ผลของเจนิสตีนต่อการสูญเสียการทำหน้าที่ของเอนโดทีเลียม ในหนูที่ถูกตัดรังไข่ทั้งสองข้าง

พันตำรวจตรีหญิง ศิริมา เขมะเพชร

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EFFECTS OF GENISTEIN ON ENDOTHELIAL DYSFUNCTION IN BILATERAL OVARIECTOMIZED RAT

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ศิริมา เขมะเพชร: ผลของเจนิสตีนต่อการสูญเสียหน้าที่ของเอนโดทีเลียมในหนูที่ถูกตัดรังไข่ทั้งสองข้าง (EFFECTS OF GENISTEIN ON ENDOTHELIAL DYSFUNCTION IN BILATERAL OVARIECTOMIZED RAT) อ.ที่ปรึกษา: รศ.นพ. ประสงค์ ศิริวิริยะกุล, อ.ที่ปรึกษาร่วม: รศ.ดร.สุทธิลักษณ์ ปทุมราช; 108 หน้า ISBN 974-17-1990-6

วิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของเจนิสตีนด้านการรักษาและป้องกันเอนโดทีเลียมที่สูญเสียหน้าที่การทำงาน รวมทั้งกลไกการออกฤทธิ์ของเจนิสตีน โดยใช้หนูเพศเมียพันธ์ Wistar นำมาตัดรังไข่ทั้งสองข้าง (OVX rat) หนูที่ถูกผ่าตัดแต่ไม่ถูกตัดรังไข่เป็นกลุ่มเปรียบเทียบ แบ่งหนูออกเป็น 2 กลุ่มใหญ่ ได้แก่ กลุ่มที่ศึกษาผลของเจนิสตีนด้านการรักษา และกลุ่มที่ศึกษาผลของเจนิสตีนด้านการป้องกัน หนูกลุ่มที่ใช้ศึกษาผลของเจนิสตีนด้านการรักษา แบ่งออกเป็น 5 กลุ่ม โดย 2 กลุ่มแรก คือ หนูที่ถูกตัดรังไข่ทั้งสองข้าง ($OVX_{3\text{-week}}$) และกลุ่มที่ผ่าตัดแต่ไม่ถูกตัดรังไข่เป็นกลุ่มเปรียบเทียบ ($Sham_{3\text{-week}}$) แล้วเลี้ยงต่ออีก 3 สัปดาห์ เพื่อศึกษาการสูญเสียหน้าที่ของเอนโดทีเลียม จากนั้นจึงใช้ model นี้ศึกษาต่อในหนู 3 กลุ่มที่เหลือ ดังนี้ คือ หนูกลุ่มเปรียบเทียบที่ได้รับตัวทำละลายเจนิสตีน (DMSO 100 μl, sc; $Sham_{veh}$) หนูที่ถูกตัดรังไข่แล้วได้รับตัวทำละลายเจนิสตีน (DMSO 100 μl, sc; OVX_{veh}) และ หนูที่ถูกตัดรังไข่แล้วได้รับเจนิสตีน (genistein 0.25 mg / Kg BW, sc; OVX_{gen}) หนูกลุ่มที่ใช้ศึกษาผลของเจนิสตีนด้านการป้องกันการสูญเสียหน้าที่ของเอนโดทีเลียม แบ่งเป็น 3 กลุ่ม คือ $Sham_{veh}$ OVX_{veh}, และ OVX_{gen} การให้เจนิสตีนหรือตัวทำละลายเจนิสตีนนั้นให้ด้วยขนาด และวิธีการเดียวกับกลุ่มที่ศึกษาผลการรักษา กลุ่มรักษาจะเริ่มให้ภายหลังทำการผ่าตัด 3 สัปดาห์ต่อเนื่องทุกวันเป็นเวลา 4 สัปดาห์ และให้ทันทีภายหลังจากการผ่าตัด ต่อเนื่องทุกวันเป็นเวลา 7 สัปดาห์ในกลุ่มป้องกัน จากนั้นจึงศึกษาตัวแปรต่างๆ

ผลการศึกษาวิจัยพบว่า หนูที่ถูกตัดรังไข่ทั้งสองข้าง ($OVX_{3\text{-week}}$, OVX_{veh}) ค่าเฉลี่ยความดันหลอดเลือดแดง (MAP) จะสูงกว่ากลุ่มเปรียบเทียบ อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ซึ่งไม่พบการเปลี่ยนแปลงดังกล่าวในกลุ่มที่ได้รับเจนิสตีน นอกจากนี้ยังพบว่าการตอบสนองต่อ Ach ลดลงอย่างมีนัยสำคัญทางสถิติแต่ไม่พบการเปลี่ยนแปลงในการตอบสนองต่อ SNP อย่างไรก็ตามเจนิสตีนนั้นมีผลในการบรรเทาความผิดปกติที่เกิดขึ้นนี้ ($p <0.001$) ผลด้านการป้องกันคล้ายกับด้านการรักษา ได้แก่การตอบสนองต่อ Ach ลดลงในกลุ่ม OVX_{veh} เปรียบเทียบกับ $Sham_{veh}$ และ OVX_{gen} ตามลำดับ ($p < 0.001$) ในขณะที่การตอบสนองต่อ SNP ไม่มีการเปลี่ยนแปลงเช่นกัน นอกจากนี้ INDO (cyclooxygenase inhibitor) สามารถยับยั้งการออกฤทธิ์ของเจนิสตีนต่อเอนโดทีเลียมได้ แสดงว่าเจนิสตีนออกฤทธิ์ผ่านวิถี cyclooxygenase

