal adaptation to chronic endurance training, yet if present, it could significantly increase the ability of the heart to pump blood (Wilmore JH.,1999). With endurance-type training, left ventricular filling would increase. This would be largely due to a training-induced increase in plasma volume that would increase left ventricular end-

diastolic volume. It was postulated that the heart would adapt to this by increasing the internal dimensions of the left ventricle, thus increasing chamber size which change only in the left ventricle caused by endurance training (Wilmore JH.,1999).

Although it is generally advised that exercise may a beneficial adjuvant in the management of diabetes mellitus, experimental evidence supporting this view point appears limited. Nevertheless, various studies have shown that exercise training will diminish basal glucose levels in experimental diabetic animals (Hall JL.,1995 , Osborn BA.,1997 , Angelis KLD.,2000). However, some studies in diabetic exercise training did not observe significant alteration of hyperglycemia (Paulson DI.,1998 , Deblieux PM.,1993). In the present study, which had identical dose of STZ as the latter report, we found no effect of exercise training on plasma glucose and HbA_{1C}. However, this result is not necessarily inconsistent with exercise training reducing the severity of the diabetic state for two reasons: 1) the disparity is unlikely to be related simply to dose of exercise training, including mode, intensity, duration and frequency of training as well as the severity of diabetes and 2) it is possible for exercise training to increase peripheral insulin sensitivity but have no effect on blood glucose concentration (Paulson DJ.,1987).

In the current study, exercise training can significantly prevent the diabetic induced lipid abnormalities as which demonstrated by plasma triglycerol levels (Table 14 and Figure 29). Cholesterol levels tended to be lower but the decrease was not significant. Because high plasma lipid levels have been associated with increased evidence of cardiovascular disease (Kahn CR.,1994), it is conceivable that the exercise-induced reduction in plasma lipid level is beneficial in the management of the diabetic and in preventing the cardiac complications

of diabetes (Defronzo RA.,1998). This results suggested that exercise training decreased the severity of the diabetic state. As exercise continues, fat rather than carbohydrate becomes the predominant fuel that is burned. In the present study, we found a decreased MDA level in liver homogenate of DM rats after 12 and 24 wk exercise training ($p<0.01$). We also showed a decrease MDA level in muscle homogenates of DM rats after 24 wk exercise training significantly. The mechanism for this beneficial effect is not completely understood, however, it appears to involve an decrease of lipid profile, because lipid is the main source of ROS. The decrease of ROS by exercise training could prevented diabetic endothelial dysfunction as described following.

At the venular site: in current study, using intravital microscopy of the rat mesentery venules, we showed **that exercise training reduced leukocyte adhering in diabetic rats** (Table 17). To our knowledge, this is the first report that studied the effects of exercise training on leukocyte-endothelium interaction in the intact mesenteric vessels of diabetic rats. There are some evidences suggests that oxidants, including superoxide and H_2O_2 may contribute to the initiating step of this cascade of leukocyte-endothelial interactions (Patel KD.,1991 , Johnston B.,1996). Consistent with Wood JG. et al. (1999), they reported that the increased leukocyte-endothelial adherence is the result of ROS generation. Therefore, based on our experimental data, we believed that the effect of exercise on decreasing diabetic induced leukocyte-endothelium interaction was actually through its ability to reducing ROS. As we have described previously that the tryglycerial level was significantly decreased in DM+Ex rats. Therefore, MDA-represent ROS level was less than DM rats.

At the arteriolar site: our results showed that exercise training did not improved the impaired vasodilatory response to the endothelium-

dependent vasodilator, Ach, in diabetic rats (Table 18 and 19). Even though there was no significant different in those between DM+Ex and Con rats. In this study, we also demonstrated a tendency decreased in eNOS protein after exercise training at 12- and 24-wk in diabetic rat. We suggested that NO production did not increased after exercise training resulted in failed to improve the impaired vascular response to Ach. In contrast to our study, Tatchum-Talom R. (2000) reported that swim training (60 min twice/day for 3-4 wk) in rats enhances NO generation in the lower limbs and up regulates eNOS activity and protein expression in aorta, lung and atria. This finding consistent to Kojda G. et al.,2001 , they found that aorta eNOS protein level in mice was induced by exercise training (treadmill running for 30 minute at 15 m/min).

The reasons whereby our exercise training did not able to protect the decrease in eNOS expression in STZ rats have not been fully defined. There are several factors that may account for the different results obtained from the various studies. The strain and/or sex of the animals studied may have been a factor. Moreover, the type and intensity (speed, % inclination and duration) of the training programs employed were also different among studied. Furthermore, we studied the effect of exercise training in the model which different from other investigators that studied in normal, hypertensive and heart failure animals. Probably there are some factor resulted from hyperglycemia in diabetic model affect the beneficial of exercise training to enhance eNOS expression. Indeed, inhibition of enzyme SOD in vascular tissue in vitro impairs relaxation to the endothelium-dependent vasodilator Ach, suggesting that the biological activity of NO depends on SOD activity (Rush JWE.,2000). Therefore, less SOD activity is one factor for supporting our result that eNOS was found to be decreased after exercise training.

In the present study, the elevated blood pressure (EC) in diabetic rats were significantly prevented by exercise training. This finding is consistent with previous studies of long term exercise both in animals (Chu TF. et al.,2000) and human (Higashi U.,1999). Such those findings have demonstrated the efficacy of exercise regimen in the ameliorated hypertension. One could raise the possibility that the reduced blood pressure caused by exercise improved endothelial dysfunction. Aerobic exercise training has been shown to alter the blood flow distribution among various tissues during acute bouts of exercise. The redistribution of blood flow in trained animals and humans indicates that vascular regulation has also been altered as a result of aerobic training (Lash JM.,1997). The stimulus produced by exercise that induces adaptation of the endothelium could be physical (e.g., increased blood flow) and/or chemical (e.g., metabolites, catecholamines, and other vasoactive substances) (Delp MD.,1995). Increases in blood flow and the corresponding increases in wall shear stress in blood vessels have been shown to be potent stimuli for adaptation of the endothelium.

However, in our study, the endothelial dysfunction resulted from diabetic ROS were still occurred in DM+Ex group as demonstrated by Ach response. Even though exercise training could decrease ROS through its triglyceride reduction, but it was not enough to keep balance between oxidative stress that was continuous generated through hyperglycemia loaded intra-cellularly. For the result of blood pressure (BP), we believe that exercise training have just delay the abnormality. It is not like local endothelial vasodilation response, BP is normally regulated by multiple factors and it has compensatory system involving as well. Therefore, in 24 wk period, we have not seen hypertension induced by diabetic state in DM+Ex yet.

4. The proposed mechanisms for the combined effects of vitamin C supplementation and exercise training on diabetic endothelial dysfunction.

As the overall results of the study, we would like to propose the possible mechanisms of vitamin C supplementation and exercise training as shown in Figure 43 and 44. Based on intravital microscopic studies on mesenteric microvasculature in diabetic rats, we demonstrated that STZ-induced diabetes rats is associated with endothelial dysfunction including increased leukocyte adhesion and impaired endothelium-dependent relaxation. The consequence of elevated blood pressure followed the endothelial dysfunction was demonstrated in the present study. From the result, there is substantial evidence to support the idea that hyperglycemia-induced endothelial dysfunction is mediated by ROS produced through glycation of proteins and hyperlipidaemia. Moreover, it was found that diabetic rats has impaired activity of the enzymatic antioxidant, SOD and importantly with the decrease in plasma vitamin C as well. Therefore, the imbalance of oxidant and antioxidant system may be involved in mediating the vascular endothelial dysfunction, especially by quenching NO production. This line of evidence has been widely documented clearly suggests that ROS production could be a common mechanism accounting for impaired endothelial function in diabetes.

Interestingly, vitamin C supplementation can significantly prevent the endothelial dysfunction in STZ diabetic rats. The abnormal leukocyte endothelium interaction and the impaired endothelium-dependent vasodilation were markedly protected by vitamin C. We suggested that the prevention of diabetic induced endothelial dysfunction in this study is probably, mediated by the supplementation of exogenous vitamin C to

scavenge excess ROS and, thereby, decrease nitric oxide inactivation as demonstrate in Figure 43.

The beneficial effects of exercise training on diabetic rats were also shown in Figure 43. However, we demonstrated that the effects of exercise training on endothelial dysfunction differed between arterioles and venules in diabetic mesenteric microcirculation. We found that the decrease of ROS by exercise training could be beneficial factor to attenuate leukocyte adherences in diabetes. However, exercise training fail to prevent the impairment of endothelium-dependent vascular relaxation in diabetes. Therefore, we suggested that exercise alone is not sufficient to prevent endothelial dysfunction in diabetes.

From the beneficial effect of vitamin C and exercise training as discussed above, **therefore in DM+Vit.C+Ex had shown the most efficacy for preventing the diabetic cardiovascular complications**. It is concluded from these results that vitamin C supplementation may be considered to be an effective antioxidant in the prevention of hyperglycemia induced oxidative stress when apply to diabetic rats. However, it does not improve the physical fitness includings heart weight, heart rate and triglyceride levels. It implied that exercise training alone has beneficial effects focus on cardiovascular fitness but less effect than vitamin C, especially for arteriolar protection. **Interestingly, treatment with vitamin C supplementation combined with exercise training have more benefit to both physical characteristics and endothelial function in diabetic rats** (Figure 44). We found the combination roles of vitamin C supplementation and exercise training in HbA_{1C}, plasma triglyceride, SOD activity, eNOS protein as well as vascular reactivity to Ach. Since exercise alone was without effects on HbA_{1C}, SOD and eNOS protein production and vasodilatation and vitamin C alone failed to

decreased triglyceride. These findings support our hypothesis that vitamin C supplementation combined with regular exercise training can significantly greater protection from the diabetic endothelial dysfunction by collaboration in ameliorating risk factors of these abnormalities phenomenon.

In conclusion, our observations indicated that the endothelial dysfunction of diabetic rats is associated with increased leukocyte adhesion impaired endothelium-dependent relaxation. However, vitamin C supplementation and regular exercise training could prevent these deleterious effects by ameliorating the diabetic-induced imbalance of oxidants/antioxidants. Our finding suggest that vitamin C supplementation combined with regular exercise training might be an effective therapeutic modality in preventing diabetic cardiovascular complications which involve crucially endothelial dysfunction in diabetes.

