ผลของเจนนิสตีนต่อระดับไขมันในพลาสมาและการทำงานของหลอดเลือดในหนูที่ถูกเหนี่ยวนำให้เป็นเบาหวานโดยสเตรปโตโซโตซิน

นางสาวอภิชญา   พงษ์สุขเวชกุล

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF GENISTEIN ON PLASMA LIPID PROFILES AND VASCULAR FUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS.

Miss Apitchaya   Pongsukwetchakul

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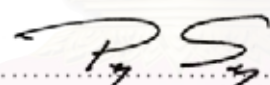
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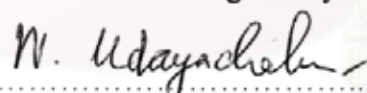
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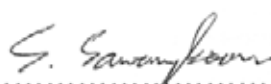
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อภิชญา พงษ์สุขเวชกุล: ผลของเจนนิสตีนต่อระดับไขมันในพลาสมาและการทำงานของหลอดเลือดใน หนูที่ถูกเหนี่ยวนำให้เป็นเบาหวานโดยสเตรปโตโซโตซิน (EFFECTS OF GENISTEIN ON PLASMA LIPID PROFILES AND VASCULAR FUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS.) อ.ที่ปรึกษา: รศ.นพ.วสันต์ อุทัยเฉลิม, อ.ที่ปรึกษาร่วม: ผศ.พญ. ดร.อรอนงค์ กุละพัฒน์, รศ.ดร.สุทธิลักษณ์ ปทุมราช; 95 หน้า ISBN974-14-2150-8

เบาหวานเป็นภาวะที่ก่อให้เกิดปัจจัยเสี่ยงด้านความดันสูง หลอดเลือดตีบ และความผิดปกติของหลอดเลือดแดงขนาดเล็ก โดยเฉพาะอย่างยิ่งมีหลักฐานทางการทดลองพบว่า การสูญเสียหน้าที่ของเอ็นโดทีเลียลเซลล์ จะมีบทบาทต่อการเกิดภาวะแทรกซ้อน ในโรคเบาหวานทางด้านต่างๆ เช่น ความผิดปกติทางตา ทางระบบประสาท ทางไต ทางกล้ามเนื้อหัวใจ เจนนิสตีนเป็นสารที่ได้จาก ถั่วเหลือง ซึ่งมีผลในการเป็นสารต้านอนุมูลอิสระและสามารถทำให้การทำงานของเอ็นโดทีเลียลเซลล์ดีขึ้น

วัตถุประสงค์ ในการทดลองครั้งนี้ผู้วิจัยมีความสนใจที่จะศึกษาผลของเจนนิสตีนต่อ Endothelial dependent และ Endothelial independent vasorelaxation ของหลอดเลือดแดง mesenteric ระดับไขมันในพลาสมา น้ำตาล และ HbA$_{1c}$ ในสัตว์ทดลองที่ ถูกเหนี่ยวนำให้เป็นเบาหวาน
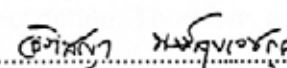
การทดลอง หนูเพศผู้พันธุ์ Wistar น้ำหนัก 180-200 กรัม โดยจะทำการสุ่มเลือกแล้วแบ่งเป็น 2 กลุ่มใหญ่ ได้แก่ หนูที่ไม่เป็นเบาหวาน กับหนูที่เป็นเบาหวาน หลังจากนั้นแบ่งเป็น 3 กลุ่มย่อย 1) กลุ่มหนูที่ไม่เป็นเบาหวาน ที่ได้รับ 0.9% โซเดียมคลอไรด์ จำนวน 100 ไมโครลิตร 2) กลุ่มหนูเบาหวาน ที่ได้รับ DMSO (Dimethyl sulfoxide) จำนวน 100 ไมโครลิตร 3) กลุ่มหนูเบาหวาน ที่ได้รับ เจนนิสตีน 0.25 มิลลิกรัมต่อน้ำหนักตัว ฉีดเข้าทางชั้นใต้ผิวหนังทุกวันเป็นระยะเวลา 4 และ 8 สัปดาห์ ในวันทำการทดลอง ทำการศึกษาการทำงานของเอ็นโดทีเลียลเซลล์ โดยเทคนิค Intravital fluorescent videomicroscopy ทำการฉีดสาร FITC-Dx-250 ซึ่ง เป็นสารเรืองแสง เพื่อดูขนาดของหลอดเลือดแดงใน mesentery และใช้โปรแกรม Image analysis ในการวัดการเปลี่ยนแปลงของ หลอดเลือดแดงรอง
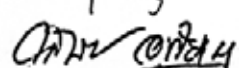
ผลการทดลอง พบว่า เมื่อให้เจนนิสตีน มีการลดลงของระดับน้ำตาลในสัปดาห์ที่ 4 (หนูกลุ่มที่ได้รับ DMSO = 346.16±18.39 มิลลิกรัมต่อเดซิลิตร, หนูกลุ่มที่ได้รับเจนนิสตีน = 276.50±20.01 มิลลิกรัมต่อเดซิลิตร; p<0.05) และ 8 (หนูกลุ่มที่ได้รับ DMSO = 465.83±32.72 มิลลิกรัมต่อเดซิลิตร หนูกลุ่มที่ได้รับเจนนิสตีน = 165.66±25.46 มิลลิกรัมต่อเดซิลิตร; p<0.05) อย่างมีนัยสำคัญทาง สถิติ อีกทั้งยังสามารถลดระดับของ HbA$_{1c}$ ในสัปดาห์ที่ 8 (หนูกลุ่มที่ได้รับ DMSO = 10.08±0.45 % หนูกลุ่มที่ได้รับเจนนิสตีน = 7.71±0.40 %; p<0.05) อย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับหนูเบาหวานที่ได้รับ DMSO แต่อย่างไรก็ตามเจนนิสตีนไม่มีผล ต่อไขมันในพลาสมา และยังพบว่าเจนนิสตีน สามารถป้องกันการสูญเสียหน้าที่ของเอ็นโดทีเลียลเซลล์ในโรคเบาหวานได้ โดย สามารถตอบสนองต่อ Ach 10^{-5} M ได้ดี ในสัปดาห์ที่ 4 (หนูกลุ่มที่ได้รับ DMSO = 6.59±0.56 % หนูกลุ่มที่ได้รับเจนนิสตีน = 18.48±1.16 %; p<0.05) และ 8 (หนูกลุ่มที่ได้รับ DMSO = 8.05±0.41 % หนูกลุ่มที่ได้รับเจนนิสตีน = 14.97±1.40 %; p<0.05)

สรุป จากการวิจัยพบว่า เจนนิสตีนสามารถป้องกันความเสื่อมของ endothelial dependent and endothelial independent vasodilation ในหนูเบาหวาน ยิ่งกว่านั้นเจนนิสตีนมีผลลดระดับน้ำตาลในเลือด อาจจะเป็นผลจากทางตรงหรือทางอ้อมในการออกฤทธิ์ของ เจนนิสตีน ดังนั้นเจนนิสตีน อาจจะมีผลในการป้องกันภาวะแทรกซ้อนทางระบบหัวใจและหลอดเลือดของโรคเบาหวานได้
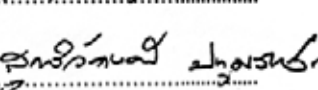
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KEYWORD: DIABETES MELLITUS/ENDOTHELIAL DYSFUNCTION/
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APITCHAYA PONGSUKWETCHAKUL: EFFECTS OF GENISTEIN ON PLASMA LIPID PROFILES AND VASCULAR FUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS. THESIS ADVISOR: ASSOC. PROF. WASAN UDAYACHALERM, M.D. THESIS CO-ADVISOR: ASSIS. PROF. ONANONG KULAPUTANA, M.D., Ph.D. ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D.

Background Diabetes mellitus is associated with increased risks of hypertension, atherosclerosis, and microcirculation disorders. Especially, a number of evidence has suggested that vascular endothelial dysfunction play a major role as underlying cause of a number of diabetic complications. Genistein, the active ingredient of soy product has been suggested for its antioxidant and its endothelial functional improvement.

Objectives The objective of this study was to determine the treatment effects of genistein on endothelium-dependent and endothelium-independent vasorelaxation of mesenteric arterioles, plasma lipid profiles, blood glucose, and HbA_{1C} level in animal models of diabetes.

Methods Male Wistar rats weighing 180-200 g were divided randomly into two major groups of diabetes (DM) and non diabetes (CON). In diabetic group, four subgroups were further randomly divided as follow: 1.) Diabetic groups received 100 µl of dimethylsulfoxide for 4 and 8 weeks (4-wk DM+DMSO and 8-wk DM+DMSO). 2) Diabetic groups received daily injection of 0.25 mg /kg bw genistein for 4 and 8 weeks (4-wk DM+Gen and 8-wk DM+Gen). On the experimental day, endothelial function of each animal was examined using intravital fluorescent videomicroscopy. FITC-Dx-250 was used as a vascular labeling in mesenteric microcirculation. Image analysis was used to measure arteriolar diameter changes.

Results The results demonstrated that blood glucose level was significantly decreased at both 4 (DM+DMSO = 346.16±18.39 mg/dl, DM+Gen = 276.50±20.01 mg/dl; $p<0.05$) and 8 weeks (DM+DMSO = 465.83±32.72 mg/dl, DM+Gen = 165.66±25.46 mg/dl; $p<0.05$) of genistein administration, whereas HbA_{1C} level was significantly attenuated only at 8 weeks. (DM+DMSO = 10.08±0.45 %, DM+Gen = 7.71±0.40 %; $p<0.05$). However genistein did not have any effects on plasma lipid profiles. In addition, it was found that genistein could prevent diabetes-induced endothelial dysfunction which was characterized by increased response to 10^{-5} M Ach response in both groups of 4 (DM+DMSO = 6.59±0.56 %, DM+Gen = 18.48±1.16 %; $p<0.05$) and 8 weeks (DM+DMSO = 8.05±0.41 %, DM+Gen = 14.97±1.40 %; $p<0.05$).

Conclusion Our findings implied that genistein may protect against damage of both endothelial dependent and independent vasodilation in diabetic rats. Moreover, genistein showed the hypoglycemic effect that might be a direct or indirect action. Therefore, genistein might be used to prevent diabetic cardiovascular complications.

Field of study..............physiology........ Student's signature....................

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Co-advisor's signature....................

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LIST OF ABBREVIATIONS

AGEs = advanced glycosylation end-products

Ach = acetylcholine

BG = blood glucose

CAD = coronary artery disease

DM = diabetes mellitus

EDRF = endothelium-derived relaxation factor

ED = endothelial dysfunction

EDV = endothelial-dependent vasodilation

ER = estrogen receptors

ERβ = estrogen receptor β

eNOS = endothelial nitric oxide synthase

Gen = genistein

GDM = gestational diabetes mellitus

HbA$_{1C}$ = hemoglobin A$_{1C}$

HDL-C = high density lipoprotein cholesterol

HR = heart rate

IDDM = insulin dependent diabetes mellitus

IFG = impaired fasting glucose

IGI = impaired glucose tolerance

i.e. = id est (that is)

iNOS = inducible nitric oxide synthase

i.p. = intraperitoneal

i.v. = intravenous (i.v.)

LDL-C = low density lipoprotein cholesterol

M = molar

MAP	=	mean arterial blood pressure
μg	=	microgram
mg/dl	=	milligram per decilitre
mg/kg bw	=	milligram per kilogram body weight
ml/min	=	milliliter per minute
mmHg	=	millimeter of mercury
NE	=	norepinephrine
NIDDM	=	non insulin dependent diabetes mellitus
NO	=	nitric oxide
NOS	=	nitric oxide synthase
nm	=	nanometer
PGI_2	=	prostacyclin
PTK	=	protein tyrosine kinase
SEM	=	standard errors of mean
SNP	=	sodium nitroprusside
STZ	=	streptozotocin
TC	=	total cholesterol
VLDL	=	very low density lipoprotein
VSMC	=	vascular smooth muscle cell

CHAPTER I
INTRODUCTION

Diabetes mellitus is characterized by several metabolic and hemodynamic abnormalities. Alteration in carbohydrate, fat, and protein metabolism in diabetes mellitus is closely related to hyperglycemia. Dyslipidemia is commonly evident in individuals with poorly controlled IDDM (Defronzo et al. 1998). Abnormal lipid and lipoproteins metabolism, high concentration on of triglyceride-lipoprotein in particular, is common in poor glycemic control (Pickup, 1997). Endothelial dysfunction plays a major role in these abnormalities.

Phytoestrogens are naturally occurring plant based diphenolic compounds that are similar in structure and function to estradiol. There are many types of phytoestrogens, but the major categories include isoflavones, lignans, and coumestans. Common and significant sources of phytoestrogens are soybeans (isoflavones), cereals, and oilseeds such as flaxseed (lignans) and alfalfa sprouts (coumestans). The animals were grazing on clover (*Trifolium* sp) a plant with a high content of formononetin, which is converted by ruminal bacteria to isoflavones, the predominant type of phytoestrogens.

The most common and well studied phytoestrogen is the class of isoflavones. The most abundant active components of isoflavones are genistein and daidzein. These agents appear to have selective estrogenic actions, i.e., in some tissues they display proestrogenic responses,

whereas in others, they inhibit estrogenic effects. The recent identification of a second subtype of estrogen receptor lends support to the theory of selective estrogenic action (Paech *et al.* 1997, Foegh *et al.* 1998). Compared with estradiol, genistein, and daidzein bind estrogen receptors with 100 and 1,000 times less affinity, respectively (Adlercruetz *et al.* 1995). Nevertheless, in the quantities that can be consumed in the diet, isoflavones can have biological effects.

Favorable effects of phytoestrogens on lipid profiles, vascular reactivity, thrombosis, and cellular proliferation have been reported. It is plausible that the lower incidence of CAD (coronary artery disease) in populations ingesting diets high in phytoestrogen is due to an improved lipid profile. When patients with type II hyperlipoproteinemia (mean TC (total cholesterol) 409 mg/dl) were placed on high soy diets for four weeks, the total cholesterol and LDL decreased by 16%. A later study designed specifically to examine diets differing only in their protein source randomly assigned healthy men to diets either: 1) high in fat, 2) low in fat with soy protein, or 3) low in fat with animal protein. Both of the low fat diets decreased total cholesterol levels and blood pressure compared with the high fat diet, but the soy protein had a more potent hypocholesterolemic effect (10% vs. 5% decline in total cholesterol) (Kestin *et al.* 1989).

Preclinical studies suggest that vascular reactivity may be favorably influenced by phytoestrogens. Primates manifest an improvement in endothelium mediated vasodilation when treated with phytoestrogens. Specifically, postmenopausal monkeys on a

phytoestrogen-rich diet for six months exhibited normal coronary artery vasodilation in response to locally-administered acetylcholine, whereas a vasoconstrictive response was seen in male animals as well as female monkeys with a low intake of phytoestrogens (Honore *et al.* 1997). In vitro studies of isolated vessels have examined the mechanisms of phytoestrogen-induced vasodilation (Nevala1 *et al.* 1998). Estradiol-17β, genistein, and daidzein were all found to relax mesenteric arterial rings of rats in a dose dependent manner, independent of gender. The vasorelaxation was endothelium-independent and was not blocked by antagonists of nitric oxide or prostacyclin production.