สรุปจากผลการศึกษาแสดงให้เห็นว่าเจนิสตีนอาจมีผลทั้งด้านการรักษาหรือป้องกันการสูญเสียหน้าที่การทำงานของเอนโดทีเลียมโดยผ่านวิถี cyclooxygenase เป็นหลัก ซึ่งอาจพัฒนาเป็นแนวทางเลือกใหม่สำหรับการป้องกันการสูญเสียหน้าที่ของเอนโดทีเลียมในสตรีวัยหมดประจำเดือนในอนาคตได้

สหสาขาวิชา.....……. ลายมือชื่อนิสิต.....

สาขาวิชา..... ลายมือชื่ออาจารย์ที่ปรึกษา.....……..

ปีการศึกษา..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4289986920: MAJOR PHYSIOLOGY
KEYWORD: GENISTEIN, ENDOTHELIAL DYSFUNCTION, OVARIECTOMIZED RAT

SIRIMA KHEMAPECH: EFFECTS OF GENISTEIN ON ENDOTHELIAL DYSFUNCTION IN BIALTERAL OVARIECTOMIZED RAT. THESIS ADVISOR: ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D. THESIS CO-ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D 108 pp. ISBN 974-17-1990-6

The objective of our study was to examine the treatment and preventive effects of genistein on endothelial dysfunction in bilateral ovariectomized rats and also clarify the mechanism(s) of action of genistein on endothelial cells. Female Wistar rats were subjected to a bilateral ovariectomy (OVX rat). Sham-operated animals were used as control. These animals were divided into two major groups; treatment and preventive groups. Treatment group was subdivided into five groups. After 3 weeks of washout period, the endothelial dysfunction was confirmed in ovx group ($OVX_{3\text{-week}}$) comparing with the normally response to vasodilators in the control ($Sham_{3\text{-week}}$) group. The other animals were further divided into three groups; sham with vehicle (DMSO 100 µl / day, sc; $Sham_{veh}$), ovx with vehicle (DMSO 100 µl / day, sc; OVX_{veh}), and ovx with genistein (0.25 mg / kg / day, sc; OVX_{gen}). The preventive group was divided into three subgroups of sham with vehicle ($Sham_{veh}$), ovx with vehicle (OVX_{veh}) and ovx with genistein (OVX_{gen}). The treatment and preventive groups were received genistein or vehicle after washout period and immediately after surgery everyday for 4 and 7 weeks, respectively. Vehicle (DMSO) or genistein were given by the same procedure for both preventive and treatment groups. All parameters were monitored after treatment for 4 weeks and 7 weeks, respectively.

The experimental results of treatment group indicated that mean arterial pressure (MAP) of both $OVX_{3\text{-week}}$ and OVX_{veh} groups were significantly increased as compared to their sham groups ($p < 0.05$). Interestingly, that significantly increased MAP was not observed in OVX_{gen}. Moreover, the studies of vasodilator responses have demonstrated only for the significant decrement of the response to Ach, not for SNP, in ovx rats. However, the tretment effect of genistein could significantly attenuate this abnormality ($p < 0.001$). The results of preventive group demonstrated as similar to the treatment group, especially Ach-induced vasorelaxation. It was markedly decreased in OVX_{veh} as compared to $Sham_{veh}$ and OVX_{gen}, respectively ($p< 0.001$). But the studied for SNP-induced vasorelaxation was unchanged. Moreover, our results demonstrated that INDO (cyclooxygenase inhibitor) could block the effect of genistein on endothelial cells. This result indicated that genistein acted via cyclooxygenase pathway.

In conclusion, genistein supplementation might be therapy or prevent endothelial dysfunction mainly via cyclooxygenase pathway. The findings suggested that genistein might be able to used as a new trend for preventing menopausal vascular endothelial dysfunction.

Inter-department…… Student's signature……

Field of study…….. Advisor's signature……

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

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LIST OF ABBREVIATIONS

Ach	=	acetylcholine
ADMA	=	asymmetrical dimethylarginine
ADP	=	adenosine diphosphate
AT	=	angiotensin
bFGF	=	basic fibroblast growth factor
BMI	=	body mass index
cAMP	=	cyclic 3′,5′-adenosine monophosphate
cGMP	=	cyclic3′,5′-guanosine monophosphate
CHD	=	coronary heart disease
COX	=	cyclooxygenase
CVD	=	cardiovascular disease
DNA	=	deoxyribonucleic acid
E_2	=	estradiol
ECE	=	endothelin-converting enzyme
EC_{50}	=	effective concentration dose
ECs	=	endothelial cells
ECHF	=	endothelium-derived hyperpolarizing factor
EGF	=	epidermal growth factor
ER	=	estrogen receptor
ERT	=	estrogen replacement therapy
ET	=	endothelin
ET_A	=	endothelin receptor subtype A
ET_B	=	endothelin receptor subtype B
HDL-c	=	high-density lipoproteins cholesterol
HRT	=	hormone replacement therapy
IFS	=	isoflavone synthase

LIST OF ABBREVIATIONS (Cont.)