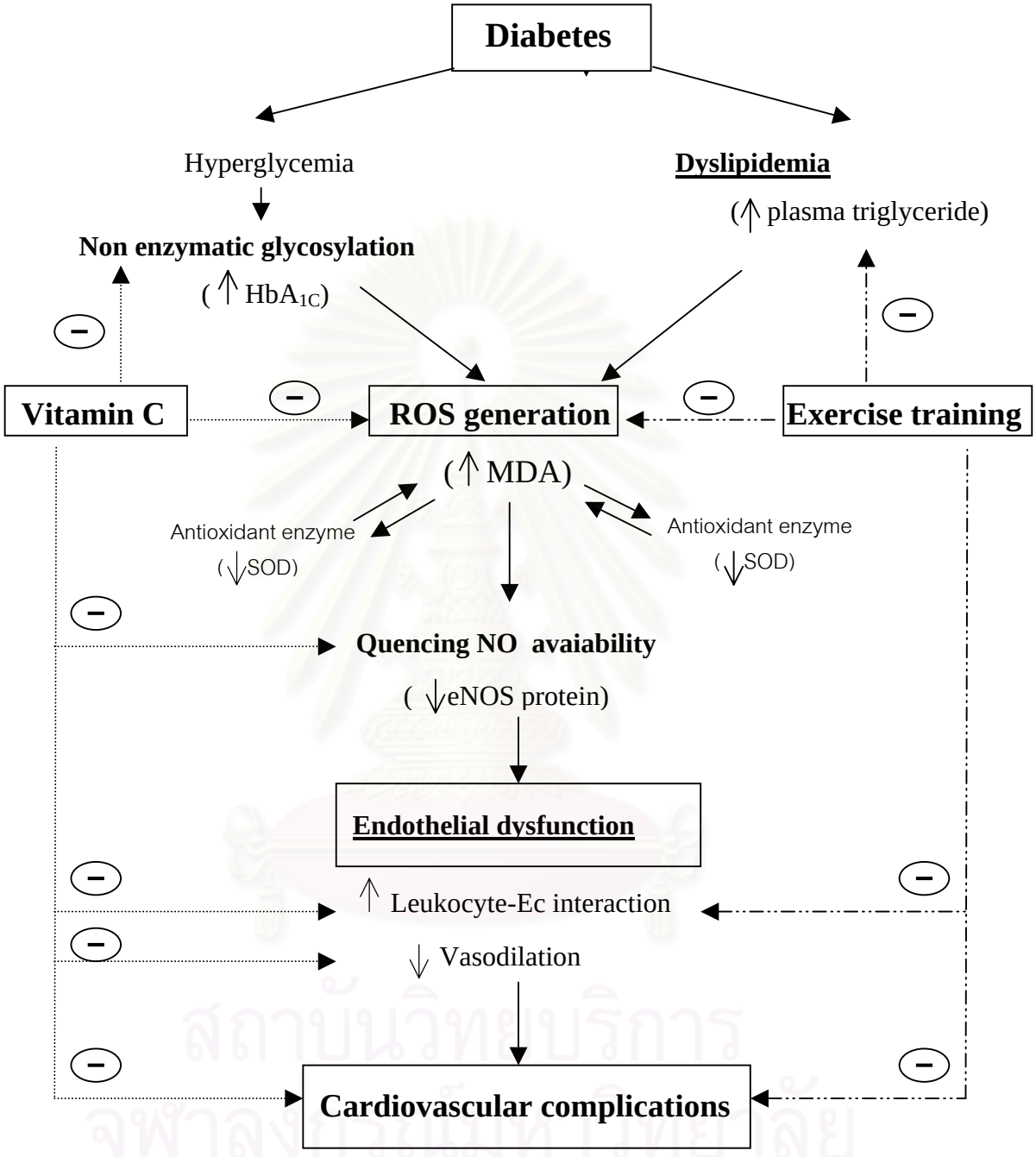


Figure 43 Protective effects of vitamin C supplementation (·····) and exercise training (–··–··) on diabetic induced endothelial dysfunction.

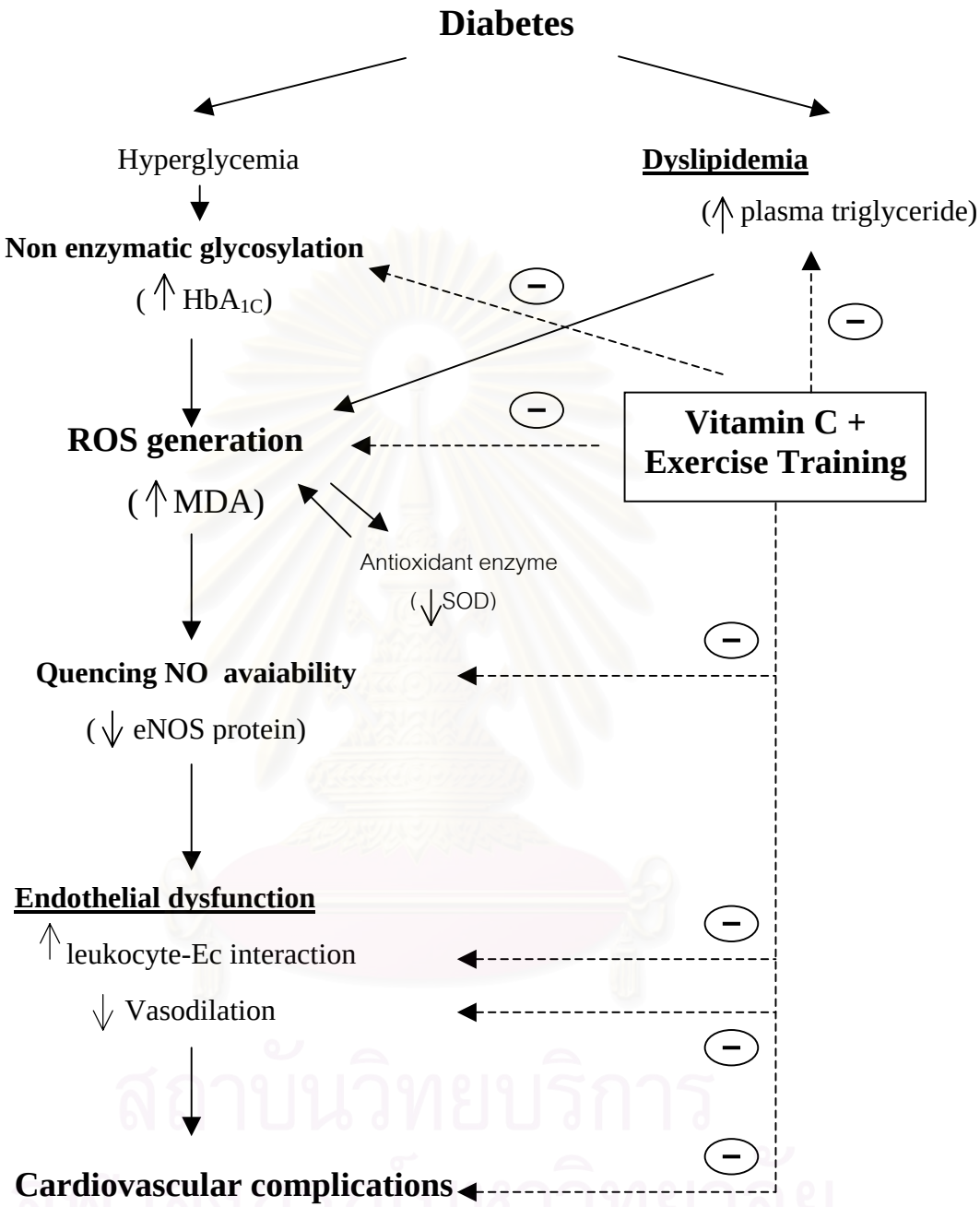


Figure 44 Purposed mechanisms for combined effects of vitamin C supplementation and exercise training (-----) on cardiovascular complications.

CHAPTER VI

CONCLUSION

In the present study, using the intravital fluorescent microscopic techniques, the effects of vitamin C supplementation and regular exercise training on diabetic-induced endothelial dysfunction were studied. Physiologic characteristics including blood pressure, heart rate, HbA_{1C}, lipid profiles, oxidant MDA levels, antioxidant SOD levels, and eNOS protein levels were also determined. The experimental data were determined for each group : Con, DM, DM+Vit.C, DM+Ex and DM+Vit.C+Ex, for both periods of 12 and 24 weeks.

From the present results, the conclusions are as follows;

1. The injection of STZ 50 mg/kg BW into Spraque Dawley rats resulted in polydipsia, polyuria, polyphagia and persistent hyperglycemia throughout the experiment. The elevated blood glucose and HbA_{1C} were demonstrated in all groups of diabetic rats. However, it was found that the reduction of HbA_{1C} levels were indicated in 24-wk exercise trained groups both with and without Vitamin C supplementation, DM+Ex and DM+Vit.C+Ex.

2. The levels of plasma vitamin C in diabetic rats was decreased when compared with control rats. Further, there was no significant difference between Con and DM+Vit.C at either 12 or 24 weeks.

3. The plasma triglyceride levels in diabetic rats were significantly higher than the other rats at both monitored time points. However, exercise improved these lipid abnormalities in this animal model.

4. The MDA levels were significantly increased in diabetic rats when compared with the other groups indicated that diabetic state had

markedly generated reactive oxygen species (ROS); this rise was prevented by both vitamin C supplementation and regular exercise training.

5. It was found that the diabetic rats had significantly lower in SOD activity of both liver and muscle homogenates indicating reducion of endogenous antioxidant in long term diabetes. However, there were no decreases in SOD activity in DM+Vit.C and DM+Vit.C+Ex groups indicating that the decreased antioxidant enzyme in diabetic rats was improved by vitamin C supplementation.

6. At 12 weeks, eNOS protein levels were found to be decreased significantly in diabetic rats. However, there were significantly higher eNOS protein levels of both diabetic rats receiving vitamin C supplementation with or without exercise (DM+Vit.C and DM+Vit.C+Ex) when compared with diabetic rats.

7. In the intravital fluorescent microscopic study, endothelial dysfunction was observed in both 12 and 24 wk of diabetes as demonstrated by the increased number of leukocyte adhering in mesenteric venules and the impaired responses to the dependent vasodilation in mesenteric arterioles (d=15-45 μm). In mesenteric venules, at both 12 and 24 wk, diabetic rats had significantly higher in the number of adhering leukocytes to endothelium of postcapillary venules relative to control rats. We found that all treatment groups in this study (DM+Vit.C, DM+Ex and DM+Vit.C+Ex) reduced this abnormalities of endothelial function. In mesenteric arterioles, the ability to dilate via endothelial-mediated mechanisms was improved in the diabetic animals by supplementation of vitamin C, independent of exercise (in both DM+Vit.C and DM+Vit.C+Ex groups). Interestingly, treatment with

exercise training alone was without beneficial effect on the impairment of endothelial dependent vasodilation in diabetic rats.

8. Both vitamin C supplementation and exercise training have beneficial, but distinctly different effects on diabetic condition. Vitamin C supplementation has beneficial effects on HbA_{1C}, arterial blood pressure, reducing leukocyte adherence, preventing the impairment of relaxation, preventing the generation of ROS as well as maintaining the antioxidant enzyme SOD and eNOS protein levels in diabetes. The beneficial influence of exercise training, by contrast, focuses on general physiological characteristics including heart weight, heart rate and triglyceride levels. Exercise training has also prevented the elevated arterial blood pressure as well as reduced the leukocyte-endothelial interaction and the generation of ROS as well.