According to the literature review as indicated above, it has been demonstrated that phytoestrogen has its good role on vascular function and lipid profiles. However, metabolic and vascular effects of phytoestrogens have not yet been clarified in diabetes. Therefore, the objectives of the present study were:

1. To study the effect of genistein on lipid profiles, plasma glucose, and HbA_{1c} in diabetic rats.

2. To study the effect of genistein on vascular function in diabetic rats.

CHAPTER II
REVIEW LITERATURE

DEFINITION OF DIABETES MELLITUS

Diabetes mellitus, one of the most important world health problem, is characterized by alterations in carbohydrate, fat, and protein metabolism which best characterized as a state of chronic hyperglycemia (World health Organization 1985), secondary to absent or markedly diminished insulin secretion and/or to ineffective insulin action. The result of this metabolic disorder, the cell lacks of fuel, makes the body recognize not enough food resulted in triggering a sense of increase hunger, polyphagia (Cotran *et al.* 1999). The glucose level in blood increase, excess glucose circulates through the kidney and appears in the urine. The body knows when the urine is too loaded with high concentrated glucose, the body will try to dilute it by allowing large amount of fluid to flow through the kidney; polyuria (Cotran *et al.* 1999). When the fluid is lost, a sense in the thirst center is triggered making the individual drinks more fluid; polydipsia (Cotran *et al.* 1999). Fundamental to all types of diabetes is impairment of insulin secretion by the pancreatic beta cells. Chemical substances e.g. streptozotocin (STZ), alloxan and vacor, pancreatitis, or surgical pancreatectomy can damage beta cells.

In 1995, an International Expert Committee of the American Diabetes Association proposed a classification system that can be divided in to five groups as follows:

(1) Insulin-dependent diabetes mellitus (IDDM) or type I diabetes

(2) Non insulin-dependent diabetes mellitus (NIDDM) or type II diabetes

(3) Gestational diabetes mellitus (GDM)

(4) Impaired glucose tolerance (IGI) and impaired fasting glucose (IFG)

(5) Other specific types of diabetes

INSULIN-DEPENDENT DIABETES MELLITUS (IDDM)

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

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 population. Individuals with poorly controlled IDDM also frequently present with a dyslipidemia, but the pattern differs from that in NIDDM (Defronzo *et al.* 1998).

Lipid and lipoprotein disturbances occur more frequently in NIDDM patients than is IDDM patients, and the characteristic dyslipidemia is already present at the prediabetic stage of impaired glucose tolerance. The most common abnormality is hypertriglyceridemia, often associated with low HDL cholesterol, while total and LDL cholesterol concentrations are similar to non-diabetic levels.

In well-controlled IDDM patients serum lipid and lipoprotein concentrations are similar to those in non-diabetic people. 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 (Table 2.1). 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 lipidemia with chylomicronaemia. These abnormalities are rapidly corrected with improved insulin therapy, through decreased hepatic lipoprotein production and increased lipoprotein lipase activity (Pickup *et al.* 1997).

Table 2.1 Characteristic dyslipidemia in diabetes.
(From Pickup *et al.* 1997)

	Serum Lipid						
	Chol	Tgi	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	↑	↑	↑	↑	↓	↑	↓

Cho, cholesterol; Tgi, triglyceride; N. normal; ↓ , ↑ , lower, higher than normal, respectively.

Recently, it was found that after 12 weeks of a single injection of streptozotocin (STZ; 45 mg/kg bw, i.p.) in rats, there were significant increase in blood glucose, plasma cholesterol and triglyceride level of diabetic animals (Ozansoy *et al.* 2001). In a separate study, Idzior-Walus B. et al. (2001) assessed the determinants and prevalence of hyperlipidemia 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 HbA_{1c} in both sexes.

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 *et al.* 2001). It was suggested that the decreasing of circulating lipids may be effective strategy to minimize increased oxidative stress in diabetic plasma vasculature (Ozansoy *et al.* 2001).

Endothelial dysfunction

Endothelial dysfunction (ED) exists in many arterial diseases and is characterized by deterioration of endothelial vasodilator function. It can manifest either by decreasing secretion of vasodilatory mediators, increasing production of vasoconstrictors, increasing sensitivity to vasoconstrictors and low resistance of VSMC (vascular smooth muscle cell) to endothelial vasodilators (Vapaatalo *et al.* 2001). Moreover, ED is also characterized by vasospasm, inflammation, platelet aggregation, thrombosis, abnormal vascular proliferation, and leukocyte adhesion resulting in atherosclerosis and hypertension. It is important to realize the

values of indicators for endothelial functional tests to quantify the severity of disease in individual subjects. Indicators or markers which can be related in ED are NO (nitric oxide) metabolites, functional test of endothelial-dependent vasodilation (EDV), circulating markers of endothelial function, and adhesion molecules (Vapaatalo *et al.* 2001).

Endothelial dysfunction and diabetes mellitus

The endothelial function may be impaired by risk factors for cardiovascular diseases such as hypertension, hyperlipidemia, and especially diabetes. Accumulating evidence suggests that insulin-dependent and non insulin-dependent diabetes mellitus are associated with impaired endothelial function (Johnstone *et al.* 1993; Williams *et al.* 1996). The impairment of EDV in response to a vasodilator, such as Ach, has been defined as one type of diabetic induced ED both in isolated arteries, and in diabetic rats (Johnstone *et al.* 1993; Kario *et al.* 1995; Wong *et al.* 1996).

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

Mechanism of endothelial dysfunction in diabetes mellitus

At least three possible mechanisms could result in free radical generation in hyperglycemia. As which, those mechanisms are polyol pathway, nonenzymatic glycosylation, and glucose autooxidation (Vanderjagt *et al.* 2001; Baynes *et al.* 1991; Kashiwagi *et al.* 1996; Giugliano and Ceriello 1996).

1. The polyol pathway

The polyol pathway is governed by aldose reductase which is found in tissues such as nerve, retina, lens, glomerulus and blood wall, in which glucose uptake does not required insulin (Pickup and Gareth 1997). In the polyol pathway (Figure 2.1), sorbitol is formed from glucose under the influence of aldose reductase and is further metabolized to fructose by polyol dehydrogenase. Sorbitol does not diffuse easily across cell membrane and may accumulate sufficiently within certain cells to cause osmotic damage and swelling. Furthermore, it was indicated that the polyol pathway associated with the generation of oxygen free radicals. A local excess of those molecules in the vascular system can induce profound endothelial cell dysfunction leading to macro and microangiopathy in diabetes.

$$\text{Glucose} \xrightarrow[\text{NADPH} \quad \text{NADP}]{\textbf{Aldose Reductase}} \text{Sorbitol} \xrightarrow[\text{NAD+} \quad \text{NADH}]{\textbf{Sorbitol Dehydrogenase}} \text{Fructose}$$
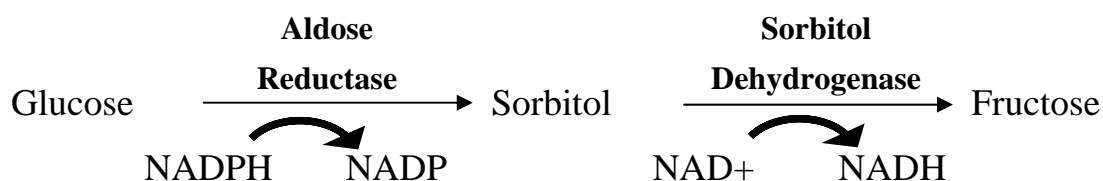
(from Barnett *et al.* 1991)

Figure 2.1 The polyol pathway

2. Nonenzymatic glycosylation

Glucose can form nonenzymatic glycosylation products such as glycosylated hemoglobin via a nucleophilic addition on glucose to the amino groups of proteins and possibly DNA (Hunt *et al.* 1990; Mullarkey *et al.* 1990). This refers to the process by which glucose chemically attaches to the amino group of proteins without the aid of enzymes. Glucose forms chemically reversible glycosylation products with protein (named Schiff bases) that may rearrange to form more stable Amadori-type early glycosylation products, which are also chemically reversible (Cotran *et al.* 1999). The production of these intermediate glycosylated compounds eventually can lead to the formation of advanced glycosylation end-products (AGEs) in a chemical reaction that irreversible (Hunt *et al.* 1990; Mullarkey *et al.* 1990) (Figure 2.2). 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. Vascular and neural tissues also may be particularly susceptible to the

accumulation of nonenzymatic glycosylation products because of their slow turnover (Hunt *et al.* 1990; Mullarkey *et al.* 1990).

Protein + Glucose $\rightleftharpoons$ Schiff Base $\rightleftharpoons$ Amadori Product $\rightarrow$ 3-Deoxyglucosone $\rightarrow$ Advanced Glycosylation End-products (AGEs)
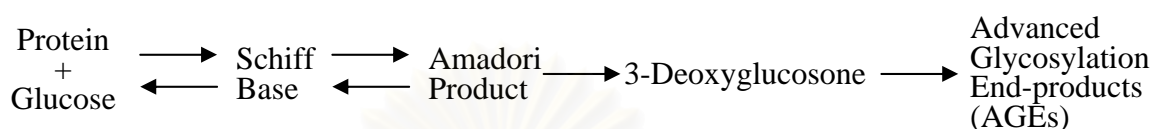
Figure 2.2 Nonenzymatic glycosylaion of proteins.
(Modified form Pickup and Gareth 1997)

2.1 Glycated hemoglobin

A familiar example of a glycated protein in non enzymatic glycylation is glycated hemoglobin A_1 (HbA$_1$). Considerable attention has been given recently to the post-transcriptional glycosylation of proteins in diabetes, particularly with respect to hemoglobin. Chromatography of adult hemoglobin yields a major fraction (more than 90% of the total) of hemoglobin A in front of which are three fast fractions, HbA$_{1a}$, HbA$_{1b}$ and HbA$_{1c}$ - the glycosylated hemoglobins (Keen *et al.* 1999). These three hemoglobins accumulate during the life span of the red blood cell. HbA$_{1c}$ comprises 4% to 6% of HbA, with the other fractions conprising 1-2% each. These glycosylated hemoglobins are formed nonenzymatically at a rate dependent on the ambient glucose concentration.

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

3. Glucose autooxidation

The term glucose autooxidation describes the capability of glucose to enolize, thereby reducing molecular oxygen and yielding oxidizing intermediates (Giugliano and Ceriello A. 1996). It has been suggested that glucose autooxidation and nonenzymatic glycation, together termed glycoxidation, are the major contributors to the increase in free radicals in diabetes (Lee *and* Chung 1999). 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 individualy susceptibility to damage, an observation that might yield insight into the basis for individual differences in susceptibility to development of complications (Baynes *et al.* 1991).

<u>Streptozotocin induced diabetes</u>

In the present study, streptozotocin (STZ) was used to experimentally induce diabetes in rats. STZ [2-deoxy-2- (3-methy1-3-nitrosoureido-D-gluco pyranoside)] is a nitrosourea derivative isolated from the mould *Streptomyees grisevus* (Figure 2.3). 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, miniature pigs, pigs and rabbits (Porte *et al.* 1997).

$$CH_2OH$$
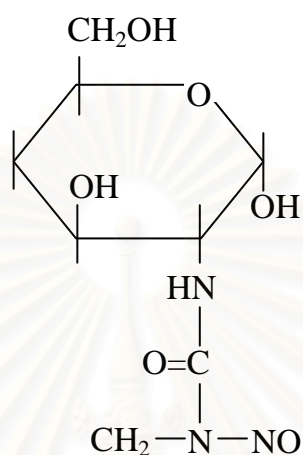
Figure 2.3 Molecular structures of streptozotocin, 2-deoxy-2-
(3-methy1-3-nitrosoureido)-D-glucopyranose.
(From Porte *et al.* 1997)

STZ 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 (Pick up *et al.* 1997). STZ-streated 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 *et al.* 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 *et al.* 1997). After the intravenous administration of STZ, an early hyperglycemic phase appears, followed by a hypoglycemic phase and then a permanent diabetic phase occurring at approximate 4, 7 and 24 hours, respectively (Wong *et al.* 1996). The precise mechanism of STZ diabetogenicity has been described. STZ may act on both the membrane and the interior of the β cell. It damages β cell membrane and also induce fragmentation of DNA (Pickup *et al.* 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 *et al.* 1995).

PHYTOESTROGENS

Phytoestrogens are a group of biologically active plant substances with a chemical structure that is similar to that of estradiol, an endogenous estrogen (**Figure** 2.4). This structural similarity accounts for the ability of these compounds to bind to estrogen receptors in various cells (Martin *et al.* 1978; Wang *et al.* 1996; Kuiper *et al.* 1998; Miksicek *et al.* 1994) and exert estrogenic or antiestrogenic effects. The 3 major classes of phytoestrogens are isoflavones, lignans, and coumestans. The major bioactive isoflavones are genistein and daidzein, which are derived from the precursors biochanin A and formononetin, respectively. Lignans are constituents of many plants and form the building blocks for the formation of lignin in the plant cell wall (Ross *et al.* 1997). They are more prevalent in the plant kingdom than are isoflavones. The 2 major lignans, enterolactone and enterodiol, are produced from matairesinol and secoisolariciresinol, respectively. Coumestrol is the most important form of coumestan consumed by humans.

In recent years, phytoestrogens have attracted increased attention among the public and the medical community because of accumulated evidence from a large body of literature (Adlercreutz *et al.* 1997; Anderson *et al.* 1998; Setchell *et al.* 1998; Anthony *et al* 1998; Tham *et al.* 1998; Lissin *et al*. 2000; Velasquez *et al*. 2001; Ranich *et al*. 2001) suggesting that consumption of plant-based foods rich in these phytochemicals may benefit human health. Substantial data from epidemiologic surveys and nutritional intervention studies in humans and animals suggest that dietary phytoestrogens have protective effects against

menopausal symptoms and a variety of disorders, including cardiovascular disease, cancer, hyperlipidemia, osteoporosis, and various forms of chronic renal diseases (Adlercreutz *et al.* 1997; Anderson *et al.* 1998; Setchell *et al.* 1998; Anthony *et al* 1998; Tham *et al.* 1998; Lissin *et al*. 2000; Velasquez *et al*. 2001; Ranich *et al*. 2001). The Food and Drug Administration of the United State authorized the use on food labels of health claims associated with soy protein and the reduced risk of cardiovascular disease (FDA. 1999). Several studies in humans and animals have shown that soy protein reduces plasma total cholesterol and LDL cholesterol. Evidence is also emerging that consumption or supplementation of foods rich in phytoestrogens may have beneficial effects on diabetes mellitus and obesity in animals and humans.
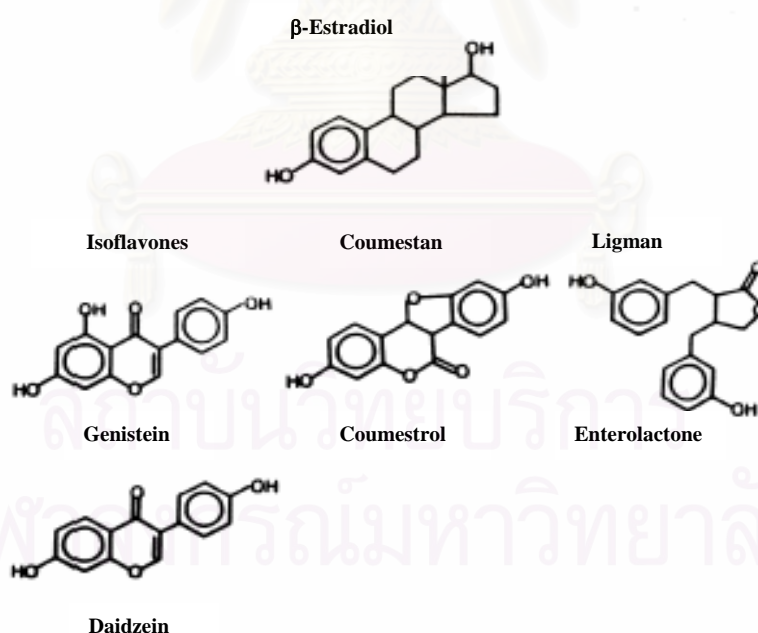
Figure 2.4 Structures of 17 ß-estradiol, isoflavones (daidzein, glycitein, and genistein), coumestrol, and lignans (secoisolariciresinol and matairesinol).