INDO	=	indomethacin
LDL-c	=	low-density lipoproteins cholesterol
L-NAME	=	N^{ω}-nitroarginine methyl ester
L-NMMA	=	L-N^{G}–monomethyl arginine
M	=	molar
MAP	=	mean arterial pressure
NADH	=	nicotinamide adenine dinucleotide
NADPH	=	nicotinamide adenine dinucleotide phosphate
NE	=	norepinephrine
NO	=	nitric oxide
NOS	=	nitric oxide synthase
NS	=	non significant
OVX	=	ovariectomy
PA	=	plasminogen activator
PAF	=	platelet-activating factor
PAI-1	=	plasminogen activator inhibitor 1
PEs	=	phytoestrogens
PGI_2	=	prostacyclin
PHS-II	=	prostacyclin H synthase-II
SERM	=	selective estrogen receptor modulator
SNP	=	sodium nitroprusside
TGFβ1	=	transforming growth factor beta 1
veh	=	vehicle
VLDL-c	=	very low density lipoproteins cholesterol

CHAPTER I

INTRODUCTION

Rationale

Menopause designates the permanent cessation of menses, which women experience at the average of 51 years.[1] This is but one aspect of the climateric, the end of woman's reproductive potential, when she experiences endocrinologic, somatic and psychological changes. The changes that occur at menopause are related both to aging and to decreased estrogen levels, and it is difficult to quantify the respective effects of each.[2]

At the beginning of the 21st century, a woman who reaches the menopause can be expected to live until age 85. Thus, a woman can anticipate living about one-third of her life without ovarian hormones; this fact alone makes the well-being of postmenopausal women a major medical concern.[1]

Cardiovascular disease (CVD) is the leading cause of death among women in industrial country. Premenopausal women have approximately one-fifth the CVD mortality of men, but after menopause their mortality exponentially rises to approach that of men.[2] One of the explanation is that estrogen of a premenopausal women confers protection, which is lost at menopause; this is supported by the observation that women who undergo a premature surgical menopause and who do not use postmenopausal estrogens have twice as much CVD as age-matched premenopausal controls.[2]

It is widely known that postmenopausal estrogen replacement therapy (ERT) can considerably improve the quality of life and contribute to a woman's well-being after her reproductive life cycle. Not only can it eliminate the vasomotor symptoms such as hot flushes, sweating and sleep pattern disturbance, the

administration of estrogens can also have positive effects on lipid profiles, endothelial cell function, vascular reactivity, hemostatic factors and bone.[3] ERT initially alleviates specific symptoms associated with the decline in estrogens production after menopause. However, while ERT has beneficial effects on its target tissue, it also produces some adverse effects, for example, hypertriglyceridemia, hypercoagulable states, endometrial hyperplasia, breast cancer and angiogenesis.[3]

Therefore, many investigators have been trying to develop other modalities to suit properly for the menopausal women such as adding the progestrogen to prevent endometrial cancer,[4, 5] adding SERM (Selective Estrogen Receptor modulator) to prevent breast and endometrial cancer[6] or using other substances to replace estrogen, such as phytoestrogen.

Plant-derived estrogens or phytoestrogens (PEs) have a similar structure to estradiol and have weak affinity for the estrogen receptors.[3] They, possibly selective estrogen agonists, may be superior to conventional estrogens. Epidemiological data indicated that women taking high amount of phytoestrogens have fewer cardiovascular diseases, breast and uterine cancer and menopausal symptoms than those taking Western diet.[7,8,9] Human and animal models studies have shown that phytoestrogens, particularly isoflavones, influence lipid profiles. Moreover, they have been shown to normalize vascular reactivity in estrogen-derived primates. Furthermore, they have antineoplastic effects with inhibition of cellular proliferation as well as angiogenesis which protect cancer development. Finally, menopausal symptoms and bone density may beneficially be influenced by phytoestrogens.[10] In long term treatment, phytoestrogens have not been reported about adverse effects yet, for example, enhancing vascular function without accelerating pathological angiogenesis. Therefore, phytoestrogens are

presented in the new paradigm to be one of the nutritional approaches to cardiovascular protection.