9. Consistent to our initial hypothesis the present study demonstrated a protective role of vitamin C supplementation plus exercise training on diabetic condition. The combination of the separate effects of these two treatments though suggests that the combination of these treatments are likely to constitute an effective and inexpensive therapeutic modality that should lesson, if not prevent, the cardiovascular complications of diabetes.

10. These finding indicated that antioxidant vitamin C improves endothelial function in diabetic rats, probably by directly scavenging hyperglycemia-induced ROS production, and strongly suggested that ROS production is a likely candidate to account for endothelial dysfunction by quenching NO availability in diabetes.

REFERENCES

Adams, MR., Jessup, W. and Celermajer, DS. Cigarette smoking is associated with increased human monocyte adhesion to endothelial cell; reversibility with oral L-arginine but not vitamin C. J. Am. Coll. Cardiol. 29 (1997): 491-497.

Ahmed, I., Lakhani, MS., Gillett, M., John, A. and Raza, H. Hypotriglyceridemic and hypocholesterolemic effects of anti-diabetic Momordica charantia (karela) fruit extract in streptozotocin-induced diabetic rats. Diabetes Res Clin Pract. 51(2001): 155-61.

Alessio, HM. Exercise-induced oxidative stress. Med Sci Sports Exerc. 25 (1993): 218-24.

Alessio, HM., Goldfarb, AH., and Cao, G. Exercise-induced oxidative stress before and after vitamin C supplementation. Int. J. Sport Nutr. 7 (1997): 1-9.

American College of Sports Medicine Position Stand. The recommended quantity and quality of exercise for developing and maintaining cardiorespiratory and muscular fitness, and flexibility in healthy adults. Med Sci Sports Exerc Jun 30 (1998): 975-91.

Angelis, KLD., Gadonski, G., Fang, J., Dall'Ago, P., Albuquerque, VL. et al. Exercise reverses peripheral insulin resistance in trained L-NAME-hypertensive rats. Hypertension 34 (1999): 768-772.

Angelis, KLD., Oliveira, AR., Dall'Ago, P., Peixoto, LRA., Gadonski, G. et al. Effects of exercise training on autonomic and myocardial dysfunction in streptozotocin-diabetic rats. Braz. J. Med. Biol. Res. 33 (2000): 365-641.

Angelis, KLD., Oliveira, AR., Werner, A., Bock, P., Bello-Klein, A. et al. Exercise training in aging hemodynamic, metabolic, and oxidative stress evaluations. Hypertension 30 (1997): 767-771.

Angulo, J., Rodriguez-Manas, L., Peiro, C., Neira, M., Marin, J. et al. Impairment of nitric oxide-mediated relaxations in anaesthetized autoperfused streptozotocin-induced diabetic rats. Naunyn Schmiedebergs Arch Pharmacol. 356 (1998): 529-537.

Arouma, OI. Review: Free radicals and antioxidant strategies in sports. J. Nutr Biochem. 5 (1994): 370-81.

Ashton, T., Young, IS., Peters, JR., Eleri, J., Jackson, SK. et al. Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. J. Appl. Physiol. 87 (1999): 2032-2036.

Asmus, KD. and Bonifacic, M. Free radical chemistry. In: Sen CK, Packer L, Hanninen O, editors. Exercise and oxygen toxicity. Ansterdam: Elservier Science p. 1-47, 1994.

Avila, KAL., Gadonski, G., Fang, J., Ago, PD., Albuquerque, VL. et al. Exercise reverses peripheral insulin resistance in trained L-NAME-hypertensive rats. Hypertension 34 (1999): 768-772.

Aydin, A., Orhan, H., Sayal, A., Ozata, M., Sahin, G. et al. Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effects of glycemic control. Clin Biochem. 34 (2001): 65-70.

Balon, TW. and Nadler, JL. Evidence that nitric oxide increases glucose transport in skeletal muscle. J. Appl. Physiol. 82 (1997): 359-63.

Barnett, AH. Pathogenesis of Diabetic Microangiopathy. Am. J. Med. 90 (June 1991): 67S-73S.

Baynes, JW. Role of oxidative stress in development of complications in diabetes. Diabetes 40 (Apr 1991): 405-12.

Beckman, JA., Goldfine, AB., Gordon, MB. and Creager, MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. Circulation 103 (2001): 1618-1623.

Bedford, TG., Tipton, CM., Wilson, NC., Oppliger, RA. and Gisolfi, CV. Maximum oxygen consumption of rats and its changes with various experimental precedures. J. Appl. Physiol. 47 (1979): 1278-1283.

Bonnardel-Phu, E. and Vicaut, E. Reactive oxygen species and acute modulation of albumin microvascular leakage in the microcirculation of diabetic rats in vivo. J. Vasc. Res. 37 (2000): 32-8.

Booth, G., Stalker, TJ., Lefer, AM. and Scalia, R. Elevated ambient glucose induces acute inflammatory events in the microvasculature: effects of insulin. Am. J. Physiol Endocrinol Metab. 280 (2001): E848-E856.

Bray, RC. and Cockle, SA. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. Biochem J. 139 (1974): 43-8.

Brody, T. Nutritional Biochemistry. 1st ed. Academic Press: California, 1994.

Brune, B., Knethen, AV. and Sandau, KB. Nitric oxide and its role in apoptosis. Eur. J. Pharmacol. 351 (1998): 261-272.

Busse, R. and Fleming, I. Endothelial dysfunction in atherosclerosis. J. Vase. Res. 33 (1996): 181-4.

Carlos, TM. and Hatlen, JM. Leukocyte-endothelial adhesion molecules. Blood 84 (1994): 2068-2101.

Carr, AC., Zhu, BZ. and Frei, B. Potential antiatherogenic mechanisms of ascorbate (vitamin C) and α-tocopherol (vitamin E). Circ. Res. 87 (2000): 349-354.

Chan, NN., Vallance, P. and Colhoun, HM. Nitric oxide and vascular responses in type I diabetes. Diabetologia 43 (2000): 137-147.

Child, RB, Wilkinson, DM, Fallowfield, JL and Donnelly, AE. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. Med Sci Sport Exerc. 30 (1998): 1603-1607.

Chu, TF., Huang, TY., Jen, CJ. and Chen, HI. Effects of chronic exercise on calcium signaling in rat vascular endothelium. Am. J. Phsiol Heart Circ Physiol. 279 (2000): H1441-H1446.

Clark, IA. Tissue damage caused by free oxygen radicals. Pathology 18 (1986): 181-186.

Cominacini, L., Garbin, U. and Cascio, LV. The need for a “free radical initiative”. Diabetologia 39 (1996): 364-366.

Consentino, F., Hishikawa, K., Katusic, ZS. and Luscher, TF. High glucose increase nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 96 (1997): 25-8.

Cotran, RS. Robbins Pathologic basis of disease 6th ed. W.B. Saunders Company: Philadelphia., 1999.

Crijns, FR., Wolffenbuttel, BH., Mey, JG. and Boudier, HA. Mechanical properties of mesenteric arteries in diabetic rats: consequences of outward remodelin. Am. J. Physiol. 276 (1999): H1672-H1677.

Cruz, JWMC., Oliveira, MA., Hohman, TC. and Fortes, ZB. Infuence of tolrestat on the defective leukocyte-endothelial interaction in experimental diabetes. European Journal of Pharmacology 391 (2000): 163-174.

Dai, S. and McNeill, JH. Ascorbic acid supplementation prevents hyperlipidemia and improves myocardial performance in streptozotocin-diabetic rats. Diabetes Res. Clin. Pract. 10 (1995): 91-97.

Damiano, ER., Westheider, J., Tozeren, A. and Ley, K. Variation in the velocity, deformation, and adhesion energy density of leukocytes rolling within venules. Circ. Res. 79 (1996): 1122-1130.

Davison, GW., George, L., Jackson, SK., Young, IS., Davies, B. et al. Exercise, free radicals, and lipid peroxidation in type I diabetes mellitus. Free Radical Biology & Medicine. 33;11 (2002): 1543-1551.

DeBlieux, PMC., Barree, RW. Mcdonough, KH. and Shepherd, RE. Exercise training improves cardiac performance in diabetic rats. Proc Soc Exp Biol Med. 203 (June 1993): 209-13.

Defronzo, RA. Classification and diagnosis of diabetes mellitus 1st edition. Mosby: St.Louis, 1998.

Delp, MD. Effects of exercise training on endothelium-dependent peripheral endothelium-dependent peripheral vascular responsiveness. Med. Sci. Sports Exerc. 27 (1995): 1152-1157.

Delp, MD. and Laughlin, MH. Time course of enhanced endothelium-mediated dilation in aorta of trained rats. Med. Sci. Sports Exerc. 29 (1995): 1454-61.

Duffy, SJ., Gokce, N., Holbrook, M., Huang, A., Frei, B. et al. Treatment of hypertension with ascorbic acid. Lancet 354 (1999): 2048-49.

Durstine, JL., Grandjean, PW., Davis, PG., Ferguson, MA., Alderson, NL. et al. Blood lipid and lipoprotein adaptations to exercise: a quantitative analysis. Sports Med. 31 (2001): 1033-62.

El-Missiry, MA. Enhanced testicular antioxidant system by ascorbic acid in alloxan diabetic rats. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 124 (Nov 1999): 233-237.

Enevoldsen, LH., Stallknecht, B., Fluckey, JD. and Galbo, H. Effects of exercise training on in vivo insulin stimulated glucose uptake in

intra-abdominal adipose tissue in rats. Am. J. Physiol. 278 (2000): E25-E34.

Fiebig, RG., Hollander, JM. and Ji, LL. Exercise down-regulates hepatic fatty acid synthase in streptozotocin-treated rats. J. Nutr. 131 (2001): 2252-2259.

Ford, I., Cotter, MA., Cameron, NE. and Greaves, M. The effects of treatment with alpha-lipoic acid or evening primrose oil on vascular hemostatic and lipid risk factors, blood flow, and peripheral nerve conduction in the streptozotocin-diabetic rats. Metabolism 50 (2001): 868-75.

Foss, ML. and Keteyian, SJ. Fox's Physiological basis for exercise and sport 6th ed. United State of America: The McGraw-Hill; 1998.

Fox, LE., Bowers, RW. and Foss, ML. The physiological basis for exercise and sport 5th ed. Madison (WI): Brawn & Benchmark; 1993.

Freeman, BA. and Crapo, JD. Biology of disease: Free radicals and tissue injury. Lab Invest. 47 (1983): 412-26.

Fu, L-x., Bergh, C-H. and Liang, O-M. Diabetes-induced changes in the Gi-modulated muscarinic receptor-adenylyl cyclase system in rat myocardium. Pharmacol Toxicol. 75 (1994): 186-93.