Absorption and metabolism of isoflavones

Isoflavones exist primarily in plants in the inactive form as glycosides. Once ingested, isoflavone glycosides (genistin and daidzin) are hydrolyzed in the intestines by bacterial ß-glucosidases and are converted to corresponding bioactive aglycones (genistein and daidzein). Further fermentation proceeds in the distal intestine with the formation of specific metabolites. The aglycones are then absorbed from the intestinal tract and conjugated mainly in the liver to glucuronides, which are either reexcreted through the bile and reabsorbed by enterohepatic recycling or excreted unchanged in the urine. Daidzein may be further metabolized to equol, dihydrodaidzein, or *O*-desmethylangolensin, whereas genistein may be metabolized to *p*-ethylphenol in the colon. Daidzein, genistein, equol, and *O*-demethylangolensin are the major isoflavones that have been detected in the blood and urine of animals and humans. Dihydrodaidzein, *p*-ethylphenol, and glycetin have also been detected in human plasma.

Concentrations of phytoestrogens and their metabolites in plasma and urine have been reported in several studies of humans and animals. In healthy humans consuming diets without soy, plasma concentrations of isoflavones are usually in the nanomolar range (eg, <40 nmol/L) (Morton *et al.* 1994). In contrast, plasma isoflavone concentrations increase markedly in the micromolar range after ingestion of isoflavones from soybean milk (Xu *et al.* 1994), soy meal (King *et al.* 1998), or baked soybean powder (Watanabe *et al.* 1998). Plasma isoflavone concentrations of 1–4 µmol/L have been reported in various population

groups consuming foods rich in isoflavones (Morton *et al.* 1994; Xu *et al.* 2000; Adlercreutz *et al.* 1993; Markkanen *et al.* 1993). Similarly, urinary excretion of isoflavones increases markedly after ingestion of isoflavone-rich diets (Markkanen *et al.* 1993).

Isoflavones and lipid metabolism

Soy isoflavones, structurally similar to estrogens (Anderson *et al.* 1996), interact with estrogen receptors and may decrease serum cholesterol concentrations by similar mechanisms (Anderson *et al.* 1996). Anthony et al (1996) reported that monkeys fed isoflavone rich soy protein isolate diets had significantly better serum lipid values (lower total cholesterol and higher soy isoflavones have many important biochemical effects that may mediate some of the health benefits outlined above (Dwyer *et al.* 1994). Huff et al. (1984) showed an increased turnover rate of VLDL apolipoprotein B in men fed soy protein diets compared with animal protein diets. Lovati et al. (1987) reported that soy-protein diets stimulated up-regulation of LDL-receptors and observed an 8-fold increase in LDL-cholesterol degradation compared with animal-protein diets. An early study at the University of Milan (Lovati *et al.* 1987) showed an 8-fold increase in LDL receptor activity in isolated lymphomonocytes from severely hypercholesterolemic patients treated with soy proteins. A similar finding, but with lesser increase of LDL receptors was reported by Baum et al. (1998) in post-menopausal women with less dramatic elevations of LDL cholesterolemia.

Phytoestrogen have been reported for their good effects on lipid profiles. Soy protein inhibits atherosclerosis in animals. This effect seems to be mediated in large part by effects on plasma lipoprotein concentrations. That is, by reducing LDL-C by about 13%, lowering plasma triglycerides by about 10%, and possibly increasing HDL-C by 2% (Anderson *et al.* 1995). These benefical effects of soy protein on plasma lipoprotein concentrations culminated recently in the U.S.Food and Drug Adiministration's approval of a health claim that 25g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease.

Isoflavones and vascular function

In addition to its effects on lipid metabolism, isoflavones influence the function of the endothelium. In the atherosclerotic macaque, dietary isoflavones enhance endothelium-dependent relaxation to acetylcholine (Ach) in the coronary arteries (Honoré *et al.* 1997) and treatment with genistein augments endothelium-dependent arterial relaxations in OVX rats (Squadrito *et al.* 2000).

Phytoestrogens have been shown to affects VSMCs. VSMCs contribute to pathological structural changes within the vessel wall by migrating from the media into the intima, and by proliferating and depositing extracellular matrix proteins such as collagen (Dubey *et al.* 1997). Genistein, daidzein, biochanin A and equol inhibit human aortic VSMC proliferation, growth, migration and mitogen-activated protein kinase (MAP) activity (Dubey *et al.* 1999). The order of potency of these

plant-derived estrogens is biochanin A > genistein > equol > daidzein (Dubey *et al.* 1999). In menopausal and perimenopausal women, dietary isoflavones improve arterial compliance (Nestel *et al.* 1997). Genistein has also been shown to reduce renal vascular resistance and to act as a diuretic (Gimenez *et al.* 1998), which can be beneficial in regulating blood pressure.

In summary, the major role of isoflavones is likely to be the effect on endothelial function. Additional effects haves been clearly demonstrated on smooth muscle of the vessel wall. Regardless of the vascular mechanism, isoflavones have demonstrated some good benefits in clinical outcomes.

CHAPTER III
MATERIALS AND METHODS

Animal preparation

Male Wistar rats (National Laboratory Animal Center of Salaya Campus, Mahidol University, Thailand) weighing 180-200 g, 6-weeks old were divided randomly into three groups:

1. Control group (Control) (n=12): normal rats were received a single intravenous injection of citrate buffer (65 mg/kg bw). The animals were supplemented with normal saline 100 μl/day injected subcutaneously.

2. Diabetic group (DM+DMSO) (n=12): rats were induced diabetes by a single intravenous injection of STZ (65 mg/kg bw) that lead to hyperglycemia. The animals were supplemented with DMSO 100 μl/day (Sigma Chemical Co., USA) injected subcutaneously.

3. Diabetic treated genistein group (DM+Gen) (n=12): rats were induced the same way as described for the diabetic group. Forty eight hours after administration of STZ, the animals were supplemented with genistein 0.25 mg/kg bw in 100μl of DMSO/day (Sigma Chemical Co., USA) injected subcutaneously.

All animals were fed with regular dry rat chow and allowed freely access to drinking water. In this study each group was further divided into 2 subgroups according to the time of collecting the specimens i.e, at the 4 weeks (n=6) and 8 weeks (n=6).

Diabetic induction

To induce diabetes mellitus, STZ (Sigma Chemical Co., USA) was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co., USA) and immediately single injected into the tail vein of 8-hours fasted rats, at a dose of 65 mg/kg bw. Blood glucose (BG) was determined by using glucometer (Advance Glucometer, Bochringer Mannheim, Germany). Samples were analyzed by applying a drop of blood to a prepared strip. Rats treated with STZ that did not exhibit an elevation of BG level greater than 200 mg/dl (Richard *et al.* 2000) at 48 hours were excluded from the study. In addition, diabetic condition was also confirmed by rat's manifestation of polyuria, polyphagia, and polydipsia.
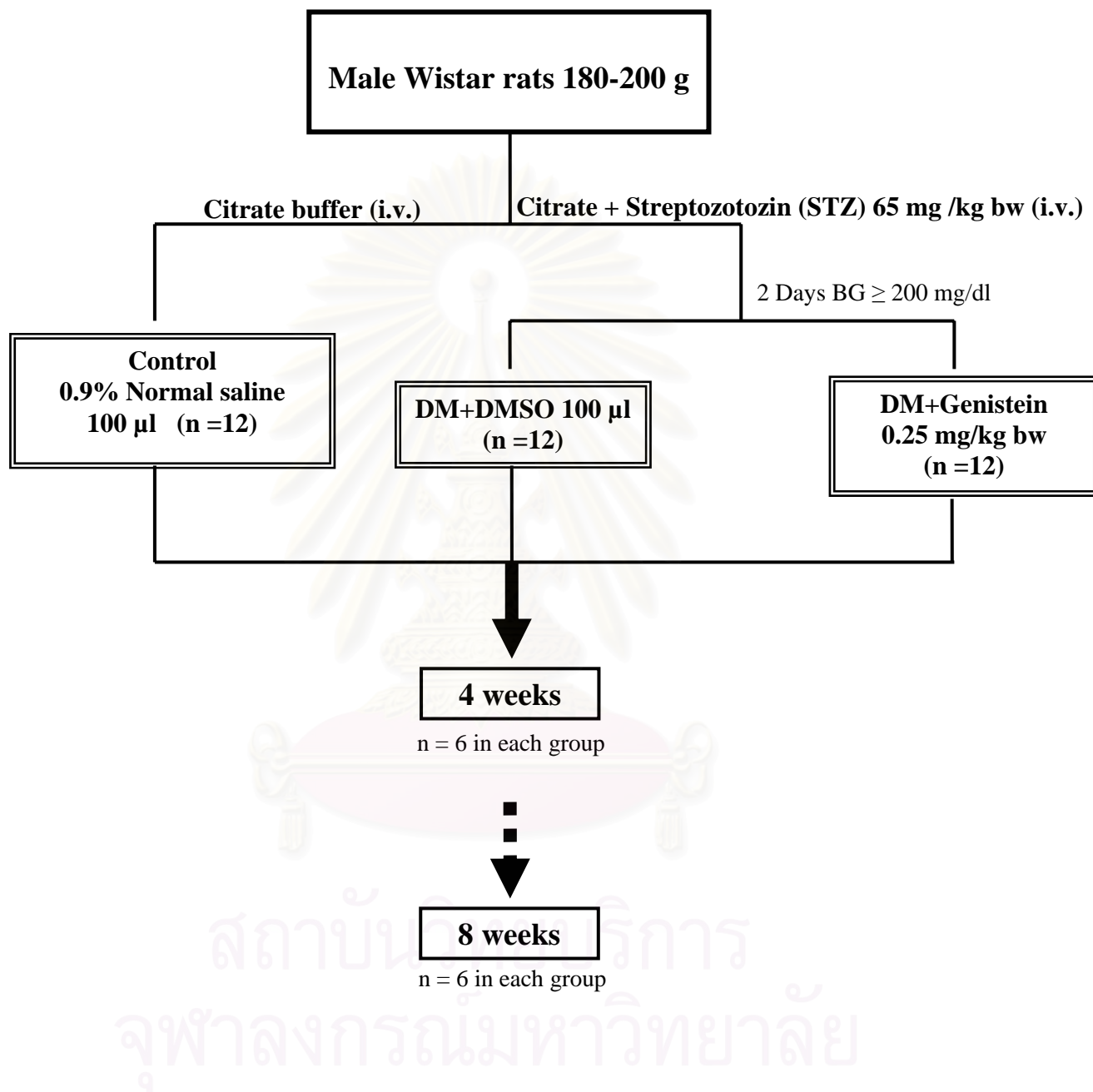
Figure 3.1 Experimental designs to study the effects of genistein on
endothelial function in diabetic rats.

Chemical substances

The list of chemical substances used in this study were given below;

Chemical	Company
Genistein	Sigma, USA.
Dimethyl sulfoxide	Sigma, USA.
Fluorescein isothiocyanate-dextran	Sigma, USA.
Heparin	Leo, Denmark.
Normal saline	Thai Nakorn Patana Co., Ltd., Thailand.
Pentobarbiturate sodium (Nembutal R)	Sonofi, Thailand.
Acetylcholine	Sigma, USA.
Sodium nitroprusside	Sigma, USA.
Norepinephrine	Sigma, USA.
Krebs-Ringer solution	
Sodium chloride (NaCl)	Merck, USA.
Potassium chloride (KCl)	Riedel-de Hach, Germany.
Calcium choloride (CaCl$_2$)	Riedel-de Hach, Germany.
Sodium bicarbonate (NaHCO$_3$)	Riedel-de Hach, Germany.
Potassium dihydrogen phosphate (KH$_2$PO$_4$)	Riedel-de Hach, Germany.
Magnesium sulphate (MgSO$_4$)	Riedel-de Hach, Germany.

The compositions of Krebs-Ringer solution are as below;

NaCl	135.7 mM/L
KCl	4.7 mM/L
$CaCl_2.2H_2O$	2.52 mM/L
$NaHCO_3$	7.14 mM/L
KH_2PO_4	1.18 mM/L
$MgSO_4.7H_2O$	1.64 mM/L

Heart rate and blood pressure recording

On the day of the experiment, rats were anesthetized with intraperitoneally injection (i.p.) of 45 mg/kg bw of sodium pentobarbital. After the induction of anesthesia, tracheostomy was performed and polyethylene catheters were inserted in the left common carotid artery (PE 90) and the left external jugular vein (PE 20) for direct arterial blood pressure monitoring and intravenous (i.v.) drug administration, respectively. After canulating common carotid artery and external jugular vein, rats rested for 5 minutes. Then, blood pressure was measured via a canular inserted into common carotid artery by polygraph system (Nihon Koden, Japan). After that, heart rate was calculated based on by choosing a constant period of heart rate extracted from the blood pressure tracing.