Possible adverse effects of estrogen replacement have directed attention to the utilization of soybean products. Soybean and other legumes contain a number of substances that have weak estrogenic activity and are consumed widely in Asian populations. Isoflavones such as **genistein is believed to be one of the key chemicals among these plant estrogens or phytoestrogens**. Previous studies showed that phytoestrogens contained in soy protein food could contribute to the cholesterol – lowering effect and involve vascular function. They also have been shown to have anti proliferative properties *in vivo*.[11,12]

Several studies have shown that genistein could inhibit basic fibroblast growth factor (bFGF) –stimulated endothelial cell proliferation and angiogenesis *in vitro*.[13] In addition, it inhibited the production of plasminogen activator (PA) and plasminogen activator inhibitor 1 (PAI – 1) in vascular endothelial cells. Moreover, Nevala et al.(1998)[14] found that genistein acted as 17β - estradiol and did not act via endothelium, in vitro, which demonstrated in age-matched non-pregnant female and male rats. This study was consistent with that of Figtree et al (2000).[15] In contrast, Squadrito et al.(2000)[16] showed that genistein improved endothelial dysfunction and acted via endothelial cells *in vivo*.

Another study in human model with randomized double-blind placebo controlled trial design, showed that dietary soy intake demonstrated cardioprotective role and lowering effects on body mass index (BMI), waist circumference, and fasting insulin[17] in postmenopausal women (aged 45-74 yrs) consuming genistein. Moreover, isoflavones intake had positive effect on high density lipoprotein cholesterol (HDL-c). This study was agreed with that of Anthony et al (1996), who demonstrated the effects of soybean isoflavones in male and female peripubertal rhesus monkey. The latter was a cross-over design

with each period lasting for 6 months. After finishing treatment period, they found that soybean isoflavones improved cardiovascular risk factors, without affecting the reproductive system, by reducing low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c) in both male and female rhesus monkey, in addition to significant increasing of HDL-c in female animals and significant lowering of total cholesterol (TC):HDL-c ratio in both sexes .[18]

However, the effects of genistein both in human and animals are controversial. In addition, there is still few experimental data for genistein's preventive effects on endothelial cell dysfunction in ovariectomized rats. The exact mechanisms also need to be clarified.

Therefore, in this study, we would like to demonstrate whether genistein can prevent and improve endothelial dysfunction in ovariectomized rats or not and to examine the mechanism(s) of action of genistein on endothelial cells.

Research question

Could genistein prevent or treat endothelial dysfunction in bilateral ovariectomized rat? And if it could, what was(were) the mechanism(s) of these effects ?

Objective

1.To study the preventive and treatment effects of genistein on endothelial dysfunction in bilateral ovariectomized rat.

2.To study the mechanism(s) of action of genistein on endothelial cells.

Hypothesis

1.Genistein may have preventive and treatment effects on endothelial dysfunction in bilateral ovariectomized rat.

2.The preventive and treatment effects of genistein on endothelial dysfunction may be mediated through the pathway(s) of;

2.1 L-argenine / NO pathway or

2.2 Cyclooxygenase pathway or

2.3 L-argenine / NO and cyclooxygenase pathways

CHAPTER II

REVIEW OF LITERATURE

Genistein

Phytochemicals are plant – derived compounds of natural origin and the number of such classes of compounds are identified as having biological activity. The expanded epidemiological studies are rapidly continued to indicate possible associations between diet and disease states. Phytoestrogens are broad group of plant – derived compounds of non steroidal structure that can behave as estrogenic mimics. They may also have beneficial effects on both prevention and treatment of estrogen deprivation status.

There are three main groups of phytoestrogens: isoflavones, coumestans, and lignans, which are present in either plant or their seeds (Fig 2.1).[19] Genistein is one of the major substance of isoflavones.

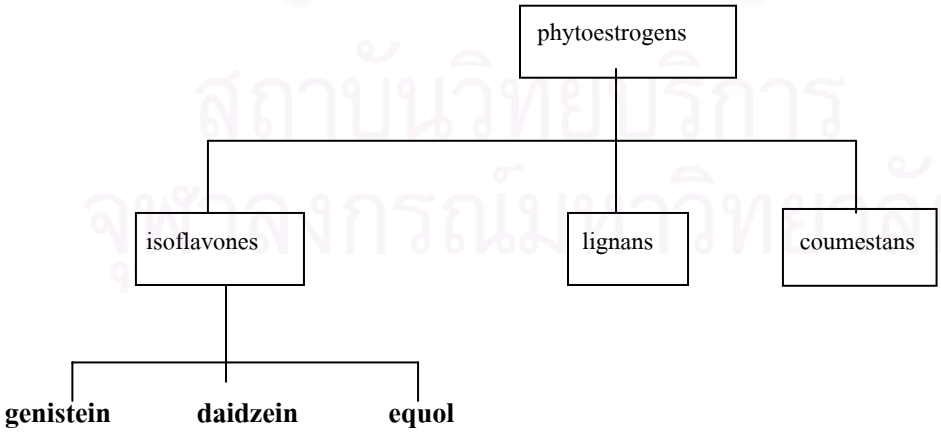


Figure 2.1 Sources and classification of dietary estrogens-like compounds. (modified from Murkies et al. 1998; 83: 297 – 303).[19]

A feature of the chemical structure of phytoestrogens is the presence of a phenolic ring that is a prerequisite for binding to the estrogen receptor (Fig 2.2). For this reason, phytoestrogens can act as estrogen agonists or antagonists[20] ; their actions at the cellular and molecular level are influenced by many factors, including receptors status, presence or absence of endogenous estrogens, and the type of target organs or cells.