Fumiko, N., Masatoshi, S., Kazuyoshi, T., Hideo, Y. and Ko, O. Mobilization of NK cells by exercise: downmodulation of adhesion molecules on NK cells by catecholamines. Am. J. Physiol Regulatory Integraive Comp. Physiol. 279 (2000): R1251-R1256.

Garvey, WT., Hardin, D., Juhaszora, M. and Dominguez, JH. Effects of diabetes on myocardial glucose transport system in rats : implications for diabetic cardiomyopathy. Am. J. Physiol. 264 (1993): H837-H844.

Gava, NS., Salvador, AV., Negrao, CE. and Krieger, EM. Low-intensity exercise training attenuates cardiac β–adrenergic tone during exercise in spontaneously hypertensive rats. Hypertension 26 (1995): 1129-1133.

Ghosh, SK., Ekpo, EB., Shah, IU., Girling, AJ., Jerkins, C. et al. A double-blind placebo-controlled parallel trial of vitamin C treatment in elderly patients with hypertension. Gerontology 40 (1994): 268-272.

Gibbons, GH. and Dzau, VJ. Molecular therapies for vascular diseases. Science 272 (1996): 689-93.

Giuglano, D. and Ceriello, A. Oxidative stress and diabetic vascular complications. Diabetes care 19 (1996): 257-267.

Goldfarb, AH., McIntosh, MK., Boyer, BT. and Fatouros, J. Vitamin E effects on indexes of lipid peroxidation in muscle from DHEA-treated and exercised rats. J. Appl. Physiol. 76 (1994): 1630-1635.

Govers, R. and Rabelink, TJ. Cellular regulation of endothelial nitric oxide synthase. Am. J. Physiol Renal Physiol. 280 (2001): F193-F206.

Gracia-Cardena, G., Oh, P., Liu, J., Schnitzer, JE. and Sessa, WC. Targeting of nitric oxide synthose to endothelial cell careolae via palmitoylation: inplications for nitric oxide signaling. Proc Natl Acad Sci USA. 93 (1996): 6448-53.

Graier, WF., Simecek, S., Kukovetz, WR. and Kostner, GM. High D-glucose increase changes in endothelial Ca^{2+} /EDRF signaling are due to generation of superoxide anions. Diabetes 45 (1996): 1386-95.

Gregory, B. The role of oxygen free radicals in human disease processes. Surgery 94;3 (1983): 407-11.

Gutteridge, JM. and Halliwell, B. The measurement and mechanism of lipid peroxidation in biological systems. TIBS. 15 (1990): 129-135.

Haendeler, J., Zeiher, AM. and Dimmeler, S. Nitric oxide and Apoptosis. Vitamins and Hormones 57 (1999): 49-77.

Hall, JL., Sexton, WL. and Stanley, WC. Exercise training attenuates the reduction in myocardial GLUT-4 in diabetic rats. J. Appl. Physiol. 78 (1995): 76-81.

Halliwell, B. and Chirico, S. Lipid peroxidation: its mechanism, measurement and significance. Am. J. Clin. Nutr. 57 (1993): 715S-725S.

Halliwell, B. and Gutteridge, JM. Antioxiant defenses. In: Free radicals in biology and medicine. 3rd ed. New York: Oxford U university Press; 139-45, 1999.

Halliwell, B. and Gutteridge, JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J. 219 (1984): 1-14.

Hames, BD., Hooper, NM. and Houghton, JD. Instant Notes in Biochemistry Bios Scientific Publishers, pp.14-16, 38-46, 1997.

Heitzer, T., Schlinzig, T., Krohn, K., Meinertz, T. and Munzel, T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 104 (2001): 2673-2678.

Heller, R., Munscher-Paulig, F., Grabner, R. and Till, U. L-Ascorbic acid potentiates nitric oxide synthesis in endothelial cells. J. Biol. Chem. 274;12 (March 1999): 8254-8260.

Heyward, VH. Evaluation of body composition. Sports Med. 22 (1996): 146-56.

Higashi, Y., Sasaki, S. and Hurisu, S. Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as

well as hypertensive subjects: role of the endothelium-derived nitric oxide. Circulation 100 (1999): 1194-1202.

Hink, UI., Li, H., Mollnau, H., Oelze, M., Skatchkov, M. et al. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circ. Res. 28 (2001): e14-38.

Hishikawa, K. and Luscher, TF. Pulsatile stretch stimulates superoxide production in human aortic endothelial cells. Circulation 96 (1997): 3610-3616.

Hornig B., Arakawa N., Kohler C., Drexler H. Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. Circulation 97 (1998): 363-368.

Howard, AB., Alexander, RW., Nerem, RM., Griendling, RR. and Taylor, WR. Cyclic strain induces an oxidative stress in endothelial cells. Am. J. Physiol. Cell Physiol 272 (1997): C421-C427.

Huang, A., Vita, JA., Venema, RC. and Keaner, JF. Fscorbic acid enhances endothelial nitric oxide synthase activity by increasing intracellular tetahydrobiopterin. J. Biol. Chem. 275 (2000): 17399-406.

Huang, WC., Juang, SW., Liu, IM., Chi, TC. and Cheng, JT. Changes of superoxide dismutase gene expression and activity in the brain of streptozotocin-induced diabetic rats. Neurosci Lett. 275 (1999): 25-8.

Huvers, FC., Leevw, PW., Houben, A., Haan, C., Hamulyak, K. et al. Endothelium-dependent vasodilatation, plasma markers of endothelial function, and adrenergic vasoconstriction responses in type 1 diabetes under near-normoglycemic conditions. Diabetes 47 (1999): 1300-7.

Idzior-Walus, B., Mattock, MB., Solnica, L. and Fuller, JH. Factors associated with plasma lipids and lipoproteins in type 1 diabetes

mellitus: the EURODIAB IDDM Complications Study. Diabetes Med. 18 (2001): 786-96.

Inoue, N., Ramasamy, S., Fukai, T., Nerem, RM. and Harrison, DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. Circ. Res. 79 (1996): 32-37.

Inukai, T., Takanashi, K., Tayama, K., Aso, Y. and Takemura, Y. High glucose concentrations abolish the superoxide dismutase response of leukocytes to ascorbic acid or troglitazone in type 2 diabetes mellitus. Life Sci. 70 (Apr 2002): 2391-2401.

Iwashima, Y., Sato, T. and Watanbe, K. Elevation of plasma thrombomodulin level in diabetic patients with early diabetic nephropathy. Diabetes 39 (1990): 983-988.

Jariyapongskul, A. Antioxidant effect of vitamin C on endothelial function in streptozotocin-induced diabetic rats Doctoral dissertation Interdepartment of physiology, Graduate school, Chulalongkorn University, 2000.

Jariyapongskul, A., Patumraj, S., Yamaguchi, S. and Niimi, H. The effect of long-term supplementation of vitamin C on leukocyte adhesion to the cerebral endothelium in STZ-induced diabetic rats. Clin Hemorheol Microcirc. 27 (2002): 67-76.

Jen, CJ., Chan, HP. and Chen, HI. Acute exercise enhances vasorelaxation by modulating endothelial calcium signaling in rat aortas. Am. J. Physiol. Heart Circ Physiol. 282 (2001): H977-H982.

Jenkins, RR. and Goldfarb, A. Introduction: oxidant stress, aging and exercise. Med Sci Sports Exerc. 25(1993): 210-2.

Jenkins, RR., Krause, K. and Schofield, LS. Influence of exercise on clearance of oxidant stress products and loosely bound iron. Med Sci Sports Exerc. 25(1993): 213-7.

Ji, LL. Exercise, oxidative stress, and antioxidants. Am. J. Sports Med 24 (1996): S20-S24.

Johnston, B., Kanwar, S. and Kubes, P. Hydrogen peroxide induces leukocyte rolling: modulation by endogenous antioxidant mechanisms including NO. Am. J. Physiol. Heart Circ. Physiol. 271 (1996): H614-H621.

Kahn, CR. and Weir, Gc. Joslin's Diabetes Mellitus 13th ed. Lea & Febiger:Pennsylvania, 1994.

Kakkar, R., Mantha, SV., Radhi, J., Prasad, K. and Kalra, J. Antioxidant defense system in diabetic kidney: a time course study. Life Sci. 60 (1997): 667-79.

Kapur, S., Bedard, S., Marcotte, B., Cote, CH. and Marette, A. Expression of nitric oxide synthase in skeletal muscle: a novel role for nitric oxide as a modulator of insulin action. Diabetes 46 (Nov 1997): 1691-700.

Kario, K., Matsuo, T., Kobayashi, H., Matsuo, M., Sakata, T. et al. Activation of tissue factor-induced coagulation and endothelial cell dysfunction in non-insulin-de-pendent diabetic patients with microalbuminuria. Arterioscler Thromb Vasc Biol. 15 (1995): 111-1120.

Kashiba, M., Oka, J. and Ichikawa, R. Impaired ascorbic acid metabolism in streptozotocin-induced diabetic rats. Free radical biology & medicine. 33;9 (2002): 1221-1230.

Kashiba, M., Oka, J., Ichikawa, R., Kageyama, A., Inayama, T. et al. Impaired reductive regeneration of ascorbic acid in the Goto-Kakizaki diabetic rat. Biochem. J. 351 (2000): 313-318.

Kashiwagi, A., Asahina, T., Nishio, Y., Ikebuchi, M., Tanaka, Y. et al. Glycation, oxidative stress, and scavenger activity: glucose

metabolism and radical scavenger dysfunction in endothelial cells. Diabetes 45 (Jul 1996): S84-6.

Keegan, A., Walbank, H., Cotter, MA. and Cameron, NE. Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta. Diabetologia 38 (1995): 1475-8.

Keen, H. Complications of Diabetes. 2nd ed. Edward Arnold, 1999.

Kim, MK., Sasaki, S., Okubu, S., Hayashi, M. and Tsugane, S. Lack of long term effect of vitamin C supplementation on blood pressure. Hypertension 40 (2002): 797-803.

Knobl, P., Schernthaner, G. and Schnack, C. Thrombogenic factors are related to urinary albumin excretion rate in Type I (insulin-dependent) and Type II (non-insulin-dependent) diabetic patients. Diabetologia 36 (1993): 1045-1050.

Kojda, G., Cheng, YC., Burchfield, J. and Harrison, DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. Circulation 103;12 (June 2001): 2839-44.

Kubes, P. and Kerfoot, SM. Leukocyte recruitment in the microcirculation: the rolling paradigm revisited. News Physiol. Sci. 16 (April 2001).

Kubes, P., Suzoki, M. and Granger, DN. Nitric oxide: an endogenous modulation of leukocyte adhesion. Proc. Natl. Acad. Sci. USA 88 (1991): 4651-4655.

Kuo, L., Davis, MJ. and Chilian, WM. Longitudinal gradients for endothelium-dependent and –independent vascular responses in the coronary microcirculation. Circulation 92 (1995): 518-525.

Laaksonen, DE., Atalay, M., Niskanen, L., Uusitupa, M., Hanninen O. et al. Increased resting and exercise-induced oxidative stress in young IDDM men. Diabetes Care 19;6 (1996): 569-574.