Studies of mesenteric arteriolar response to vasoactive agents

The abdomen was opened and the small intestine was displaced to expose a segment of the mesentery. The distal part of ileum was exteriorized (Iafrati *et al.* 1997; Jun *et al.* 1998; Karas *et al.* 1999). A well-vascularized mesenteric window was selected and spreaded out flat over a small plexiglass platform. The mesenteric tissues were superfused with Krebs- Ringer solution containing (in mmol/L); 135.7 NaCl, 4.7 KCl, 2.52 $CaCl_2.2$ H_2O, 7.14 $NaHCO_2$, 1.18 KH_2PO_4, 1.64 $MgSO_4.7$ H_2O and maintained at 37^0 C in order to prevent tissues drying. Microvessels selected for study were defined according to their branch order location within the microvascular network. After mesenteric preparation, a bolus injection of 0.2 ml of 5 % FITC-dextran 250 was given through the jugular vein. Preconstriction vascular diameter with norepinephrine (NE; 10^{-5} M) before vasorelaxation mesenteric arteriolar in response to Acetylcholine (Ach; 10^{-5} M) and Sodium nitroprusside (SNP; 10^{-5} M). The arteriolar diameter was observed before and after the application of Ach; 10^{-5} M using intravital microscopic study as mentioned above. After washing three times with Krebs-Ringer solution, the vessels were equilibrated for 20 minutes to their baseline diameter. Sodium nitroprusside (SNP; 10^{-5} M), (Nakayama *et al.* 1991) a potent smooth muscle vasodilator, was then topically applied. The mesenteric arteriolar diameter was measured before and after applying SNP by an intravital videomicroscope in the same manner as procedure. After finishing the experiment, rats were terminated with an overdose of sodium pentobarbital.
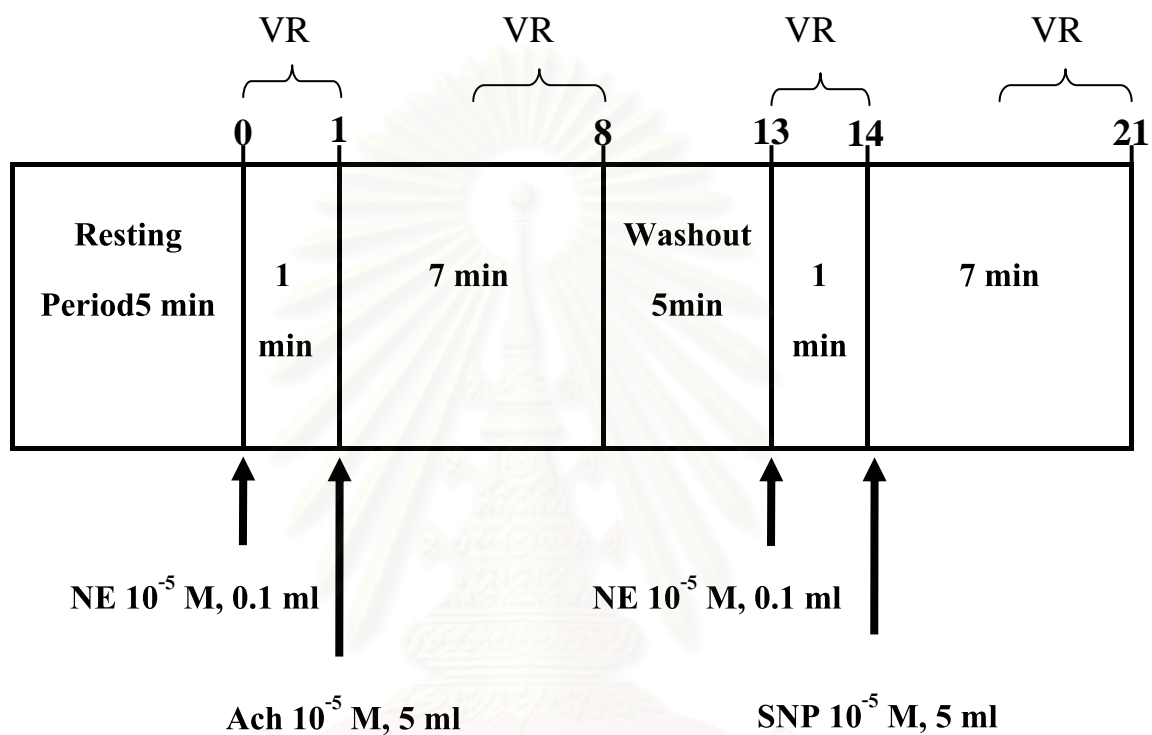
Figure 3.2 The protocol used for studies of mesenteric arteriolar

responses to vasoactive agents.

VR = Video Recording

Direct visualization of mesenteric microcirculation

Video image of the mesenteric microcirculation of the distal ileum was obtained by epi-illumination fluorescent microscopy using fluorescence isothiocyanate-labled dextran of 250,000 molecular weight (FITC- dextran – 250). In this experiment, **FITC**-dextran – 250 was dissolved to make the final concentration 5 mg/100 ml in normal saline (Enrich *et al.* 1980; Lehr *et al.*1993; Menger *et al.* 1993). After **FITC** - dextran – 250 reached the mesenteric arterioles, images of vessels could be observed by epi-illumination fluorescence microscopy (Nikon model optiphot-2). The epi-illumination system consists of a 50 W mercury lamp with 488 nm excitation filter and 515 nm emission barrier filter. The image of selected vessels could also be observed on a black and white video monitor (Sony, GM – 1411 QM) using a silicon intensified target television camera (Nikon –SIT 68) mounted on a fluorescence microscopy using a × 20 objective lens and a × 10 eyepiece (CFI Plan Fluor) which was used to observe mesenteric arteriolar diameter. Video images of microvessels were stored on videotape (Sony, DX-E 180) connected with the video timer. During the experiment, microvessel images could be printed by using video graphic printer (Sony, UP – 890 CE). Diameter of mesenteric microvessel images were measured from the fluorescence video image of FITC – labeled dextran on the video monitor using digital image of processor system (Figure 3.3) (Michelassi *et al.* 1987) with the software "Global Image". The arteriolar diameter was calculated as the mean of triple measurements form three video frames by using the same reference point as a marker for measuring each vessel in each frame (Figure 3.4). Measurements of arteriolar diameter in response

to NE were performed immediately before Ach or SNP application. Arteriolar diameters were measured at about 7 minute after Ach or SNP application. Vasodilation responses were expressed as the percentage of maximal relaxation after preconstriction with norepinephrine (NE; 10^{-5} M) (Sun *et al.* 1992).

Plasma total cholesterol, triglyceride, HDL-C, LDL-C, HbA_{1C} and blood glucose levels.

Blood samples were collected at the end of the experiment. Plasma was separated from whole blood by centrifugation with 3,500 rpm at 4° C for 15 minutes, then it was stored at a temperature of 4 ^{o}C unit being analyzed at the BRiA LAB CO., LTD. The analysis of total cholesterol, triglyceride, HDL-C, LDL-C, HbA_{1C} and glucose in the blood was determined by using an automatic analysis technique on a chemical analyzer form Roche diagnostics (COBAS INTEGRA 700).

Cholesterol is determined by enzymatic, colorimetric method (CHOD/PAP) with cholesterol esterase, cholesterol oxidase, and 4-aminoantipyrine. Triglyceride is determined by enzymatic, colorimetric method (GPO/PAP) with glycerol phosphate oxidase and and 4-aminophenazone. HDL-C concentration is determined after isolation of HDL-C in the specimen. The separating reagent for HDL-C uses phosphotungstic acid and magnesium ions to precipitate the chylomicrons, VLDL, and LDL. After centrifugation, HDL remaining in the supernatant is quantitated by its cholesterol content. The HDL-C concentration is determined using an enzymatic, colorimetric method

(CHOD/PAP). LDL-C concentration is determined using an enzymatic, colorimetric method (CHOD/PAP).

Total Hb and HbA_{1C} concentrations are determined after hemolysis of the anticoagulated whole blood specimen. Total Hb is measured colormetrically. HbA_{1C} is determined immunoturbicimetrically. The ratio of both concentration yields the final percent HbA_{1C} result [HbA_{1C} (%)]. Blood glucose is determined using enzymatic reference method with hexokinase.

Data analysis

The results are expressed as means ± SEM. Statistical analysis of differences between groups at each time point was performed using one way ANOVA followed by post hoc Bonferroni's test. Probability values of less than 0.05 were considered statistically significant.

Intravital Fluorescent Microscopy and Image Analysis
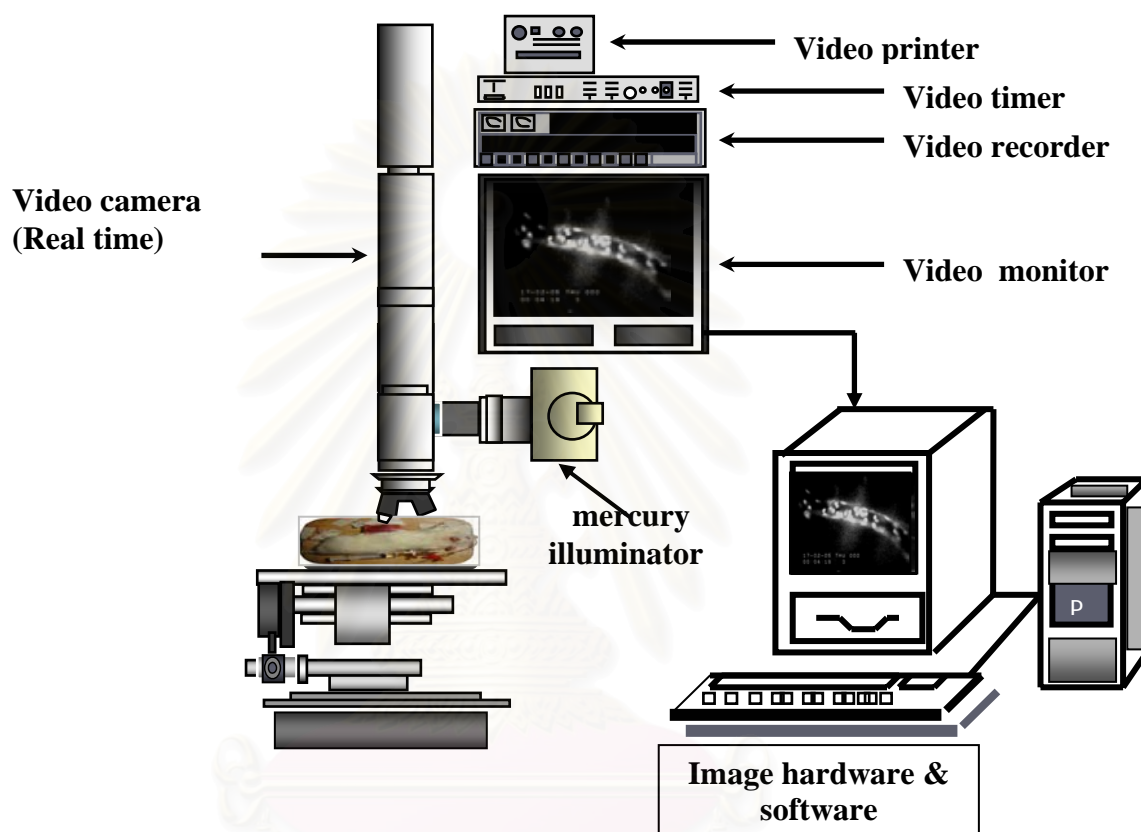
Figure 3.3 Endothelial function study using intravital fluorescent microscopy and image analysis.

A.   B.

Figure 3.4 The reference point A and the defined point B and C.

The diameter of arteriole was measured as the length of B-C.

$$\text{Mean diameter } (\overline{BC}) = \frac{B_1C_1 + B_2C_2 + B_3C_3}{3}$$

$$\% \text{ Change of Diameter} = \frac{\overline{BC_s} - \overline{BC_n}}{\overline{BC_n}} \times 100\%$$

$\overline{BC_s}$ mean diameter of mesenteric arteriolar responses to topical application of Ach or SNP.

$\overline{BC_n}$ mean diameter of mesenteric arteriolar responses to topical application of NE.

CHAPTER IV
RESULTS

Rats with intravenous injection of STZ 65 mg/kg bw significantly resulted in hyperglycemia within 48 hours and shown persistent hyperglycemia throughout the experiment. In the present study, the criteria used for diabetic rats was the blood glucose level had to be higher than 200 mg/dl.

The results shown in Figure 4.1 demonstrated that heart rate of diabetic rats (DM+DMSO) and diabetic rats supplementation with genistein at 4 and 8 weeks (DM+Gen) was not significantly different compared to control group. Results of mean arterial pressure in DM+DMSO and DM+Gen groups were no significant difference compared to control group at 4 and 8 weeks. (Figure 4.2, mean±SEM. see appendix)

Body weight

In this study, the changes in body weight (g) were determined. DM+DMSO and DM+Gen groups had significantly decreased body weight compared to the control group at 4 and 8 weeks. DM+Gen group had no significant difference compared to DM+DMSO group at 4 and 8 weeks. (Figure 4.3)

Blood Glucose and HbA$_{1c}$

The results revealed that DM+DMSO and DM+Gen groups were significantly increased blood glucose and HbA$_{1c}$ compared to control group. At 4 and 8 weeks, DM+Gen group had significantly decreased blood glucose compared to DM+DMSO group (Figure 4.4). At 4 weeks, HbA$_{1c}$ in DM+Gen group had no significant difference compared to DM+DMSO group but at 8-weeks HbA$_{1c}$ in DM+Gen group had significantly decreased as compared to DM+DMSO group. (Figure 4.5)

Lipid profiles

Plasma total cholesterol, triglyceride, HDL-C and LDL-C in DM+DMSO and DM+Gen groups had no significant difference from control group at 4 and 8 weeks. All these assessed lipid profiles were also demonstrated graphically as shown in Figures 4.6-4.9.

Studys of mesenteric arteriolar responses to vasoactive agent

There were no significant differences of NE-preconstriction in mesenteric arteriolar among 3 groups (Figure 4.10).

Endothelium dependent relaxation

Endothelium dependent relaxation determined by measurement the mesenteric arteriolar responses to topical application of Ach (10^{-5} M).

Intravital fluorescent microscopy was used to examine those arteriolar responses in control, DM+DMSO and DM+Gen groups at 4 and 8 weeks of treatment. The changes of arteriolar diameter in DM+DMSO group at 4 and 8 weeks were significantly decreased compared to control groups. The changes of arteriolar diameter in DM+Gen and control groups at 4 and 8 weeks were no significant difference, but when compared to DM+DMSO groups at 4 and 8 weeks, DM+Gen groups had significant improvement of Ach induced relaxation in mesenteric arterioles. The results were summarized in Figure 4.11.