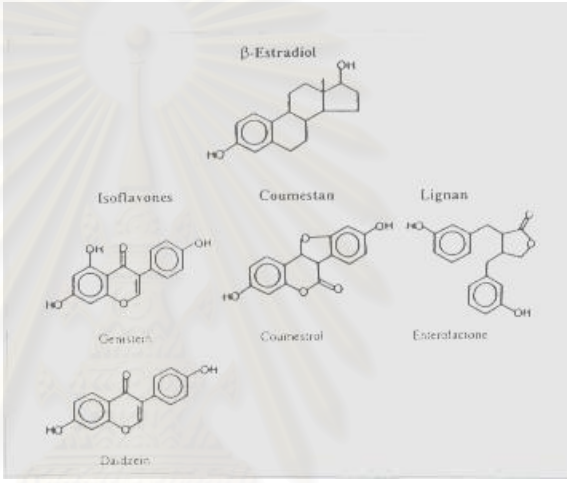


Figure 2.2 Structure of phytoestrogens
(modified from Lissin et al. 2000; 35(6): 1403 –1410).[3]

Genistein is biosynthetically the simplest of the isoflavonoid compounds of the leguminosae. It is a central intermediate in the biosynthesis of more complex isoflavonoids with role in establishment or inhibition of interactions between plants and microbes. Previous studies have focused on the pharmacological activities of genistein as a tyrosine kinase inhibitor,[21] its chemoprotectant activities against cancer and cardiovascular diseases, and its phytoestrogen activity. Genistein is a major subject of discussion in the context of nutraceuticals and functional foods, and may soon provide a case study for evaluating the delivery of health-promoting compounds through genetically modified plants.

Distribution of genistein and its metabolites

The isoflavonoids, a restricted distribution in the plant kingdom, are mostly limited to the subfamily papilionoideae of the leguminosae. Their structural variation is surprisingly large and involves not only the number and complexity of substituents on the 3-phenylchroman framework, but also different oxidation levels of the heterocycle and the presence of additional heterocyclic rings. The number of known isoflavone glycosides, e.g. genistin (genistein 7-O-β-D-glucopyranside) is, however, small when compared to the vast range of known flavonoid glycosides. O-glycosides predominate but a considerable number of C-glycosides have also been documented.[22]

Genistein as a phytoestrogen

Genistein shares structural feature with the potent estrogen 17β-estradiol, particularly the phenolic ring and the distance between its 4′- and 7-hydroxyl groups (Fig 2.3). These features confer ability to bind estrogen receptors and sex hormone binding proteins, and genistein can thus exert both estrogenic and antiestrogenic activity, the latter by competing for receptor binding by estradiol. Structural similarities have also been noted between genistein and tamoxifen (Fig 2.3), a synthetic anti-estrogen that has been clinically tested as a chemopreventive agent in women with high risk of breast cancer. The potent estrogen equol, a major metabolite of dietary isoflavonoids formed by the gastrointestinal flora (Fig 2.3), and genistein can displace bound estrogen testosterone from human sex steroid binding protein. Moreover, It should be noted that genistein binds differentially to human α and β estrogen receptors[23] and this should be carefully considered when extrapolating the results of phytoestrogen administration experiments in animals to hormone-related diseases in humans.

The majority dietary sources of isflavonoids for humans are soy products. One gram of powdered soybean chips contain nearly 800 μg of daidzein and

over 500 μg of genistein (primarily as glycosides), whereas one gram of soy protein has approximately 150 μg daidzein and 250 μg of genistein. Highly processed soy products such as miso and soy souce contain lower level of genistein than does tofu, the major source of isoflavones in the Asian diet. In human eating a soy-rich diet, ingested isoflavone levels can be very high, as determined by urinary excretion. Their levels of urinary equol can be approximately 100-fold higher than those observed in adults who consume little soy products in their diet. A high dietary consumption of genistein has been linked to a number of potential health benefits, as summarized in Figure 2.4 and discussed below.

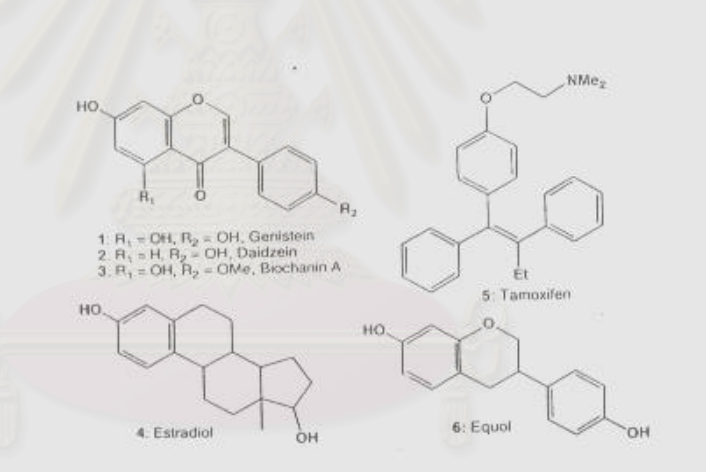


Figure 2.3 Structures of isoflavone phytoestrogens in relation to estradiol and tamoxifen. (modified from Dixon et al. 2002; 60: 205-211).[22]