Lailerd, N., Pongchaidecha, A., Boonnayathap, U., Chaiwan, S. and Mattayabun, S. Effects of exercise and diabetic condition of gastrointestinal transit and glucose homeostasis in rats. Thai J. Physiol. Sci. 9 (1996): 45-62.

Lamb, DR., Editor. Physiology of exercise response and adaptations. 2nd ed. New York: Macmillan; 1984.

Lash, JM. Exercise training enhances adrenergic constriction and dilation in the rat spinotrapezius muscle. J. Appl. Physiol. 85 (1998): 168-174.

Lash, JM. and Bohlen, HG. Time- and order-dependent changes in functional and NO-mediated dilation during exercise training. J. Appl. Physiol. 82 (1997): 460-468.

Laughlin, MH. and Muller, JM. Vasoconstrictor responses of coronary resistance arteries in exercise-trained pigs. J. Appl. Physiol. 84 (1998): 884-889.

Lee, AY. and Chung, SS. Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J. 13 (1999): 23-30.

Lee, DM. Malondialdehyde formation in stored plasma. Biochem Biophy Res Com. 95;4 (1980): 1663-72.

Lefer, DJ., Janes, SP. and Girod, WG. Leukocyte-endothelial cell interactions in nitric oxide snthase-deficient mice. Am. J. Physiol. 276 (1999): H1943-50.

Lehninger, AL. Oxidative phosphorylation and photophosphorylation. In: Lehninger AL, Nelson DL, Cox MM, editors. Principles of biochemistry 2nd ed. New York: Worth; 1993. p. 542-70.

Lehr, HA., Weyrich, AS., Saetzler, RK., Jurek, A., Arfors, KE. et al. Vitamin C blocks inflammatory platelet-activating factor minetics created by cigarette smoking. J. Clin. Invest. 99 (1997): 2358-2364.

Lekakis, JP., Anastasiou, EA., Papamichael, CM., Stamatelopoulos, KS., Dagre, AG. et al. Short-term oral ascorbic acid improves endothelium-dependent vasodilatation in woman with a history of gestational diabetes mellitus. Diabates care 23 (2000): 1432-1433.

Ley, K. and Gaehtgens, P. Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. Cir. Res. 69 (1991): 1034-1041.

Lindsay, RM., Jamieson, NSD., Waller, SA. and McGuigan, CC. Tissue ascorbic acid and polyol pathway metabolism in experimental diabetes. Diabetologia 41 (1998): 516-523.

Liu, J., Yeo, Hc., Overvik-Douki, E., Hagen, T., Doniger, SJ., et. al. Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. J. Appl. Physiol.. 89 (2000): 21-28.

Lowry, OH., Rosebrough, NJ., Farr, AL. and Randall, J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193 (1951): 265-75.

Lund, DD., Faraci, FM. Miller, FJ. and Heistad, DD. Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. Circulation 101 (2000): 1027-1033.

Lutty, GA., Cao, J. and McLeod, DS. Relationship of polymorphonuclear leukocytes to capillary dropout in the human diabetic choroid. Am J. Pathol. 151 (1997): 707-714.

Makino, A. and Kamta, K. Possible modulation by endothelin–1, nitric oxide, prostaglandin I_2 and thromboxane A_2 of vasoconstriction

induced by an 2-agonist in mesenteric arterial bed from diabetic rats. Diabetologia 41 (1998): 1410-1418.

Manore, M. and Thompson, J. Sport nutrition for health and performance. Human kinetic : USA, 2000.

Margaritis, I., Tessier, F., Richard, MJ. and Marconnet, P. No evidence of oxidative stress after a triathlon race in highly trained competitors. Int J. Sport Med. 18 (1997): 186-190.

Marzatico, F., Pansarasa, O., Bertorelli, L., Somenzini, L. and Della, VG. Blood free radical antioxidant enzymes and lipid peroxides following long-distance and lactacidemic performance in highly trained aerobic and sprint athletes. J. Sports Med Phy. Fitness. 37 (1997): 235-239.

Mathovics, B., Kotoman, M., Varga, SZ., Hai, DG. and Varga, CS. Oxidative stress in experimental diabetes induced by streptozotocin. Acta Physiol Hung. 85 (1997): 29-38.

McArdle, WD., Katch, FI. and Katch, VL., editors. Exercise physiology 4th ed. Maryland: Williams & Wilkins: 1996.

McCord, JM. and Fridovich, T. Superoxide dismutase. J. Biol. Chem. 244 (1969): 6049-55.

Menger, MD. and Lehr, HA. Scope and perspectives of intravital microscopy-bridge over from in vitro to in vivo Immunology Today. Elsevier Science Publishers Ltd, UK: 1993.

Michael, T., Jchnstare, MD., Shelly, J., Creager, BSN., Kathleen, M. et al. Impaired endothelium dependent vasodilation in patients with insulin-dependent diabetes mellitus. Circulation 88 (1993): 2510-2516.

Midaoui, AE.,Tancrede, G. and Nadeau, A. Effect of physical training on mitochondrial function in skeletal muscle of normal and diabetic rats. Metabolism 45;7 (1996): 810-816.

Misato, K., Jun, O.., Rumi, I. and Asako, K. Impaired reductive regeneration of ascorbic acid in the Goto-Kakizaki diabetic rat. Biochem. J. 351 (2000): 313-318.

Molecular Probes, Inc. Products (Online). (n.d.). Available from http://www.probes.com/servlets/structure?item=1301

Molecular Probes, Inc. Products (Online). (n.d.). Available from http://www.probes.com/servlets/structure?item=143

Moraska, A., Deak, T., Spencer, RL. Roth, D. and Fleshner, M. Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. Am. J. Physiol. Regulatory Integrative Comp Physiol. 279 (2000): R1321-R1329.

Morigi, M., Angioletti, S., Imberti, B., Donadelli, R., Micheletti G. et al. Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. J. Clin. Invest. 101 (1998): 1905-1915.

Mullan, BA., Young, IS., Fee, H. and McCance, DR. Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. Hypertension 40 (2002): 804-809.

Murohara, T., Scalia, R. and Lefer, AM. Lysophosphatidylcholine promotes P-Selectin expression in platelets and endothelial cells. Cir. Res. 78 (1996): 780-789.

Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T. et al. Does superoxide underline the pathogenesis of hypertension? Proc Natl Acad Sci USA. 88 (1991): 10045-10048.

Naziroglu, M., Dilsiz, N. and Cay, M. Protective role of intraperitoneally administered vitamins C and E and selenium on the levels of lipid

peroxidation in the lens of rats made diabetic with streptozotocin. Biol. Trace Elem. Res. ; 70;3 (Dec 1999):223-232, .

Neviere, R., Guery, B., Mordon, S., Zerimech, F., Charre, S., Wattel, F. and Chopin, C. Inhaled NO reduces leukocyte-endothelial cell interactions and myocardial dysfunction in endotoxemic rats. Am. J. Physiol. Heart Circ. Physiol. 278 (2000): H1783-H1790.

Ng, LL., Ngkeekwong, FC., Quinn, PA. and Davies, JE. Uptake mechanisms for ascorbate and dehydroascorbate in lymphoblasts from diabetic nephropathy and hypertensive patients. Diabetologia 41 (1998): 435-442.

Niess, AM., Hartmann, A., Grenert-Fuchs, M., Poch, B. and Speit, G. DNA damage after exhaustive treadmill running in trained and untrained men. Int. J. sports Med. 17 (1996): 397-403.

Ohgawa, H., Ohishi, N. and Yaki, K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. Anal Biochem 95 (1979): 351-8.

Ohkuwa, T., Sato, Y. and Naoi, M. Hydroxyl radial formation in diabetic rats induced by streptozotocin. Life Sci. 56;21 (1995): 1789-98.

Opie, LH. The Heart, Physiology, from cell to Circulation. 3rd ed. Lippincott-Raven : California., 1998.

Ortenblad, N., Madsen, K. and Djurhuus, MS. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 272 (1997): R1258-1263.

Osborn, BA., Daar, JT., Laddaga, RA., Romano, FD. and Paulson, DJ. Exercise training increases sarcolemmal GLUT-4 protein and mRNA content in diabetic heart. J. Appl. Physiol. 82 (1997): 828-834.

Ozansoy, G., Akin, B., Aktan, F. and Karasu, C. Short term gemfibrozil treatment reverses lipid profile and peroxidation but does not alter

blood glucose and tissue antioxidant enzymes in chronically diabetic rats. Mol Cell Biochem. 216 (2001): 59-63.

Palmer, AM., Gopaul, N., Dhir, S., Thomas, CR., Poston, L. et al. Endothelial dysfunction in streptozotocin-diabetic rats is not reversed by dietary probucol or simvastin supplementation. Diabetologia 41 (1998): 157-164.

Palmer, AM., Thomas, CR., Gopaul, N., Dhir, S., Poston, L. et al. Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesenteric arteries of the streptozotecin-diabetic rat. Diabetologia 41 (1998): 148-156.

Panes, J., Kurose, I., Rodriguez-Vaca, MD., Anderson, DC., Miyasaka, M. et al. Diabetes exacerbates inflammatory responses to ischemia-reperfusion. Circulation 93 (1996): 161-167.

Park, KS., Kim, CS. and Kang, SW. Impaired endothelial-dependent relaxation is mediated by reduced production of nitric oxide in the streptozotocin-induced diabetic rats. Korean J Physiol Pharmacol. 4 (2000): 263-270.

Patel, KD., Zimmerman, GA., Prescott, SM., McEver, RP. and McIntyre, TM. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophsis. J. Cell. Biol. 112 (1991): 749-59.

Paulson, DJ., Kopp, SJ., Peace, DG. and Tow, JP. Improved postischemic recovery of cardiac pump function in exercise trained diabetic rats. J. Appl. Physiol. 63(1988): 187-193.

Paulson, DJ., Kopp, SJ., Peace, DG. and Tow, JP. Myoxardial adaptation to endurance exercise training in diabetic rats. Am. J. Physiol. 252 (1987): R1073-R1081.

Perry, MA., Bienvenu, K. and Granger, DN. Leukocyte adhesion in normal and ischemic cat mesentery. (n.p.) (n.d.)

Peterson, TE., Poppa, V., Ueba, H., Wu, A., Yan, C. et al. Opposing effects of reactive oxygen species and cholesterol on endothelial nitric oxide synthase and endothelial cell careolae. Cir Res. 85 (1999): 29-37.

Pickup, J. and Gareth, W. Textbook of diabetes mellitus. 2st edition (Volume 2). Mosby : St. Louis., 1997.

Pieper, GM., Siebeneich, W., Rosa, AM., Jordan, M. and Adams, MB. Chronic treatment in vivo with dimethylthiourea, a hydroxyl radical scavenger, prevent, diabetes-induced endothelial dysfunction. J. Cardiovase Pharmacol. 28 (1996): 741-5.