Endothelium-independent relaxation

Endothelium-independent relaxation determined by measurement the mesenteric arteriolar responses to topical application of SNP (10^{-5} M). The results showed that the change of arteriolar diameter in DM+DMSO group at 4 and 8 weeks was significantly decreased compared to control group. The change of arteriolar diameter in DM+Gen and control groups at 4 and 8 weeks was no significant difference but when compares with DM+DMSO group at 4 and 8 weeks, DM+Gen group had significant improvement of Ach induced relaxation in mesenteric arterioles. (Figure 4.12)

Fugure 4.1 Heart rate (beats/min) of control treated rats, DMSO treated

rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

Figure 4.2. Mean arterial blood pressure of control treated rats, DMSO
treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

Figure 4.3. Body weight (g) of control treated rats, DMSO treated

rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).
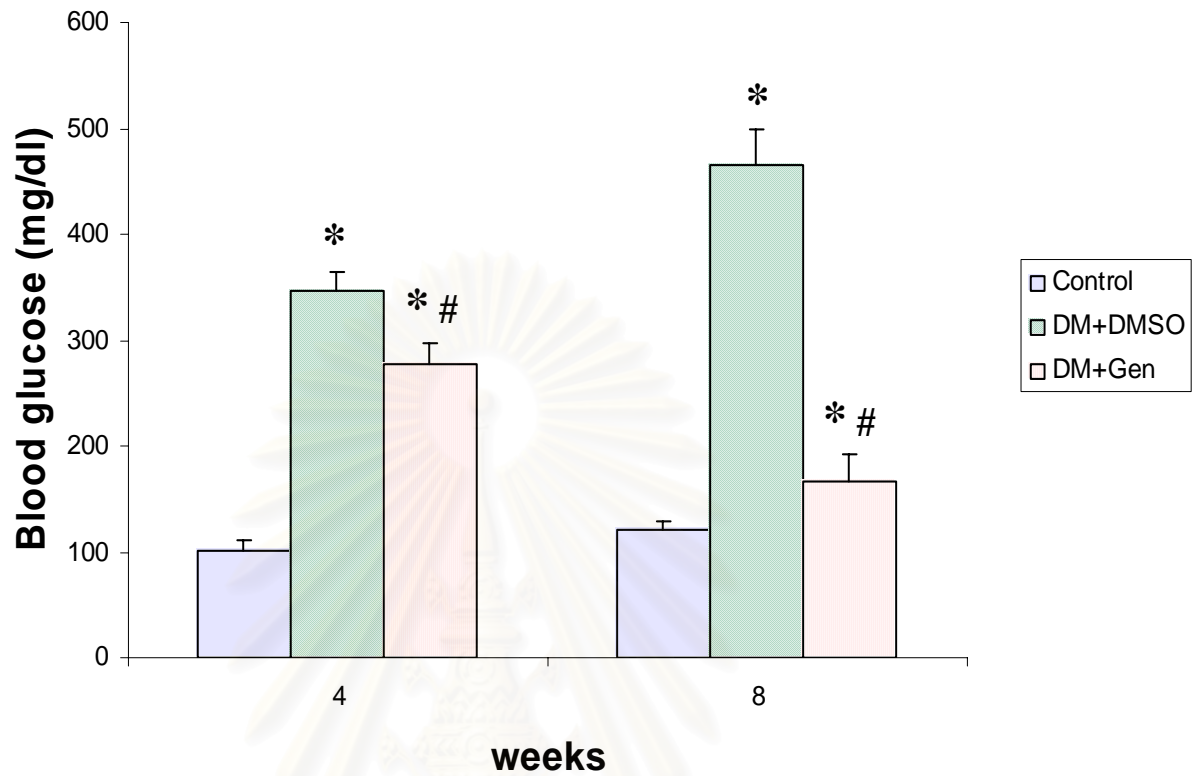
Figure 4.4 Blood glucose (mg/dl) of control treated rats, DMSO treated

rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).
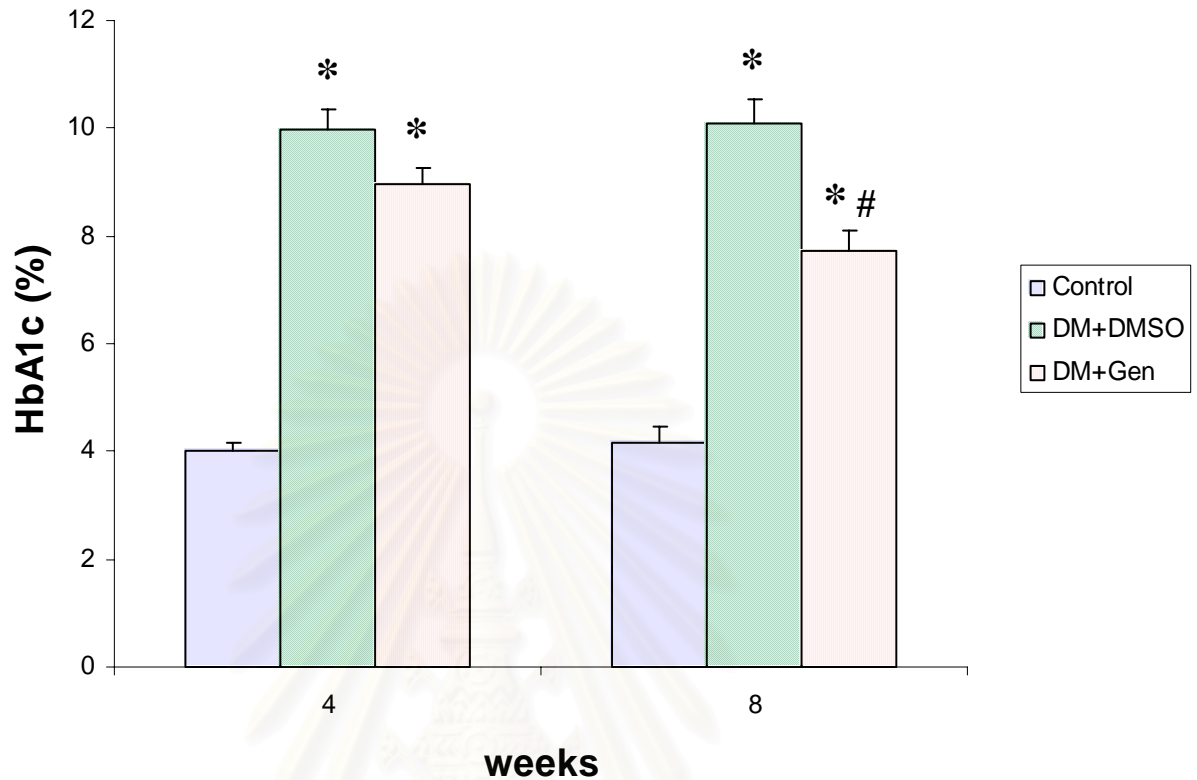
Figure 4.5 HbA$_{1c}$ (%) of control treated rats, DMSO treated rats, and
genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).
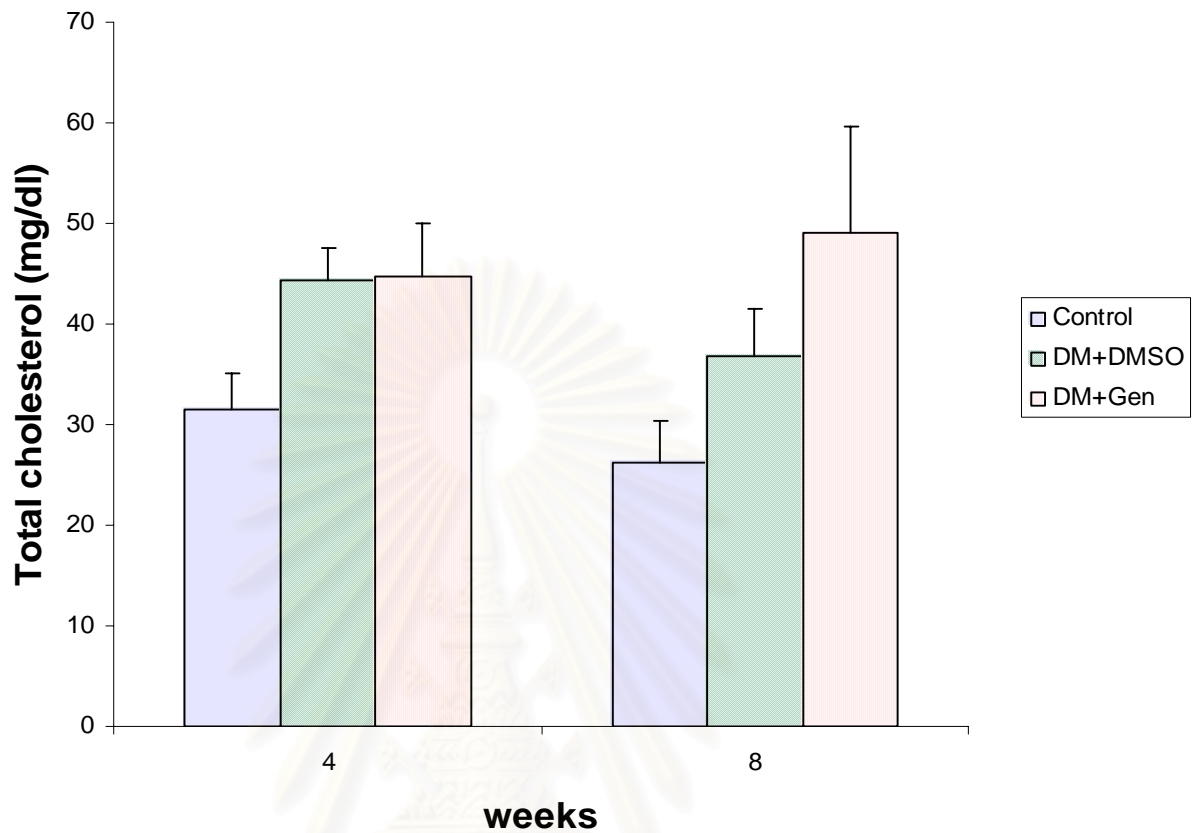
Figure 4.6 Total cholesterol (mg/dl) of control treated rats, DMSO

treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6
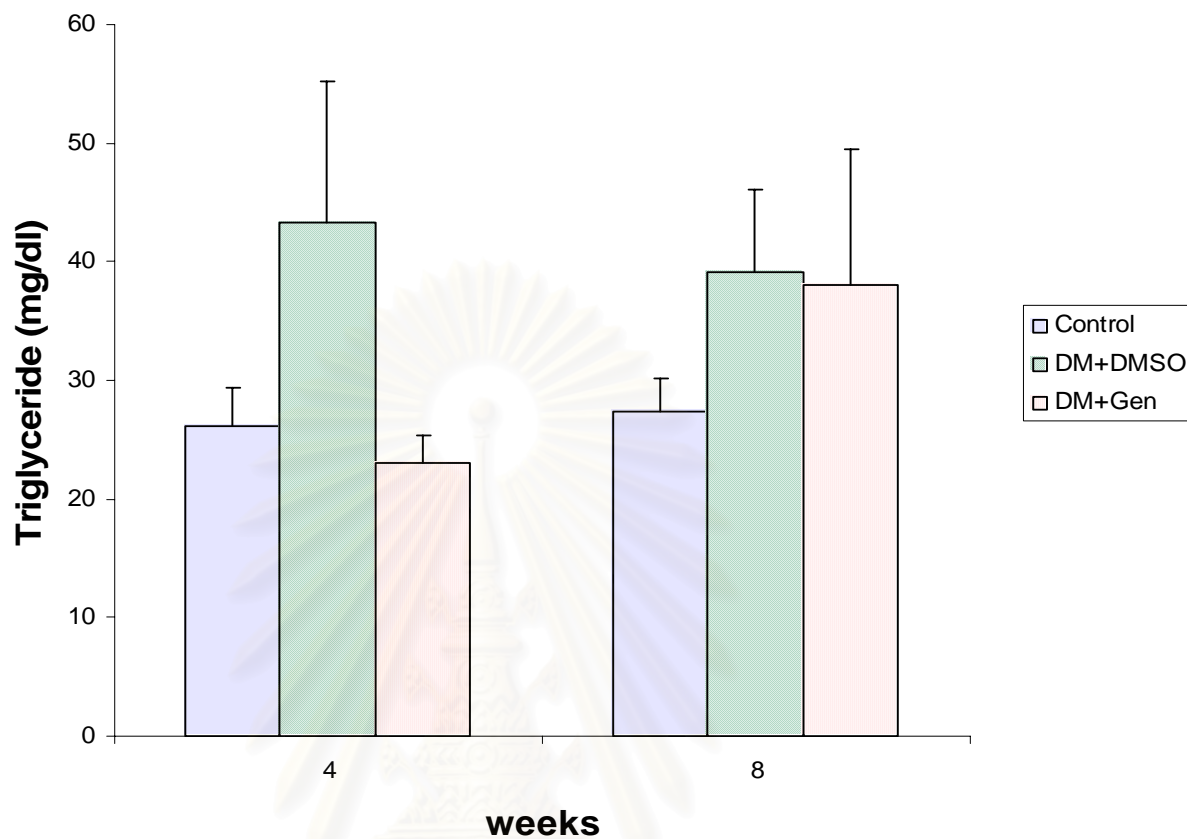
Figure 4.7 Triglyceride (mg/dl) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

44
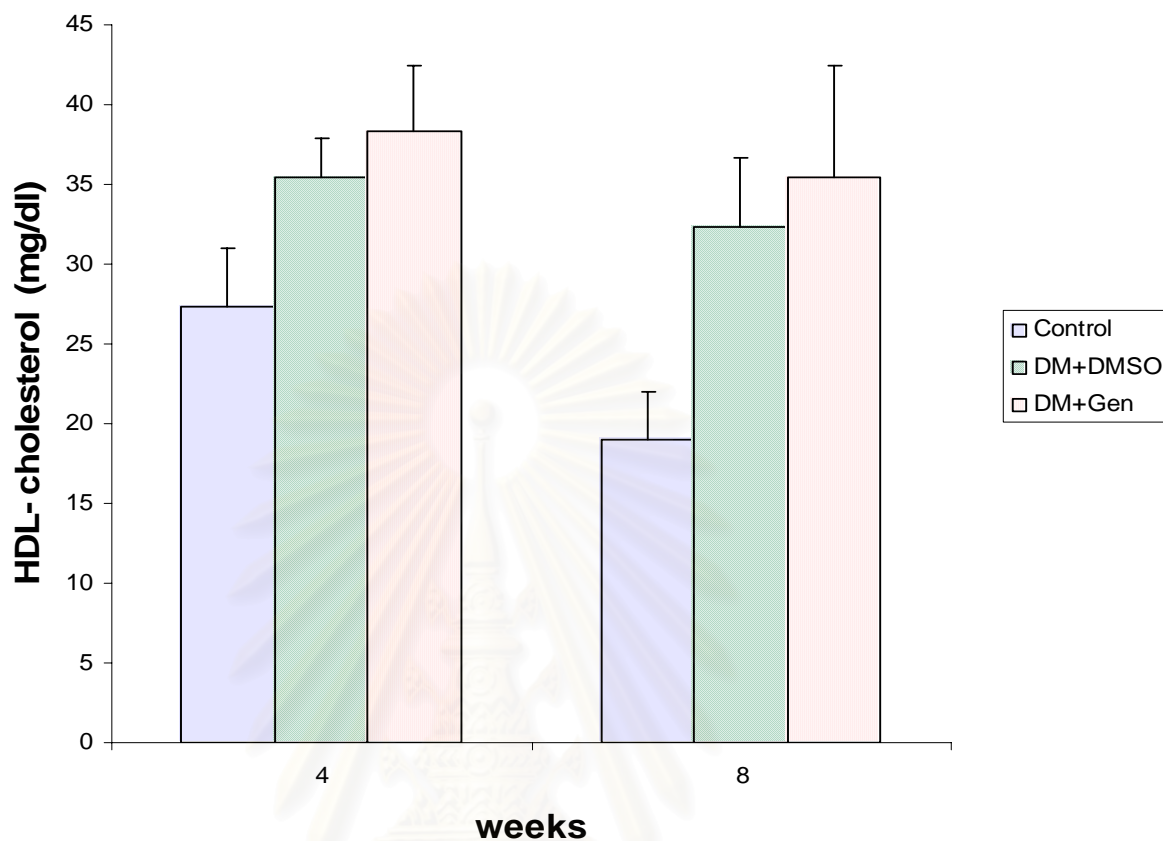
Figure 4.8 HDL-cholesterol (mg/dl) of control treated rats, DMSO
treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6
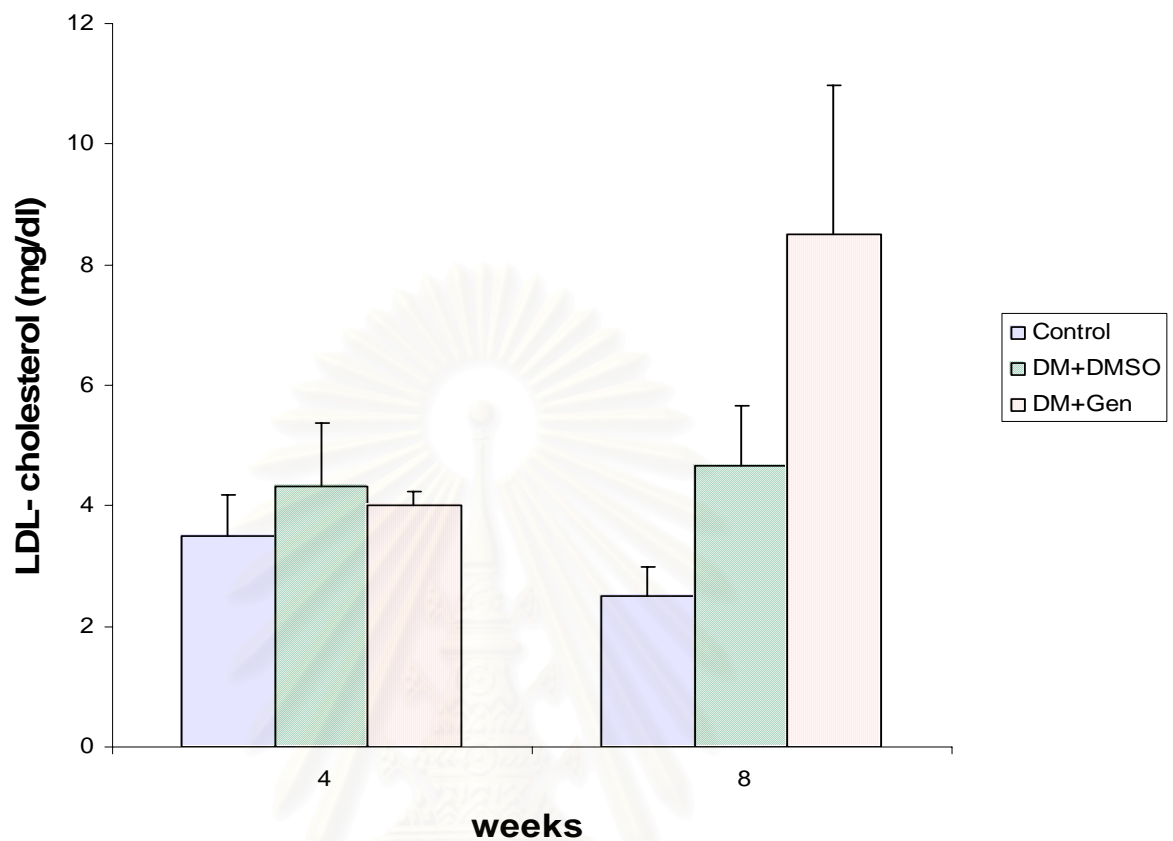
Figure 4.9 LDL-cholesterol (mg/dl) of control treated rats, DMSO

treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6
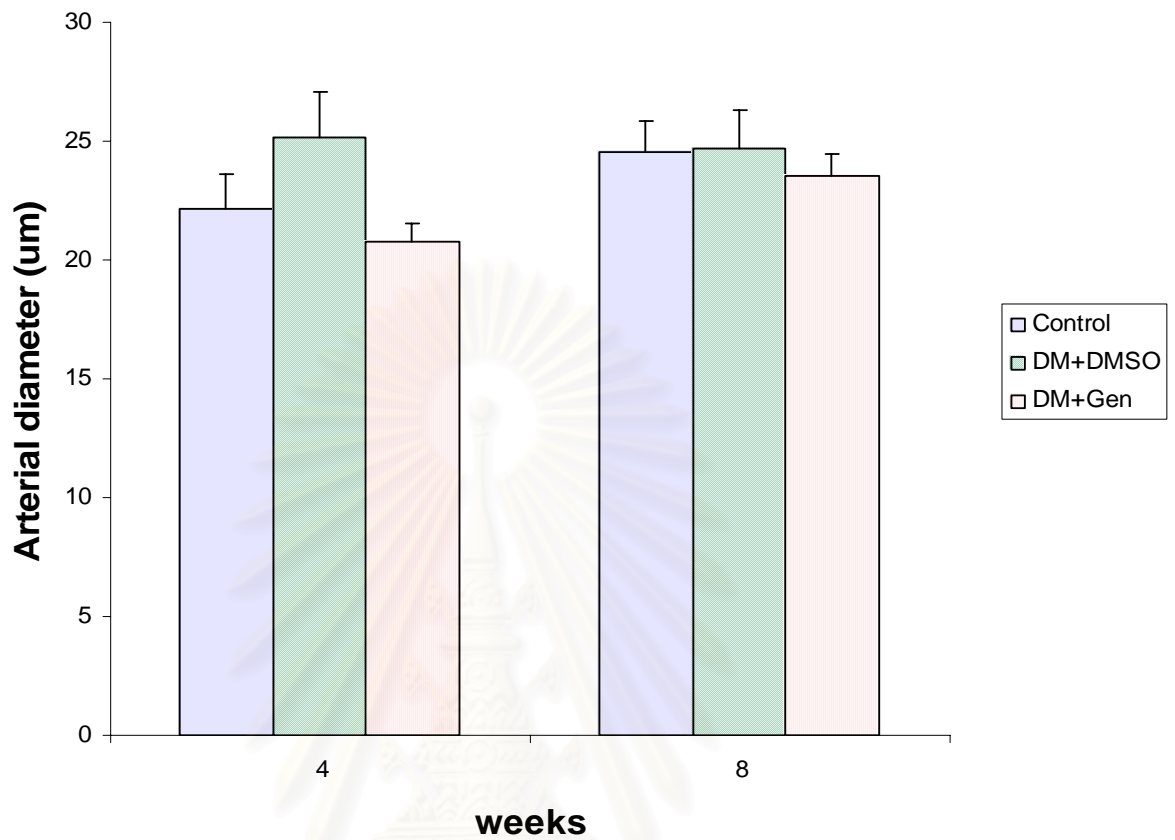
Figure 4.10 Arteriolar diameter in response to norepinephrine (10^{-5} M)
of control treated rats, DMSO treated rats, and genistein
treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6
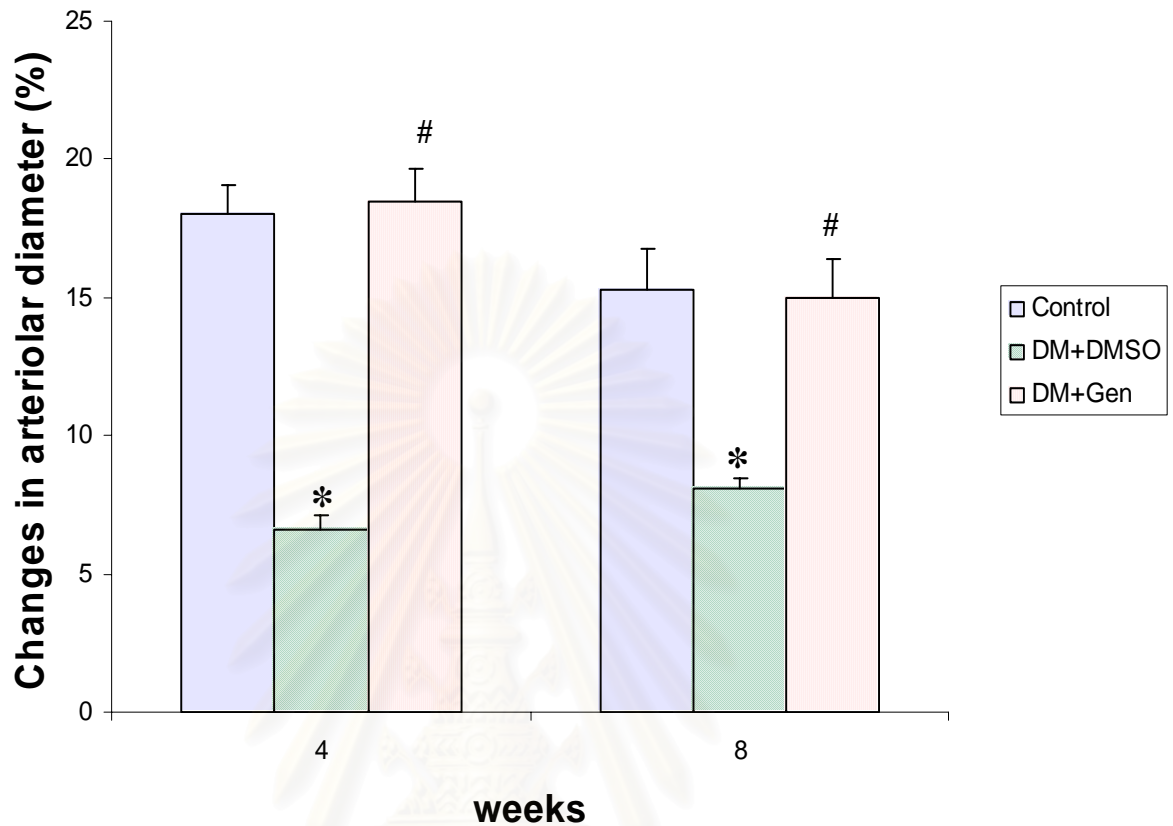
Figure 4.11 Changes in arteriolar diameter in response to

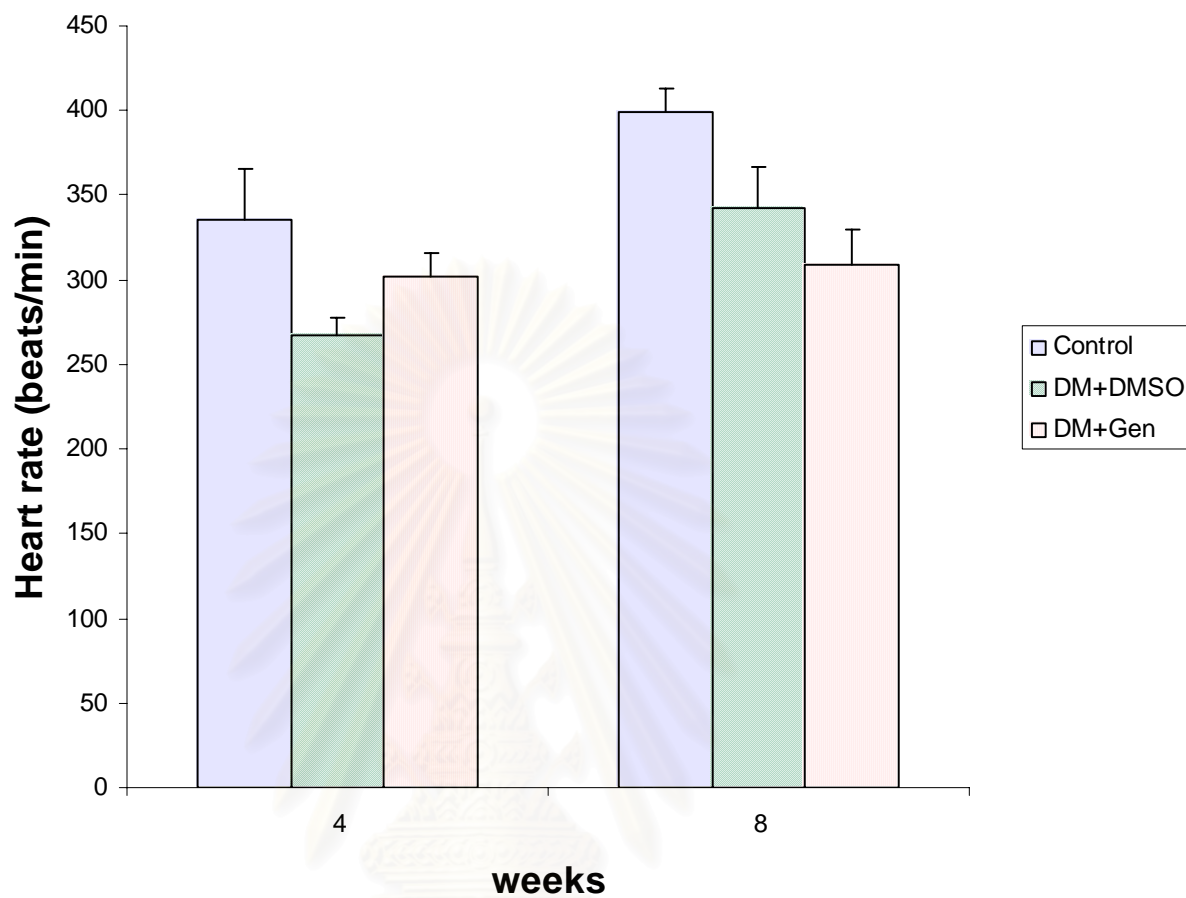acetylcholine (10^{-5} M) of control treated rats, DMSO

treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

* Significant difference as compared to control ($p < 0.05$).

Significant difference as compared to DM+DMSO ($p < 0.05$).
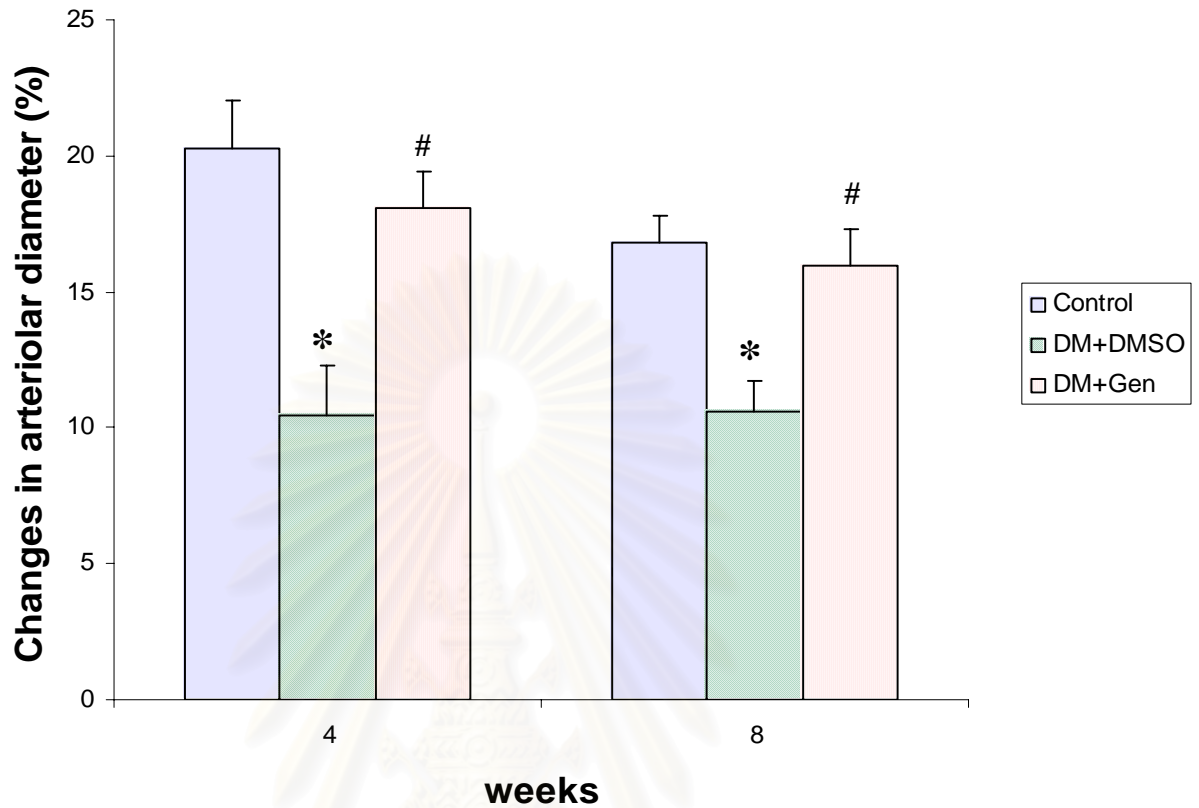
Figure 4.12 Changes in arteriolar diameter in response to

sodium nitroprusside (10^{-5} M) of control treated rats, DMSO

treated rats, and genistein treated rats at 4 and 8 weeks.