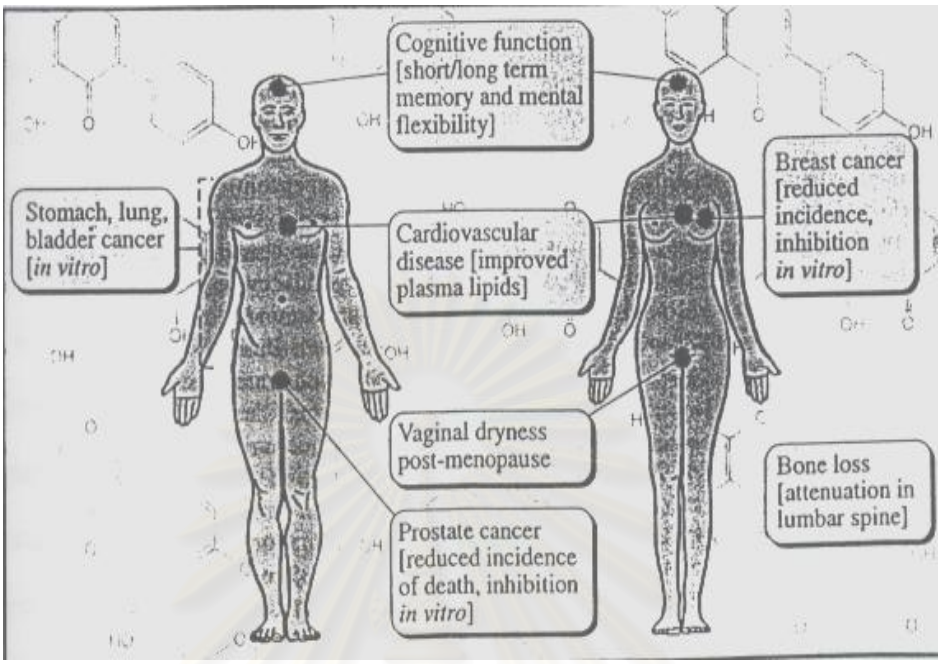


Figure 2.4 Proposed targets for beneficial effects of dietary genistein or a high soy diet on human health.
(modified from Dixon et al. 2002; 60: 205-211).[22]

Genistein as a cancer chemopreventive agent

Significant correlations exist between an isoflavone-rich soy-based diet and reduced incidence of breast cancer or mortality from prostate cancer in humans. An early epidemiological study of Singapore Chinese women that included 420 healthy controls and 200 with histologically confirmed breast cancer indicated that soy consumption was directly correlated with reduced risk of cancer[24], and the effects appeared to be dietary rather than genetic. Similar observations have been reproduced in many, but not all, subsequent studies undertaken up the present day. Based on knowledge of diet and urinary excretion levels of daidzein, genistein, and equol in Japanese as compared to American or European subjects,

the isoflavonoids found in soy products were proposed to be the agents responsible for the reduced cancer risk.[25]

When administered neonatally, genistein effectively protects against chemically-induced mammary tumors in rats.[26] The protective effects include increased latency, reduced tumor incidence and more rapid maturation of undifferentiated end buds to differentiated lobules. Biochanin A (4′-methoxygenistein), a major isoflavone component of chickpea, is likewise active as a cancer chemopreventant in animal model systems. Genistein may induce early mammary gland differentiation resulting in a less active epidermal growth factor signaling pathway in adulthood that, in turn, suppresses development of mammary cancer.[27] Although no clinical trials have been documently reported about the effects of controlled dietary supplementation with genistein on breast cancer incidence in humans, a high soy diet containing up to 45 mg of isoflavones per day can cause changes to menstrual cycle that may help reduce cancer risk.[22]

In addition to effects on breast cancers, genistein and related isoflavones also inhibit cell growth, or development of chemically induced cancer, in stomach, bladder, lung, prostate and blood. Inhibition of the growth of human stomach cancer cell lines *in vitro* by genistein and biochanin A apparently involves stimulation of a signal transduction pathway leading to apoptosis.[28] When these cancer cells were transplanted into mice, biochanin A, but not genistein, significantly inhibited tumor growth. Genistein strongly inhibits growth of leukemia cells when targeted to them by linkage to a monoclonal antibody and a prenyl isoflavone derivative (ipriflavone) has been developed as an oral treatment for acute leukemias.

In spite of the large number of studies supporting cancer chemoprevention by genistein, some studies have suggested a potential for opposite effects. These

include increased numbers of carcinogen-induced aberrant cryt foci in the colons of rats fed genistein and induced structural chromosome aberrations in human peripheral lymphocytes.