Piper, GM. Review of alterations in endothelial nitric oxide production in diabetes. Hypertension 31 (1998): 1047-60.

Plower, RW., Chambers, C. and Larsen, WJ. Diabetes-mediated decrease in ovarian superoxide dismutase activity are related to blood-follicle barrier and ovvlation defects. Endocrinology 137 (1996): 3109-10.

Pohl, U. and Wit, C. A Unique Role of NO in the Control of Blood Flow. News Physiol Sci. 14 (Apr 1999): 74-80.

Porte, D. Diabetes mellitus 5th ed. Appleton & Lange. Stamford: Connnecticut.,1997.

Power, RW., Chambers, C. and Larsen, WJ. Diabetes-mediated decrease in ovarian superoxide dismutase activity are related to blood-follicle barrier and ovvlation defects. Endocrinology 137 (1996): 3109-10.

Powers, SK. and Howley, ET. Exercise physiology: theory and application to fitness and performance 4th ed. McGraw-Hill. 2001.

Prentice, WE. Rehabilitation techniques in sports medicine 3rd ed. WCB / McGraw-Hill:134, 1999.

Radak, Z. Free radicals in exercise and aging 1st ed. Human Kinetic : USA, 2000.

Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, BA. et al. Angiotensin II-mediated hypertension in the rat increases vascular syperoxide production via mambrane NADH/NADPH oxidase activation. Contributions to alterations of vasomotor tone. (n.p.) (n.d.)

Ranjan, V., Xiao, Z. and Diamond, SL. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. Am. J. Physiol. 269 (1995): H550-H555.

Rankinen, T., Rice, T., Perusse, L., Chagnon, YC., Gagnon, J. et al. NOS 3 Glu 298 Asp genotype and blood pressure response to endurance training the heritage family study. Hypertension 36 (2000): 885-889.

Resta, TC., Kanagy, NL. and Walker, BR. Estradiol-induced attenuation of pulmonary hypertension is not associated with altered eNOS expression. Am. J. Physiol. Lung Cell Mol Physiol. 280 (2001): L88-L97.

Richard, F., Ammar, Jr., Gutterman, DD., Brooks, LA. and Dellsperger, KC. Free radicals mediate endothelial dysfunction of coronary arterioles in diabetes. Cardiovascular Res. 47 (2000): 595-601.

Rigla, M., Fontcuberta, J., Meteo, J., Caixas, A., Pou, JM. et al. Physical training decreases plasma thrombomodulin in type 1 and type 2 diabetic patients. Diabetologia 44 (2001): 693-699.

Robergs, RA. and Roberts, SO. Exercise physiology: exercise, performance and clinical applications. Mosby: St. Louis., 1996.

Robinson, LE. and Field, CJ. Dietary long-chain (n-3) fatty acids facilitate immune cell activation in sedentary, but not exercise-trained rats. J. Nutr. 128 (1998): 498-504.

Rodriguez, JA., Grau, A., Eguinoa, E., Nespereira, B., Rerez-Ilzarbe M. et al. Dietary supplementation with vitamin C and E prevents downregulation of endothelial NOS expression in hypercholesterolemia in vivo and in vitro. Atherosclerosis 165 (2002): 33-40.

Rodriguez-Manas, L., Angulo, J. and Peiro, C. Endothelial dysfunction and metabolic control in streptozotocin-induced diabetic rats. Br. J. Pharmacol. 123 (1998): 1495-1502.

Rush, JWE., Leughlin, MH., Woodman, CR. and Price, EM. SOD-1 expression in pig coronary arterioles increased by exercise training. Am. J. Physiol. Heart Circ Physiol. 279 (2000): H2068-H2076.

Salas, A., Panes, J., Elizalde, JI., Casadevall, M., Anderson, DC. et al. Mechanisms responsible for enhanced inflammatory response to ischemia-reperfusion in diabetes. Am. J. Physiol. Heart Circ. Physiol. 275(1998): H1773-H1781.

Santini, SA., Marra, G., Giardina, B., Cotroneo, P., Mordente, A., et. al. Defective plasma antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM. Diabetes 46 (1997): 1853-1858.

Sanz, MJ., Johnston, B., Issekutz, A. and Kubes, P. Endothelin-1 causes P-selectin-dependent leukocyte rolling and adhesion within rat mesenteric microvessels. Am. J. Physiol. Heart Circ. Physiol. 277 (1999): H1832-H1830.

Saram, K., McNeill, KL., Khokher, S., Ritter, JM. and Chowienczyk, PJ. Divergent effects of vitamin C on relaxations of rabbit aortic rings to acetylcholine and NO-donors. Br. J. Pharmacol. 135 (2002): 1044-1050.

Schaffler, A., Arndt, H., Scholmerich, J. and Palitzsch, KD. Acute hyperglycaemia causes severs disturbances of mesenteric microcirculation in an in vivo rat model. Eur. J. of Clinical Invest. 28 (Noverber 1998): 886-893.

Schnetterer, L., Findl, O., Fasching, P., Ferber, W., Strenn, J. et al. Nitric oxide and ocular blood flow in patients with IDDM. Diabetes 46 (1997): 653-8.

Seigneur, M., Dufourc, P. and Conri, C. Levels of plasma thrombomodulin are increased in atheromatous arterial disease. Thromb Res. 71 (1993): 423-431.

Seluam, R. and Anuradha, CV. Effect of oral methionine on blood lipid peroxidation and antioxident in alloxan-induced diabetic rats. J. Nutr. Biochem. 1 (1999): 653-8.

Sen, CK. Oxidants and antioxidants in exercise. J. Appl Physiol. 79 (1995): 675-686.

Shaul, PW., Smart, EJ., Robinson, L., German, Z. and Yuhanna, IS. Acylation targets endothelial nitric-oxide synthose to plasmalemmal caveolac. J Biol Chem. 271 (1996): 6518-6522.

Shepherd, JT. Circulatory response to exercise in health. Circulation 89 (1994): 2308-2314.

Sherman, DL., Keaney, JF., Biegelsen, ES., Duffy, SJ., Coffman, JD. et al. Pharmacological concentrations of ascorbic acid are required for the beneficial effect on endothelial vasomotor function in hypertension. Hypertension 35 (2000): 936-941.

Siman, CM. and Eriksson, UJ. Vitamin C supplementation of the maternal diet reduces the rate of malformation in the offspring of diabetic rats. Diabetologia 40 (1997): 1416-1424.

Siu, SM. and Draper, HH. Metabolism of malonaldchyde in vivo and in vitro. Lipids 17 (1982): 349-55.

Slater, TF., Cheeseman, KH., Davies, MJ., Proudfoot, K. and Xin, W. Free radical mechanisms in relation to tissue injury. Proc Nutr Soc. 46 (1987): 1-12.

Slatter, DA., Bolton, CH. and Bailey, AJ. The improtance of lipid-derived malondialdehyde in diabetes mellitus. Diabetologia 43 (2000): 550-557.

Smith, AR., Visioli, F. and Hagen, TM. Vitamin C matters: increased oxidative stress in cultured human aortic endothelial cells without supplemental ascorbic acid. FASEB J. (May 2002).

Smits, P., Hersbach, FMRJ., Jansen, TLTA., Thien, T. and Lutterman, JA. Impaired vasodilator response to atrial natriuretic factor in IDDM. Diabetes 42 (1993): 1454-61.

Solzbach, U., Horning, B., Jeserich, M. and Just, H. Vitamin C improves endothelial dysfunction of epicardial coronary arteries in hypertensive patients. Circulation 96 (1997): 1513-1519.

Stamler, JS., Loh, E., Roddy, MA., Currie, KE. and Creager, MA. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthly humans. Circulation 89 (1994): 2035-40.

Sun, D., Huang, A., Koller, A. and Kaley, G. Short-term daily exercise activity enhances endothelial NO synthesis in skeletal muscle arterioles of rats. J. Appl. Physiol. 76 (May 1994): 2241-7.

Sun, F., Iwaguchi, K., Shudo, R. and Nagaki, Y. Change in tissue concentrations of lipid hydroperoxides, vitamin C and vitamin E in rats with streptozotocin-induced diabetes. Clin Sci (Lond). 96 (February 1999): 185-90.

Svetkey, LP. and Loria, CM. Blood pressure effects of vitamin C what's the key question? Hypertension 40 (2002): 789-791.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A. and Salvetti, A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. Circulation 97 (1998): 2222-2229.

Tapan, KB. Vitamins in Human Health and Disease CAB international: Guildford UK. 1996.

Tappel, AL. Lipid peroxidation damage to cell components. Fed Proc. 32;8 (1973): 1870-4.

Tatchum-Talom, R., Schulz, R., McNeil, JR. and Khadour, FH. Upergulation of neuronal nitric oxide synthase in skeletal muscle by swim training. Am. J. Physiol Heart Cir. Physiol. 279 (2000): H1757-66.

Taylor, PD., Graves, JE. and Poston, L. Selective impairment of acetylcholine-mediated endothelium-dependent relaxation in isolated resistance arteries of the streptozotocin-induced diabetic rat. Clin Sci. 88 (1995): 519-524.

Ting, HH., Timimi, FK., Boles, KS., Creager, SJ., Ganz, P. et al. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. J. Clin Invest. 97 (Jan 1996): 22-8.

Toskulkao, C. and Glinsukon, T. Endurance exercise and muscle damage: relationship to lipid peroxidation and scavenging enzymes in short and long distance runners. Jpn. J. Phys Fitness Sports Med. 45 (1996): 63-70.

Tribe, RM. and Poston, L. Oxidative stress and lipids in diabetes: a role in endothelium vasodilator dysfunction? Vasc. Med. 1;3 (1996): 195-206.

Tribe, RM. Thomas, CR. and Poston, L. Flow-induced dilatation in isolated resistance arteries from control and streptozotocin-diabetic rats. Diabetologia 41 (998): 34-39.

Vallejo, S., Angulo, J., Peiro, C., Nevado, J., Sanchez-Ferrer, A. et al. Highly glycated oxyhaemoglobin impairs nitric oxide relaxations in human mesenteric microvessels. Diabetologia 43 (2000): 83-90.

Van, DPS., Van, ABS., Erkelens, DW., Marx, JJM., Gispen, W-H. et al. The role of oxidative stress in neuropathy and other diabetic complications. Diabetes/Metabolism Rev. 11 (1995): 181-192.

VanderJagt, DJ., Harrison, JM., Ratliff, M. and Hunsaker, LA. Oxidative stress indice in IDDM subjects with and without long-term diabetic complications. Clin Biochem. 34 (2001): 265-270.

Vapaatalo, H. Clinically important factors influencing endothelial function. Med Sci Monit. 7;5 (2001): 1075-1085.

Venditti, P. and Meo, SD. Effect of training an Antioxidant capacity, tissue damage, and endurance of adult male rats. Int. J. Sports Med. 19 (1997): 497-502.