Values are means ± SEM; n = 6

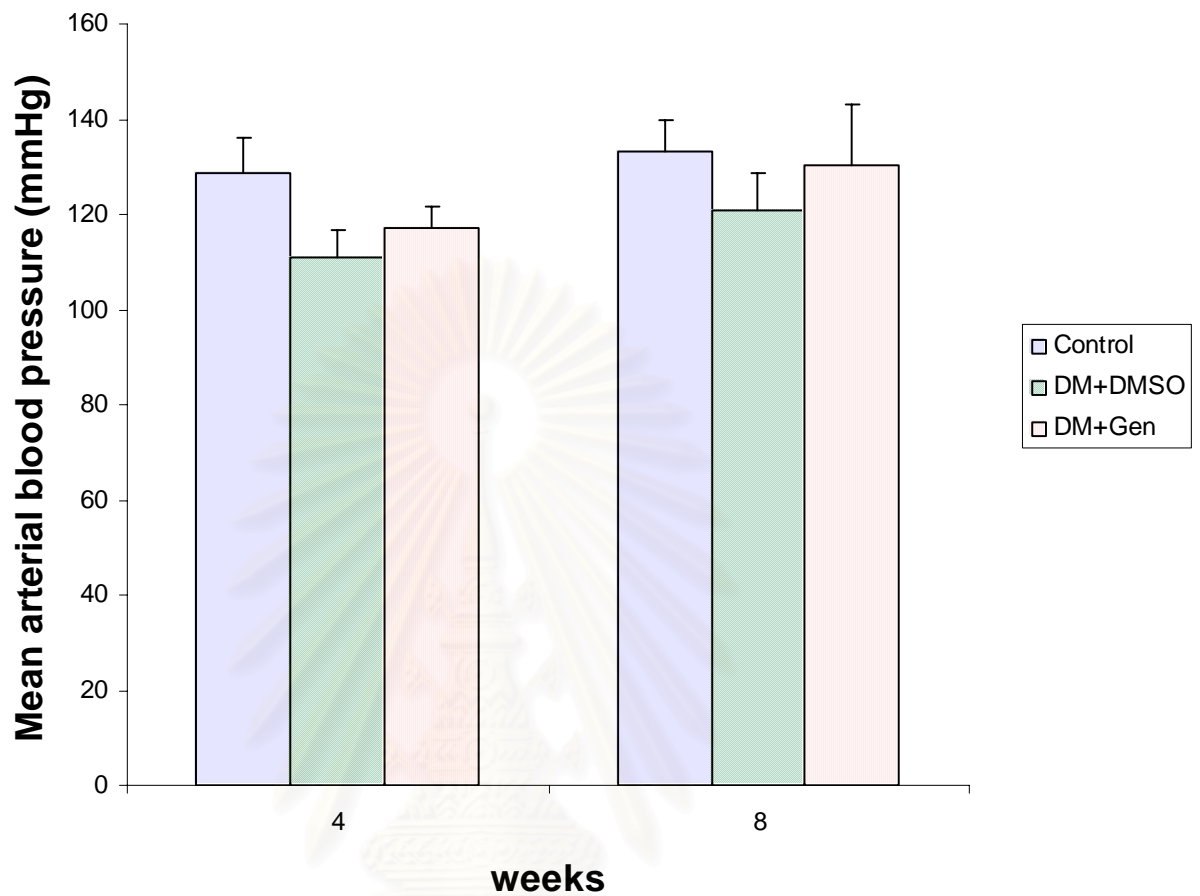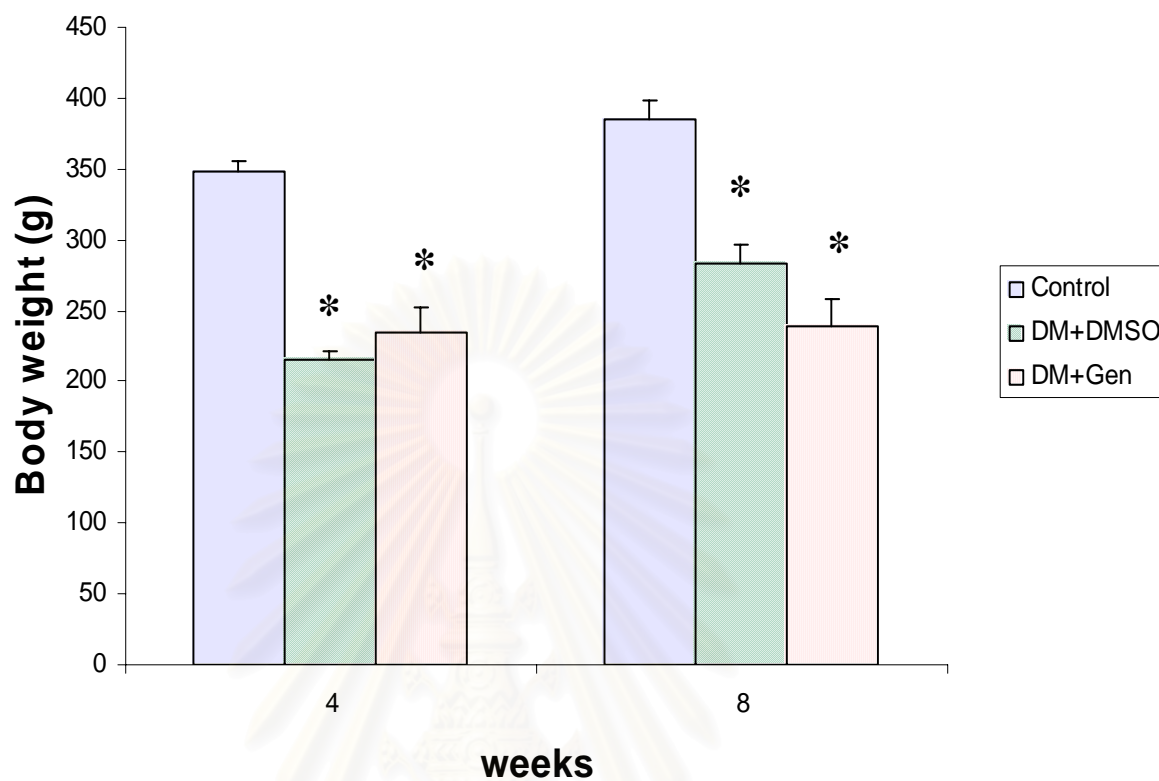* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).
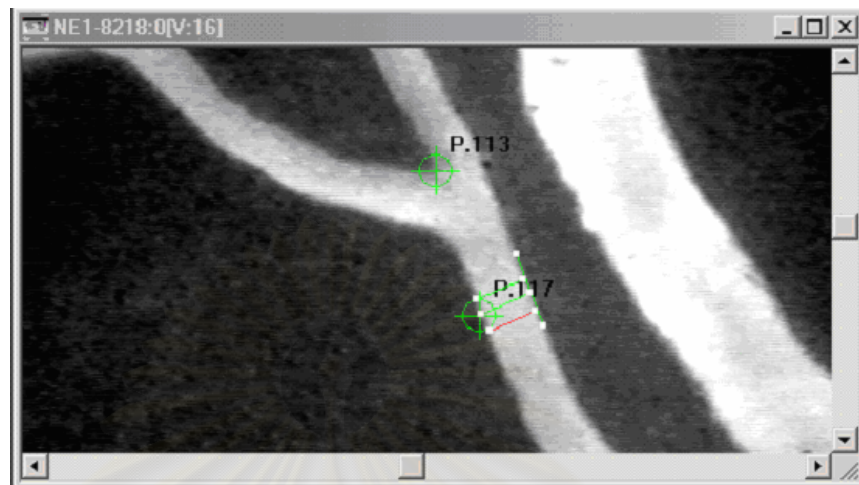
Figure 4.13 Image vascular response to norepinephrine (10^{-5}) of
genistein treated diabetic rats at 8 weeks.

Figure 4.14 Image vascular response to acetylcholine (10^{-5}) of
genistein treated diabetic rats at 8 weeks.

CHAPTER V

DISCUSSION

In the present study, the effect of genistein on diabetic induced endothelial dysfunction was studied by using intravital fluorescent video microscopy. The diabetic condition was induced by streptozotocin (STZ, iv. 65 mg/kg bw). The inclusion criteria for diabetes was confirmed by blood glucose $\geq$ 200 mg/dl (Richard *et al.* 2000). The vasorelaxation in response to endothelial dependent (10^{-5}M; Ach) and endothelial independent agents (10^{-5}M; SNP) were used to characterize the endothelial dysfunction at 4 and 8 weeks after STZ injection.

Study of blood glucose and HbA_{1c} levels

As showed in Figures 4.4-4.5, the results demonstrated that blood glucose and HbA_{1c} levels in diabetic groups were significantly increased in both 4 and 8 weeks from experimental periods. Interestingly, blood glucose and HbA_{1c} levels of 4 weeks and 8 weeks genistein treated groups were significantly decreased as compared to their age-matched STZ groups (p<0.05).

There were two earlier reports by Ohno et al. (1993) and Jonas et al. (1995) which suggested that genistein could stimulate insulin secretion from pancreatic β cells via independent protein tyrosine kinase (PTK) inhibition. In 1999, Persaud et al. had added more insight data that 50 µM genistein significantly potentiated glucose-stimulated insulin release from

rat islets through the voltage-dependent Ca^{2+} channel which regulated Ca^{2+} influx into β cells after food intake. Besides, it has been reported that genistein treatment significantly decreased fasting glucose in postmenopausal women as well (Crisafulli *et al.* 2005; Shao-Yi *et al.* 2004).

Since in our study, the model of STZ was used, as hypoinsulinemia there may have a few of β cell left and genistein may be stimulate the residual β-cells to secrete more insulin and resulted to its hypoglycemic action.

Study of lipid profiles

Our findings showed in Figures 4.6-4.9, demonstrated that levels of cholesterol, triglyceride, HDL-C and LDL-C were unchanged in both 4 and 8 weeks of diabetic rats. Similar to both 4 and 8-weeks genistein treated groups there were no significantly different as compared to their age-matched DM+DMSO groups (p<0.05).

These finding also demonstrated in previous results of Squadrito and coworkers (1999), reported that the period of endothelial dysfunction did not correlate with changing of lipid profiles. Moreover, phytoestrogens intake did not lead to change in LDL oxidizability or plasma levels of total cholesterol or triacylglycerol (Simons *et al.* 2000; Samman *et al.* 1999; Dewell *et al.* 2002). In addition, Vega-lopez et al. (2005) reported that phytoestrogens had not significantly reduced LDL oxidation.

In rats, no difference was reported in total cholesterol levels between 2 and 12 months of age but a significant increase in total cholesterol was observed at the age of 24 months (Lacko and Davis 1979). Which is in accordance with Aguila et al. (2002). Despite the difficulty in producing hyperlipidemia and atherosclerosis in rats, special diets may induce an increase in the serum levels of cholesterol, and also induce arterial hypertension (Gill *et al.* 1989).

During 1960 to 1993, several studies on the hypocholesterolemic effects of soy protein in both humans and animal model had been performed. However, the American Heart Association (AHA) had concluded that soy protein could lower serum cholesterol only in animals but not humans (Chait *et al.* 1993). Formal recognition of the cholesterol lowering properties of soy protein, had reported come one year earlier, in 1999, when the U.S. Food and Drug Adiminstration (FDA) approved a health claim for the cholesterol-lowering effects reduction (Food and Drug Adiministration, 1999). Some studies suggested that 25g soy protein was needed for cholesterol reduction (Nagata *et al.* 1998; Ho *et al.* 2000; Tonstad *et al.* 2002; Teixeira *et al.* 2000). It is clear that our dose of genistein used in our study was much less than 25g. We also demonstrated endothelial dysfunction in STZ-rats despite no significant change in lipid profile. These may support that the endothelial dysfunction in diabetes mellitus didn't result from lipid abnormalities and effect of genistein in preventing endothelial dysfunction in diabetes mellitus has other mechanism(s).

Study of endothelial function

Endothelium-dependent relaxation

In this study, STZ was used to induce diabetic state in order to imitate type I diabetes mellitus. The results showed that in DM+DMSO group, the responses to both vasodilators were significantly decreased. Hyperglycemia had led to endothelial dysfunction as which normally named as diabetes-induced endothelial dysfunction. Hyperglycemia produces free radicals through several pathways including advanced glycation end products (AGEs), polyol pathway activity, and activating NAD(P)H oxidase (Baynes 1991, Tesfamariam 1994, Inoguchi *et al.* 2000). The impaired relaxation to acetylcholine in the diabetic arterioles agreed with the majority of studies in isolated conduit arteries, but several studies have shown unchanged or augmented relaxant responses to muscarinic agonists in diabetic arteries (Ozturk *et al.* 1996; Cooper *et al.* 2001).

It has been shown that the attenuated relaxant responses to acetylcholine in diabetic rat aorta are dependent on the duration of the diabetes (Orie *et al.* 1993; Pieper 1999). Although exposure to elevated glucose levels the impairment of acetylcholine-induced relaxation had been observed that free radical scavengers, such as superoxide dismutase, could prevent this impairment (Tesfamariam and Cohen, 1992; Taylor and Poston, 1994a). Moreover, oxygen-derived free radicals had been shown to abolish endothelium-dependent relaxation in both normal (Gryglewski *et al.* 1986; Rubanyi and Vanhoutte 1986) and diabetic

blood vessels (Pieper and Gross 1988; Tesfamariam and Cohen, 1992; Chang *et al.* 1993).

Previous studies have established a role for estrogen in the regulation of vascular function. Estrogen can act directly on the vascular endothelial cells to enhance nitric oxide (NO) synthesis through genomic stimulation of endothelial NO synthase (eNOS) expression (MacRitchie *et al.* 1997) and by receptor-mediated, nongenomic, eNOS activation (Chen *et al.* 1999). However, it is unknown whether the phytoestrogen, genistein, has a similar effect or not. Genistein ingestion can increase circulating nitrate/nitrite (Squadrito *et al.* 2002) and endothelium dependent vasodilation in humans (Walker *et al.* 2001; Squadrito *et al.* 2002). In animal models, genistein induces NO-mediated relaxation of rat pulmonary arteries (Karamsetty and Klinger *et al.* 2001) and aorta (Mishra *et al.* 2000), suggested that genistein may directly act on vascular endothelium to regulate eNOS. Other studies suggested that genistein may induce vascular relaxation by cAMP-dependent mechanisms (Satake *et al.* 1999) or inhibition of tyrosine kinases (Duarte *et al.* 1997).