Genistein and cardiovascular disease

Results of epidemiological studies have suggested that high dietary intake of isoflavones and / or flavonols may contribute to a low incidence of heart disease in Japanese women. These effects may result from inhibition of low density lipoprotein oxidation by isoflavones, an effect that may be enhanced by food sources rich in vitamin C.[29] Genistein also appears to improve plasma lipids, resulting in lowered LDL cholesterol, the ratio of total cholesterol to HDL cholesterol, and the ratio of LDL to HDL cholesterol, in premenopausal women.[30] In rats, the hypocholesterolemic effect of a soy diet may involve interactions between the isoflavones and soy protein, whereas, in cholesterol fed rabbits, attenuation of atherosclerosis by isoflavones does not require the presence of soy protein. However, the direct effects of genistein on vessels wall have been less documented.

Genistein and postmenopausal problem

Estrogen deficiency in postmenopausal women can lead to unpleasant symptoms such as hot flushes and vaginal dryness and its long-term effects increase of osteoporosis and cardiovascular disease. Soy isoflavones positively help maintenance of bone mass in ovariectomized rodents, but daidzein may be more efficient than genistein in this respect. One study has indicated that isoflavone-rich soy protein may attenuate bone loss in lumbar spine of postmenopausal women, and this effect is due to isoflavones rather than soy protein.[31] An isoflavone-rich diet may help approximately two-thirds of postmenopausal women better cope with hot flushes in addition to potentially reducing the risk of cardiovascular diseases which are elevated after menopause.

Pharmacological activities of genistein

Not all the effects of isoflavones on human health are necessarily associated with their estrogenic activity. Genistein also inhibits DNA topoisomerase and tyrosine protein kinase,[32] as well as possesses antioxidant and cell cycle inhibitor activities. Kinase inhibition is generally regarded as being specific for tyrosine kinases, such as epidermal growth factor (EGF) receptor, although at higher concentrations genistein also inhibits protein histidine kinase. Other isoflvones such as daidzein do not inhibit tyrosine kinase activity, and are therefore used as controls in pharmacological experiments utilizing genistein.

Genistein blocks EGF-mediated tyrosine phosphorylation *in vivo* in human epidermal carcinoma cells. When specifically targeted to the B-cell-specific receptor CD-19 by conjugation to a monoclonal antibody, genistein selectively inhibited CD-19-associated tyrosine kinase activities, resulting in death of human B-cell precursor leukemia cells.[33] However, in several cell systems in which genistein inhibits growth, it does not appear to induce phosphorylation of EGF receptors or other tyrosine kinase substrates; in such cases, it has been suggested that the isoflavone might inhibit cell growth by modulating transforming growth factor (TGF) β1 signaling pathways.

Unlike other isoflavonoids, genistein exerts toxicity only at concentrations greatly in excess of those at which it first exerts its biological and pharmacological effects, making it a potentially important molecule for dietary cancer chemoprevention.

Biosynthesis of genistein

Isoflavonoids are formed by a branch of the flavonoid biosynthetic pathway, and originate from a central flavanone intermediate that is ubiquitously present in plants. For entry into the isoflavonoid pathway, the flavanone first undergoes abstraction of hydrogen radical at C-3 followed by B-ring migration

from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical. This reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, and is catalyzed by a microsomal cytochrome P450 enzyme (2-hydroxyisoflavanone synthase or 2-HIS, loosely termed isoflavone synthase or IFS). IFS is sterioselective, and (2R)-flavanones are not substrates. The resulting 2-hydroxyisoflavanone is unstable and undergoes dehydration to yield genistein or daidzein (the latter is formed by IFS from liquiritigenin [4′,7-dihydroxyflavanone], as shown in scheme 3. The dehydration reaction can take place non-enzymatically *in vitro*, although the reaction may be enzyme catalyzed *in vivo*.

Because of the lability and low abundance of IFS, it eluded molecular characterization for many years. However, cDNAs encoding IFS have now been cloned from soybean and other species,[34,35] largely aided by functional genomics approaches. One form of the enzyme from soybean, CYP93C1v2, converts liquiritigenin or naringenin directly to daidzein or genistein, repectively, in the presence of NADPH when expressed in insect cells.[35] It is not clear whether dehydration of the putative 2-hydroxyisoflavanone intermediate occurs on the enzyme, or results from an endogenous dehydratase activity present in the insect cell microsomes. In contrast, when IFS from licorice (Glycyrrhizaechinata) or soybean is expressed in yeast, the 2-hydroxyisoflavanone intermediate can be recovered.

Metabolism of soybean isoflavones in humans

Isoflavones make up the common form of phytoestrogens. They have a common diphenolic structure that resembles the structure of the potent synthetic estrogens diethylstilbestrol and hexestrol. Two of the major isoflavones found in humans are genistein and daidzein. Genistein and daidzein are potent compounds, which are metabolized from their plant precursors, biochanin A and formononetin, respectively. In plant, isoflavones are inactive. When the sugar residue is

removed, these compounds become activated. These plant compounds undergo fermentation by intestinal microflora, with both metabolites and unfermented parent (aglycone) compounds being liable to absorption. In the body, the parent compounds are reconjugated to glucuronides, but otherwise do not undergo any further metabolism in the body and excreted in the urine. In the colonic microflora, daidzein may be metabolized to equol or to O – Dma and genistein may be metabolized to p – ethyl phenol. Daidzein, genistein, equol and O – Dma are the major phytoestrogens detected in the blood and urine of humans and animals.[36]