Viguie, CA, Frei, B, Shigenaga, MK, Ames, BN, Packer, L et al. Antioxidant status and indexes of oxidative stress during consecutive days of exercise. J. Appl Physiol. 75 (1993): 566-572.

Vincent, HK., Power, SK., Stewart, DJ., Demirel, HA., Shanely, RA. et al. Short-term exercise training improves diaphragm antioxidant capacity and endurance. Eur J. Appl. Physiol. 81 (Jan 2000): 67-74.

Wade, CR. and Van, RA. Plasma thiobarbituric acid reactivity: Reaction conditions and the role of iron, antioxidants and lipid peroxy

radicals on the quantitation of plasma lipid peroxides. Life Sci. 43;13 (1988): 1085-93.

Wand, SP., West, MW., Dresner, LS., Fleishhacker, JF., Distant, DA. et al. Effects of diabetes and uremia on mesenteric vascular reactivity. Surgery 120 (1996): 328-36.

Wang, JS., Chow, SE., Chen, JK. and Wong, MK. Effect of exercise training on oxidized LDL-mediated platelet function in rats. Thromb Haemost. 83 (2000): 503-8.

Wang, SP., West, MW., Dresner, LS., Fleishhacker, JF., Distant, DA. et al. Effects of diabetes and uremia on mesenteric vascular reactivity. Surgery 120 (1996): 328-36.

Will, JC. and Byers, T. Does diabetes mellitus increase the requirement for vitamin C? Nutrition Reviews 54;7 (1996): 193-202.

Williams, SR. Essentials of Nutrition and Diet Therapy 6th Training on Oxidized LDL-Mediated Platelet Function in Rats. Thromb Haemost 83: 503-ed. Mosby : Missouri 1994.

Willson, DO. and Johnson, P. Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. J. Appl. Physiol. 88;5 (May 2000): 1791-6.

Wilmore, JH. and Costill, DL. Physiology of sport and exercise 2nd ed. Human Kinetice. 1999.

Winterbourn, CC., Hawkings, RE., Brain, M. and Canell, RW. The estimation of red cell superoxide dismutase activity. J. Lab Clin Med. 85 (1975): 337-41.

Wolinsky, I. and Driskell, JA. Sports Nutrition: Vitamins and Trace Elements. CRC Press, Inc Florida, 1997.

Wong, KK. and Wu, HM. Attenuation of early hyperglycaemia induced by streptozocin in fasting rats. Biochem Mol Biol Inter. 38;1 (Feb 1996): 133-139.

Wood, JG., Johnson, JS., Mattioli, LF. and Gonzalez, NC. Systemic hypoxia promotes leukocyte-endothelial adherence via reactive oxidant generation. J. Appl. Physiol. 87;5 (Nov 1999):1934-40.

Woodman, CR., Muller, JM., Rush, JWE., Laughlin, MH. and Price, EM. Flow regulation of ecNOS and Cu/Zn SOD mRNA expression in porcine coronary arterioles. Am. J. Physiol. Heart Circ Physiol. 276 (1999): H1058-H1063.

Young, IS., Torney, JJ. and Trimble, ER. The effect of ascorbate supplementation on oxidative stress in the streptozotocin diabetic rat. Free Radical Biol & Med. 13 (1992): 41-6.

Zarain-Herzberg, A., Yano, K., Elimban, V. and Dhalla, NS. Cardiac sarcoplasmic reticulum $Ca2^{+}$-ATPase expression in streptozotocin-induced diabetic rat heart. Biochem Biophys Res Commun. 203 (1994): 113-20.

Zhao, G., Zhang, X., Smith, CJ., Xu, X., Ochoa, M. et al. Reduced coronary NO production in conscious dogs after the development of alloxan-induced diabetes. Am. J. Physiol. 277 (1999): H268-H278.

Zhao, G., Zhang, X., Xu, X., Wolin, MS. and Hintze, TH. Depressed modulation of oxygen consumption by endogenous nitric oxide in cardiac muscle from diabetic dogs. Am. J. Physiol Heart Circ Physiol. 279 (2000): H520-527.

Zuluaga, M., Briggs, C., Carlisle, J., McDonald, V., McMecken, J. et. al. Sports physiotherapy: Applied science and practice. 1st ed. Churchill Living Stone, 1995.

APPENDICES

APPENDIX I

A. Fluorescence microscopy

Cell biologists need to examine the structure of cell and their components. The microscope is an indispensable tool for this purpose because most cellular structures are too small to be seen by the unaided eye. Regardless of the kind of microscope used, three elements are always needed to form an image: a source of illumination, specimen to be observed, and a system of lenses to focus the illumination on the specimen and to form the image. The past decade has seen a renaissance in light microscopy that has enabled researchers to explore new levels of the subcellular organization and physiological dynamics of cells. In the present study, we used intravital fluorescence microscopy which is one of various forms of light microscopy suitable for the observation of the mesenteric microcirculation of rats.

The term “intravital microscopy” can be summerized as an experimental approache in which the microcirculation of selected organs are made accessible to direct observation in anesthetized or conscious animals light and fluorescence microscopy. In the fluorescence mode the technigue referred to as a dye involves measurements based typically on the absorption of light by a specific fluorescent probe. To understand fluorescence microscopy and the use of fluorescent probes, it is first necessary to understand the process of fluorescence.

Fluorescence is a process that begins with the absorption of light by amole cule at ashort wave lergth subsequent and ends with emission of a photon after relaxation from an excited state at a longer wavelergth. This phenomenon is best approached by considering the quantum behavior of light, as opposed to its wavelike behavior. Figure A-1a is a diagram of the

various energy levels of a simple atom. When an atom absorbs a photon (or quantum) of light of the right energy, one of its electrons jumps from its ground state to a higher-energy, or excited, state. As the atom jiggles around, this electron often loses some of its energy and drops back down to the original ground state, in the process emitting another photon and heat. The emitted photon is always of less energy (longer wavelength) than the original photon that was absorbed. Real fluorescent molecules have energy diagrams that are more complicated than that depicted in Figure A-1a . The number of possible energy levels in real molecules are much greater, so the different energies that can be absorbed and emitted are correspondingly greater. The absorption and emission spectra of a typical fluorescent molecule are shown in Figure A-1b. Every fluorescent probe has its own characteristic absorption and emission spectra. The shapes the spectra are functions of the dye structures and can be influenced by the environment of the probe (e.g. temperature, solvent, pH, ion binding, ets.). A fluorescent microscope is constructed to facilitate observation of fluorescerce. To this end the have an exciter filter between the light source and the condenser lens that transmits only excitation light (Figure A-2). The condenser lens then focuses the rays on the specimen, causing fluorescent compounds in the specimen to absorb and this++
emit light of longer wavelength. Both the excitation light from the illuminator and the emitted light generated by fluorescent compounds in the specimen then pass through the objective lens. As the light passes through the tube of the microscope above the objective lens, it encounters a barrier filter that specifically removes the excitation wavelengths. This leaves only the emission wavelengths to form the final image, which therefore appears bright against a dark background.

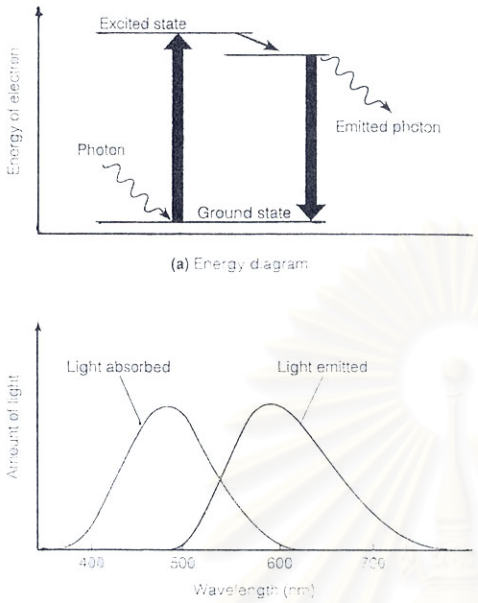


Figure A-1 Principles of Fluorescence. (a) An energy diagram of fluorescence from a simple atom. (b) The absorption and emission spectra of a typical fluorescent molecule.

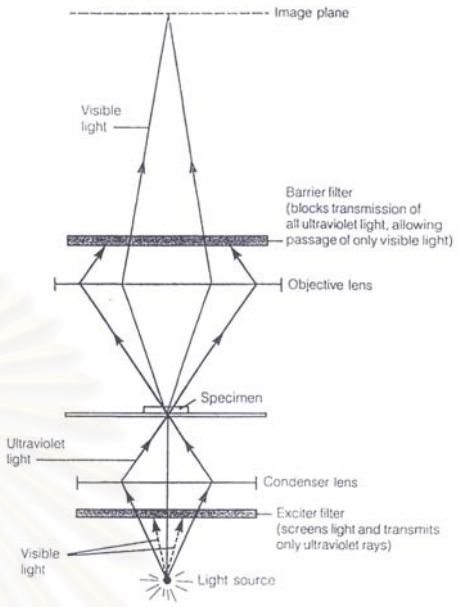


Figure A-2 The configuration of the optical elements and the path of light rays through the fluorescence microscope.

(From Hames BD.,1997)

B. Fluorescent dyes

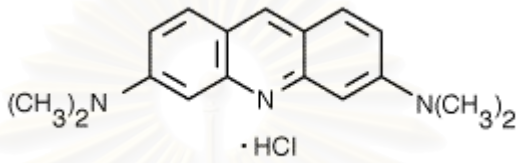
A chemical is said to be fluorescent if it absorbs light at one wavelength (the excitation wavelength) and emits light (fluoresces) at a specific and longer wavelength. The fluorescent dyes have different spectral characteristics and the fluorescence intensity obtained by excitation at the optimum wavelength. In this study, acridine orange and FITC-dextran were the two fluorescent compounds used to study the mesenteric microcirculation including leukocyte adhering to endothelial cells and the vasoactive of arterioles response to vasodilators, respectively.

Acridine orange

Molecular Formula: $C_{17}H_{20}ClN_3$

Molecular Weight: 301.82

CAS Number/Name: 65-61-2 / 3,6-Acridinediamine, N,N,N',N'-tetramethyl-, monohydrochloride



From WWW.probes.com/servlets/structure?item=1301

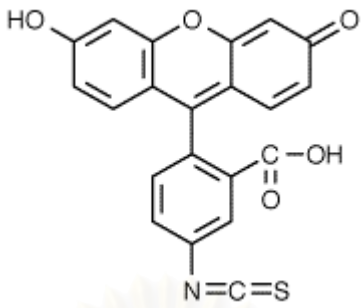
Acridine orange is a cell permeant nucleic acid binding dye. It is a dye that interacts with DNA and RNA by intercalation or electrostatic attractions. In condensed chromatin, however, the bulk of DNA is packed in a way that does not allow efficient intercalation of acridine orange. This cationic dye has green fluorescence with an emission maximum at 525 nm when bound to DNA. Upon association with RNA, its emission is shifted to ~650 nm (red fluorescence). Acridine orange is water soluable and available as a solid and, for ease of handling, as a 10 mg/mL aqueous solution.