By using genistein, several studies have show that genistein have benefit effects on preventing vascular dysfunction in different pathogenesis such as atherosclerotic female macaques (Honore *et al.* 1997) and in ovariectomized rats (Squadrito *et al.* 2000, Khemapech *et al.* 2003, Chanawirat *et al.* 2006). In vivo, genistein has also been reported to improve endothelial function in several animal models (Bermejo *et al.* 2003), including SHR, hypertensive rat model (Kitayama *et al.* 2002). However genistein or soy supplements have been proven to be effective

in protecting endothelial dysfunction in men and postmenopausal women, in some but not in all studies (Simons *et al*. 2000; Squadrito *et al*. 2002).

Yousif et al. (2005) study the role of tyrosine kinase-mediated pathways in diabetes-induced alterations in responsiveness of rat carotid artery. They found that treatment with genistein, AG1478 or AG825 resulted in a significant improvement in diabetes-induced impairment in endothelium-dependent relaxation.

Dongmin et al. (2004) found that genistein could activate endothelial nitric oxide synthase (eNOS) in intact bovine aorta and in human umbilical vein within 10 min incubation period. They indicated that the maximal eNOS activity was produced by 1 uM genistein. With this 1 uM genistein, the phosphorylation of eNOS at serine 1179 was maximally performed at 10 min. Moreover, they also demonstrated that this rapid activation of eNOS was not dependent on RNA transcription or new protein synthesis and was not blocked by estrogen receptor antagonist as well.

Besides, Ibrahim et al. (2005) showed that treatment with genistein could produce a significant normalization of the altered agonist-induced vasoconstrictor responses in diabetic rats.

By using the same dose of genistein (0.25mg/kg bw), Siriviriyakul and his co-worker (2006) has showed that effect of endothelial-dependent relaxation of genistein in ovarectomized rat was mediated through both NO and PGI_2.

Endothelium-independent relaxation

In our study, the endothelium- independent response was investigated by analyzing the relaxant effect of sodium nitroprusside. Because of sodium nitroprusside causes relaxation in a vessel via endothelium independent pathway, or by direct activation of guanylate cyclase in smooth muscle cell. This activation leads to increasing the rate of formation of cyclic GMP in the vascular smooth muscle cell which initiates the process of relaxation (Rapoport and Murad 1983).

In our study, the result showed that DM+DMSO group had significantly decrease response to sodium nitroprusside induced vasodilatation of mesenteric arteriolar. The vascular endothelium regulates the arterial tone and blood flow by affecting the vascular smooth muscle tone.

From the study of Ricardo et al. in 2001, the result showed that the dose-dependent vasorelaxant response to sodium nitroprusside (0.01–100 mM) in aortic ring segments was impaired similarly by diabetes in both genders. Moreover, Bassirat and Khalil (2000) suggested that 4-weeks diabetes induced SNP-response reduction might be due to endothelin and free radicals altered microvascular function. Several other mechanisms had been postulated such as altered guanylate cyclase activity or other down-stream NO pathways such as decreased G-kinase efficacy (Pieper1999; Ren *et al.* 1997; Head *et al.* 1987; Wakabayashi *et al.* 1987).

Therefore, our finding demonstrated that both vasorelaxant response to Ach and SNP were decreased in DM rats. It might be

explained that DM had caused both impairment of NO bioactivity and reducing the efficiency of the 2nd messenger system (cGMP) or activating protein kinase C (PKC). *(Since PKC can counteract the reduction in intracellular Ca2+ activated by cGMP (Khalil et al., 1996).*

From our results, DM+Gen group could improve mesenteric arteriolar dilation to sodium nitroprusside as compared to DM+DMSO group. Genistein is a well-established and effective nonselective tyrosine kinase inhibitor (Akiyama *et al.* 1987), and thus, may inhibit tyrosine kinase-mediated contraction of vascular smooth muscle (Liu and Sturek, 1996), in particular, the responses to 5-hydroxytryptamine (Watts *et al.* 1996). The cyclic nucleotides cAMP and cGMP shift the concentration–contraction curve to Ca2+ to the right in the rat mesenteric artery (Kawada *et al.* 1997, Simard 1997; White *et al.* 2000). There is evidence of crossover activation of cAMP-dependent protein kinase by cGMP in vascular smooth muscle (Cornwell *et al.* 1994; Ruiz-Velasco *et al.* 1998). Therefore, it may be another possibility that genistein that indeed causes vasodilatation by nitric oxide activated cAMP-dependent protein kinase has further crossed increase in cGMP level as well (Tsukada *et al.* 2002).

The idea has supported by Lee and coworkers in 2004 who indicated a novel mechanism for genistein to exert its modulatory effect through the second messenger cAMP and/or cGMP with the resultant activation of cAMP dependent protein kinase.

Besides, there were some reports indicated the direct effect of genistein on smooth muscle cells also. For example, Nevala et al. (1998)

demonstrated that the direct relaxing effect of genistein (>10uM) in the rat mesenteric artery was independent of the endothelium activity. Findings by Lee and Man (2003) showed that the enhancement of sodium nitroprusside-induced relaxation by genistein in porcine coronary arteries with intact endothelium was independent of NOS activity.

The proposed hypothesis to explain our findings is demonstrated by diagram 5.1. The diagram indicated that hyperglycemia caused the increase in free radicals which then further reduced nitric oxide bioavailablity as showed by Ach results. The endothelial dysfunction was also enhanced by diabetic reduced cGMP as well, as which it showed by the results of SNP.

Our findings has showed that genistein could attenuate these both endothelial dependent and endothelial independent abnormalities. Genistein might protect endothelial and smooth muscle cells through its hypoglycemic and antioxidative effects. The direct effect of genistein on endothelial dependent vasorelaxation has been indicated and implied by nongenomic mechanism similar to estrogen. Together with the literature reviews, therefore, we believe that genistein could increase vasodilatation via its direct action on smooth muscle cells through a cyclic-AMP dependent and/or nonselective tyrosine kinase inhibitor mechanisms as well.
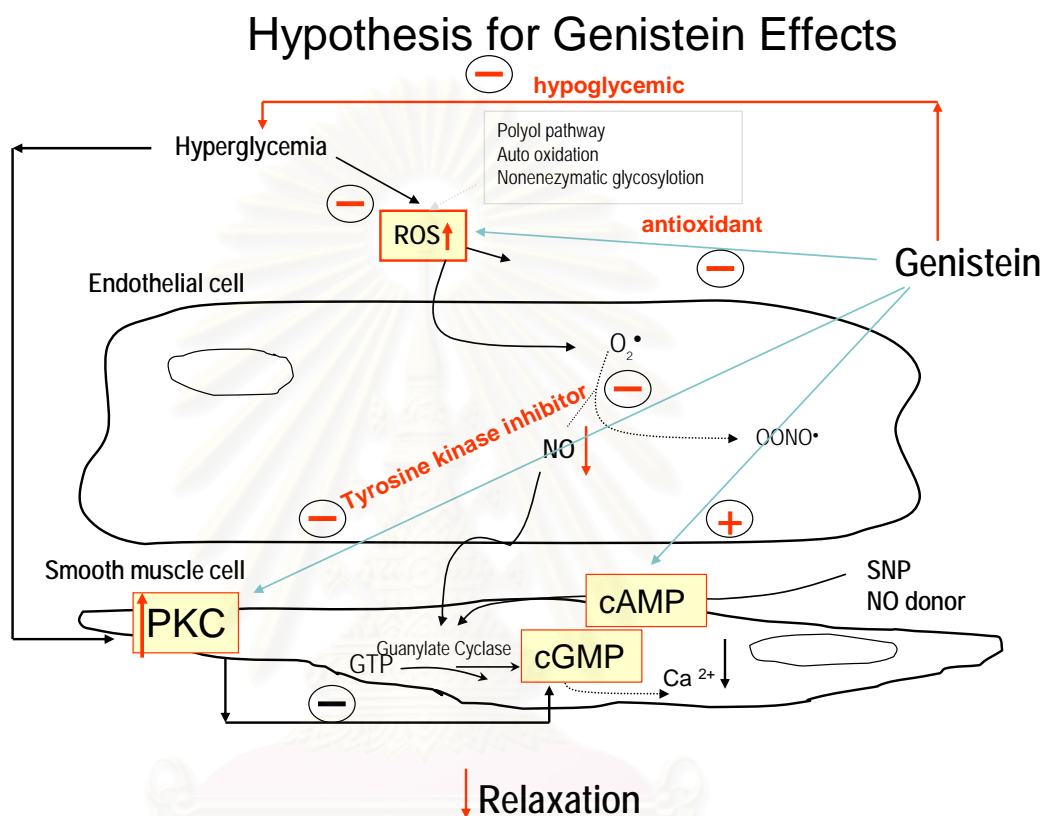
Figure 5.1 Proposed mechanisms of genistein on endothelial dependent
and endothelial independent in diabetes mellitus.

CHAPTER VI
CONCLUSION

This study investigated the effects of genistein on plasma lipid profiles, blood glucose, HbA_{1c} and endothelial function in streptozotocin induced diabetic rats. The overall results of this investigation indicated that:

1. Genistein has no effect on heart rate, blood pressure, and body weight in 4 weeks and 8 weeks diabetic rats.
2. There were no significant changes in lipid profiles in all groups at 4 and 8 weeks. However, blood glucose and HbA_{1c} was decreased significantly in 8-week genistein treated group. At this moment, therefore, it implied that the endothelial dysfunction was not primary caused by dyslipidemia.
3. The daily injection of 0.25 mg/kg body weight of genistein could attenuate the impairment of diabetes induced endothelium-dependent relaxation occurred at 4 and 8 weeks.
4. The daily injection of 0.25 mg/kg body weight of genistein could attenuate the impairment of endothelial-independent relaxation in both 4 and 8 weeks diabetic rats as well.
5. Vascular dysfunction without dyslipidemia of diabetic animals in this study indicate that of vascular dysfunction may not caused by lipid abnormalities.

Our findings have demonstrated that genistein might be a great benefit for preventing diabetes induced vascular complications in the future. The mechanism(s) of genistein to improve endothelial function in diabetes mellitus, especially, its hypoglycemic effect with no significant change in lipid profiles still needs further investigation.

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APPENDIX

Table 1 Heart rate (beats/min) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	Heart rate (beats/min)	
	4 weeks	8 weeks
Control (n=6)	336.00±29.12	398.83±14.02
DM+DMSO (n=6)	267.00±10.24	342.50±24.24
DM+Gen (n=6)	302.33±13.82	308.83±20.75

Values are means ± SEM; n = 6

Table 2 Mean arterial blood pressure of control treated rats, DMSO
treated rats, and genistein treated rats at 4 and 8 weeks.

Groups	Mean arterial blood pressure (mmHg)	
	4 weeks	8 weeks
Control (n=6)	128.81±7.18	133.36±6.37
DM+DMSO (n=6)	110.76± 6.02	120.74±7.99
DM+Gen (n=6)	117.07±4.51	130.29±12.70

Values are means ± SEM; n = 6

Table 3 Body weight (g) of control treated rats, DMSO treated rats, and
genistein treated rats at 4 and 8 weeks.

Groups	Body weight (g)	
	4 weeks	8 weeks
Control (n=6)	348.00±7.28	385.33±13.71
DM+DMSO (n=6)	215.66±6.20*	282.66±13.63*
DM+Gen (n=6)	235.00±17.76*	238.33±19.78*

Values are means ± SEM; n = 6

* Significant difference as compared to control ($p < 0.05$).

Table 4 Blood glucose (mg/dl) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	Blood glucose (mg/dl)	
	4 weeks	8 weeks
Control (n=6)	101.83±8.84	120.83±6.98
DM+DMSO (n=6)	346.16±18.39*	465.83±32.72*
DM+Gen (n=6)	276.50±20.01*#	165.66±25.46*#

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).

Table 5 HbA$_{1c}$ (%) of control treated rats, DMSO treated rats, and
genistein treated rats at 4 and 8 weeks.

Groups	HbA$_{1c}$ (%)	
	4 weeks	8 weeks
Control (n=6)	4.01±0.15	4.15±0.33
DM+DMSO (n=6)	9.98±0.38*	10.08±0.45*
DM+Gen (n=6)	8.95±0.33*	7.71±0.40*[#]

Values are means ± SEM; n = 6

* Significant difference as compared to control ($p < 0.05$).

Significant difference as compared to DM+DMSO ($p < 0.05$).

Table 6 Total cholesterol (mg/dl) of control treated rats, DMSO treated rats, and genistein treated rats at 4 and 8 weeks.

Groups	Total cholesterol (mg/dl)	
	4 weeks	8 weeks
Control (n=6)	31.50±3.65	26.16±4.24
DM+DMSO (n=6)	44.33±3.24	36.83±4.65
DM+Gen (n=6)	44.66±5.40	49.00±10.67

Values are means ± SEM; n = 6

Table 7 Triglyceride (mg/dl) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	Triglyceride (mg/dl)	
	4 weeks	8 weeks
Control (n=6)	26.16±3.19	27.33±2.78
DM+DMSO (n=6)	43.33±11.92	39.16±6.94
DM+Gen (n=6)	23.00±2.38	38.00±11.49

Values are means ± SEM; n = 6

Table 8 HDL-cholesterol (mg/dl) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	HDL- cholesterol (mg/dl)	
	4 weeks	8 weeks
Control (n=6)	27.33±3.67	19.00±3.01
DM+DMSO (n=6)	35.50±2.41	32.33±4.31
DM+Gen (n=6)	38.33±4.08	35.50±6.97

Values are means ± SEM; n = 6

Table 9 LDL-cholesterol (mg/dl) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	LDL- cholesterol (mg/dl)	
	4 weeks	8 weeks
Control (n=6)	3.50±0.67	2.50±0.50
DM+DMSO (n=6)	4.33±1.05	4.66±0.98
DM+Gen (n=6)	4.00±0.25	8.50±2.48

Values are means ± SEM; n = 6

Table 10 Arteriolar diameter in response to norepinephrine (10^{-5} M) of
control treated rats, DMSO treated rats, and genistein treated
rats at 4 and 8 weeks.

Groups	Arteriolar diameter (µm)	
	4 weeks	8 weeks
Control (n=6)	22.12±1.464	24.52±1.31
DM+DMSO (n=6)	25.18±1.877	24.67±1.64
DM+Gen (n=6)	20.75±0.784	23.54±0.91

Values are means ± SEM; n = 6

Table 11 Changes in arteriolar diameter in response to acetylcholine
$(10^{-5}$ M) of control treated rats, DMSO treated rats, and genistein treated rats at 4 and 8 weeks.

Groups	Changes in arteriolar diameter (%)	
	4 weeks	8 weeks
Control (n=6)	18.06±1.03	15.27±1.52
DM+DMSO (n=6)	6.59±0.56*	8.05±0.41*
DM+Gen (n=6)	18.48±1.16#	14.97±1.40#

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).

Table 12 Changes in arteriolar diameter in response to sodium
nitroprusside (10^{-5} M) of control treated rats, DMSO treated
rats, and genistein treated rats at 4 and 8 weeks.

Groups	Changes in arteriolar diameter (%)	
	4 weeks	8 weeks
Control (n=6)	20.26±1.74	16.78±1.03
DM+DMSO (n=6)	10.44±1.85*	10.58±1.15*
DM+Gen (n=6)	18.09±1.32[#]	15.98±1.34[#]

Values are means ± SEM; n = 6

* Significant difference as compared to control (p < 0.05).

Significant difference as compared to DM+DMSO (p < 0.05).

BIOGRAPHY

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