Fluorescence Isothiocyanate (FITC)

Molecular Formula: $C_{21}H_{11}NO_5S$

Molecular Weight: 389.38

CAS Number/Name: 3326-32-7 / Spiro(isobenzofuran-1 (3H), 9'-(9H)xanthen) –3-one, 3',6'-dihydroxy-5-isothiocyanato-



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The amine-reactive fluorescein derivatives have been the most common fluorescent derivatization reagents for covalently labeling proteins. Further the fluorescence maximum of FITC is close to the spectral line of the widely used mercury arc-lamps (494 nm), making it widely used in intravital microscopy applications. In addition, fluorescein's protein conjugates are not inordinately susceptible to preciptiation. The sysnthesis of fluorescein isothiocyanate, carboxyfluorescein (FAM, see below) and similar fluorescein-derived reagents yields a mixture of isomers at the 5- and 6- positions of fluorescein's "bottom" ring. Spectra of the two isomers are almost indistinguishable in both wavelength and intensity. However, the isomers may differ in the geometry of their binding to proteins, and the conjugates may elute under different chromatographic conditions or migrate differently in an electrophoretic gel when the dyes are used for high-resolution DNA sequencing. Thus, certain applications may require the single-isomer preparations. Many fluorescein probes are available form Molecular Probes either as a mixture of visomers or as purified single isomers. The 5-isomer of "isomer I" of FITC is the most widely used FITC isomer, probably because it is easier to isolate in pure form than the

6-isomer or “isomer II” of FITC. FITC is readily soluble in aqueous solutions that have a pH above 6 and may deteriorate during storage.

Caution must be taken with FITC, though, as its fluorescence intensity will diminish rapidly (fade) after excitation and is sensitive to pH. Further, prolonged excitation of FITC in vivo appears to produce reactive oxygen species and has been used as a means for producing focal thrombi in the microcirculation. In this study, these properties of FITC did not influence the experimental results.

APPENDIX II

Malondialdehyde (MDA)

PRINCIPLES

Oxidation of polyunsaturated fatty acids leads to numerous peroxidic and aldehydic compounds, in particular the volatile low molecular weight aldehyde, malondialdehyde (MDA). The chemical composition of the end products of peroxidation will depend on the fatty acid composition of the lipid substrate used and upon what metal ions are present. For example, the presence of copper and iron ions give different end-product distributions as measured by the thiobarbituric acid (TBA) test. This is one of the most commonly used methods for detecting and measuring lipid peroxidation. The lipid material is simply heated with TBA at acidic pH (3.5), and the formation of a pink chromogen is measured at or close to 532 nm. The chromogen is formed by reaction of one molecule of malondialdehyde (MDA) with two molecules of TBA.

REAGENTS

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

Dissolve SDS 8.1 g in distilled water and allow to stand overnight at room temperature until it is dissolved. Then make up to 100 ml.

Do not shake because this solution will produce a lot of bubbles and should stored in the refrigerator.

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

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

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

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

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

TMP is used as an external standard. The level of lipid peroxide is expressed as nmole of MDA. Prepared stock 10^3 nmole TMP with distilled water, then pipette 0.04, 0.08, 0.12, 0.16, 0.20 ml of this stock TMP solution and add distilled water to 10 ml in each concentration. These will give the following concentration of standard TMP: 4, 8, 12, 16, 20 nmole/ml. Store the stock solutions in the refrigerator.

5. 1.15% (w/v) KCl

Dissolved KCl 11.50 g in 1000 ml. Of distilled water and mix throughly.

PROCEDURE

1. After washing the isolated tissues in ice-cold 0.9% (w/v) NaCl, the liver and muscle homogenates are prepared by homogenizing each gram of wet tissue in 9 ml of 1.15% KCl.

2. Pipette the following solutions into a series of glass tubes with screw caps:

Solution	Blank (ml)	Standard (ml)	Unknown (ml)
Sample	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH 3.5)	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5
TMP stock standard	-	0.5	-
Distilled water	0.8	0.3	0.3

3. Heated the tubes in the water-bath at 95^{o}C for 60 min.

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

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

6. The content of lipid peroxide is expressed in terms of nmole MDA/g wet wt., converting the O.D. reading using the data generated as the calibration curve, below.

CALIBRATION CURVE

1. Prepare a series of tube containing TMP stock standard in water in the following concentrations: 2.0 nmole/0.2 ml, 4.0 nmole/0.2 ml, 60 nmole/0.2 ml, 8.0 nmole/0.2 ml and 10.0 nmole/0.2 ml.

2. Perform the procedure as in step 2, above. Determine the absorbance at 532 nm. Then plot the optical density versus nmole of MDA/ 0.2 ml of tissue homogenate.

APPENDIX III

Superoxide dismutase (SOD)

PRINCIPLES

Superoxide dismutase catalyzes the breakdown of the superoxide free radical ($O_2^{\bullet -}$) according to the reaction:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

The assay system is based on the ability of the enzyme SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radical which is generated by its reaction of photoreduced riboflavin and oxygen.

REAGENTS

1. 0.1 M EDTA containing 1.5 mg of sodium cyanide

   3.72 g of EDTA and 1.5 mg of NaCN is dissolved in 100 ml of distilled water.

2. 1.5 mM NBT (nitroblue tetrazolium)

   Dissolve 123 mg of NBT in 100 ml of distilled water.

3. 0.12 mM Riboflavin

   Dissolve 4.5 mg of riboflavin in 100 ml of distilled water.

4. 0.067 M Phosphate buffer pH 7.8

   Mix 0.067 M of $NaH_2PO_4 \bullet H_2O$ (1.37 gl/200 ml) in 0.067 M $NaH_2PO_4 \bullet 2H_2O$ (10.67 gl/1000 ml) to make phosphate buffer pH.7.8.

PROCEDURE

1. After washing in ice-cold 0.9% NaCl, liver and muscle tissue is homogenized in 0.1 M phosphate buffer pH 7.4 at a ratio of 1 g of wet tissue in 9 ml of buffer

2. The supernatant fraction that is subjected to centrifuge at 3,000 rpm and 4^{o}C for 15 minutes. The resultant supernatant fraction is then collected for enzyme assay.

3. For each sample to be assayed, the tubes were set up containing 0, 10, 20, 40, 60, 80, 200 and 500 μl of SOD extract from tissues. The reagents were added into these tubes as following:

0.2 ml of 0.1M EDTA + 1.5 mg of NaCN

0.1 ml of 1.5 mM nitroblue tetrazolium (NBT)

0.067 M phosphate buffer pH 7.8 was added to give a total volume of 3 ml.

0.05 ml of 0.12 mM riboflavin (lastly added)

4. The tube which contained no extract was a control for each run. All tubes were then illuminated with a light box for 12 min at room temperature (25^{o}C).

5. Optical density was measured at 560 nm.

CALCULATION

Results were expressed as unit of superoxide dismutase per mg protein of tissue. One unit is defined for a particular system as that the amount of enzyme causing half the maximum inhibition of NBT reduction. The percent inhibition of NBT reduction versus the amount of SOD extract was plotted on a linear scale. The 50% of maximum inhibition thus determined was used in the following equation.

$$E = \frac{1{,}000}{\mu l \text{ of SOD extract} \times g \text{ wet wt}}$$

where:

E = the enzyme activity expressed as 50% inhibition in unit/ g wet wt

APPENDIX IV

Western Blotting for eNOS

Hearts were pulverized in ice cold tris-HCl buffer (pH 7.4) containing 10 mM Tris-HCL, 255 mM sucrose, 2 mM EDTA, 4 μM pepstatin, 12 μM leupeptin, 1 μM aprotinin and 2mM PMSF. The tissues were homogenized on ice for 1 minute by using an homogenizer. The homogenates were then centrifuged for 10 minutes at 1500 x g at 4^{o}C to remove tissue debris. The resultant supernatant was utilized for measurement of total protein level and western blot analysis.

Total protein level of the homogenates, as determined by Lowry method (Appendix IV) utilizing BSA as a standard, was performed to adjust the concentration of homogenate for eNOS protein western blot analysis.

Western blot analysis of tissue homogenates was performed as previously described (Resta TC. et. al., 2001). Samples (18 μg protein per lane) were size fractionated by 7.5% (v/v) SDS polyocrylamide gel electrophoresis. In addition to the samples, each gel included both molecular mass (Bio-Rad) and eNOS (human endothelial lysate; Transduction Laboratories) standards. The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked overnight at 4^{o}C with 5% (w/v) nonfat milk, 3% (w/v) BSA (Sigma), and 0.05% (v/v) Tween 20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris•HCl and 50 mM NaCl (pH 7.5). Blots were incubated for 4 h at room temperature with a mouse monoclonal antibody raised against human eNOS (1:2,500;Transduction Laboratories) in TBS. Immunochemical labeling was achieved by

incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000: Bio-Rad) in TBS followed by chemiluminescence labeling (ECL detection). eNOS protein bands were detected by exposure to chemiluminescence-sensitive film (Kodak). Membranes were stained with Coomassie brilliant blue to confirm equal protein loading per lane. The density of the bands was quantified by scanning densitometry using a ScanJet 5200 C scanner (HEWLETT PACKARD, Singapore) and Global Lab image software and expressed as a percentage relative to the positive external control.

APPENDIX V

Total protein

Total protein was determined by modified lowry technique to correct the amout of MDA, SOD and eNOS protein levels.

REAGENTS

1. Solution A : alkaline tartrate reagent

Na_2CO_3	10.0 g
$Na_2C_4H_4O_6.2H_2O$	0.1 g
NaOH	1.2 g

Dissolve these chemicals in distilled water and make up to 500 ml.

2. Solution B : 0.5% (w/v) copper sulfate

Dissolve 0.5 g $CuSO_4.5H_2O$ in 100 ml distilled water.

3. Solution C

Mix 500 ml of solution A with 10 ml of solution B and use immediately.

4. Solution D : 1 N folin phenol reagent

Dilute 30 ml folin ciocalteu phenol reagent with 30 ml distilled water and use immediately.

5. Standard protein solution

Bovine serum albumin (0.010 g/DW 10ml) is used as standard protein.

PROCEDURE

1. Add solution into each tube as follows:

Solution	Blank	Standard (ml)	Unknown (ml)
Distilled water	0.5	-	-
Standard protein	-	0.5	-
Diluted sample	-	-	0.5
Solution C	5.0	5.0	5.0

Mix well and stand at room temperature for 10 min.

2. Mix throughly with 0.5 ml of solution D and let stand for 30 min at room temperature.

3. Read optical density at 650 nm against the reagent blank.

CALCULATION

Protein concentration (mg%)

$$= \frac{\text{O.D. Unknown}}{\text{O.D. Standard}} \times \text{Concentration of standard} \times \text{Dilution factor}$$

BIOGRAPHY

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