Potency and biological effects

The biological potencies of phytoestrogens vary. The majority of these compounds are non steroidal in structure and vastly less potent than the synthetic estrogens (10^{-3} to 10^{-5} fold), and they vary between species as well as route of administration.[19,36] The relative potencies determined by human cell culture bioassays (compared with estradiol, to which they give an arbitrary value of 100) are coumestrol 0.202, genistein 0.084, equol 0.016, daidzein 0.013 and formononetin 0.00016.[36]

Isoflavones, genistein, : Possible Role in Postmenopausal Hormone Replacement

A large number of observational studies have suggested that postmenopausal hormone replacement therapy (HRT) may also contribute to cardiovascular prevention. In the study of Heart and Estrogen / Progestin Replacement Therapy (HERS), increased thromboembolic complications were observed during the first year of 4.1-year follow up.[37] Additionally, estrogen could produce endometrial proliferation and slightly increase breast cancer risk during prolonged HRT use.[38] This event has promoted the search for alternative hormone therapies. The Selective Estrogen Receptor Modulators (SERMs) are

currently under intensive study. Raloxifene, the second generation SERMs, appears to exert estrogen agonist effects on bone , vascular system, while one exerts antagonist effects on endometrium and breast tissue.[39,40,41] Theoretically, isoflavones share properties with SERMs, suggesting that they could act as agonist in bone and vascular tissue, although the evidence has not been clear comparing with SERMs data. There are conflicting reports concerning isoflavones effects on these tissues. However, the beneficial effects of isoflavones, genistein, as estrogen agonists and antagonists needs further investigation. Therefore, the main objective of this study was to determine the preventive and therapeutic effects of genistein on vascular endothelial dysfunction in bilateral ovariectomized rat and also to study the mechanism(s) of genistein on vascular endothelial cells.

Role of The Endothelium in Health

The endothelium not only provides a structural barrier between the circulation and surrounding tissue, but endothelial cells (ECs) also secrete mediators that influence vascular hemodynamics in the physiologic state (Table 2.1). ECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), as well as vasoconstrictors, including endothelin (ET) and platelet-activating factor (PAF). These chemically diverse compounds are not stored in intracellular granules. Rather, their major biologic effects are regulated by localization of specific receptors on vascular cells, through their rapid metabolism or at the level of gene transcription. NO is constitutively secreted by ECs, but its production is modulated by a number of exogenous chemical and physical stimuli, whereas the other known mediators (PGI_2, ET and PAF) are synthesized primarily in response to changes in external environment.

Endothelium-derived relaxing factors

Stimulation of intact endothelial cells by neurotransmitters, hormone, and substances derived from platelets and the coagulation system causes the release of a substances that, in turn, induces relaxation of the underlying vascular smooth muscle (Fig 2.5).[42,43] Furthermore, shear forces generated by the circulating blood induce endothelium-dependent vasodilation, which is an important adaptive response of the vasculature during exercise. This endothelium-derived relaxing factor, a diffusible substance with a half-life of a few seconds,[42] has been identified as the free radical, nitric oxide (NO). NO is formed from L-arginine by oxidation of the guanidine-nitrogen terminal.[44] The NO-synthesizing enzyme exists in several forms in endothelial cells, platelets, macrophages, vascular smooth muscle cells, nerves, and the brain.[45] In endothelial cells, gene expression of NO synthase (NOS), although constitutively activated, can be upregulated by shear stress and estrogens. The activity of NO synthase can be inhibited by the circulating amino acid, asymmetrical dimethylarginine (ADMA), which accumulates in patients with renal failure.[46] This observation has been further extended to hypercholesterolemia; increased levels of ADMA were seen in hypercholesterolemic rabbits despite normal renal function, and elevated circulating ADMA was subsequently observed in patients with occlusive peripheral atherosclerotic disease. An inducible isoform of NOS exists in vascular smooth muscle and macrophages. When activated by cytokines such as endotoxin, interleukin-1β, and tumor necrosis factor α, this calcium-independent enzyme produces large amouts of NO, and hence is activated in inflammatory processes and endotoxic shock.

Endothelium-dependent relaxation due to NO involves formation of cyclic 3′,5′-guanosine monophosphate (cGMP) via the soluble enzyme guanylyl cyclase[47] (Fig 2.5). NO-induced endothelium-dependent relaxation can be

pharmacologically inhibited by analogues of L-arginine such as L-N^G-monomethyl arginine (L-NMMA) or -nitroarginine methyl ester (L-NAME), which compete with the natural precursor L-arginine at the catalytic site of the enzyme.[45] In isolated arteries, these inhibitors cause endothelium-dependent contraction, whereas in perfused hearts, inhibition of NO formation markedly decreases coronary flow. Local infusion of L-NMMA into the human forearm circulation induces an increase in peripheral vascular resistance. When infused intravenously, L-NMMA induces long-lasting increases in blood pressure. This indicates that the vasculature is in a constant state of vasodilation due to a continuous basal release of NO by the endothelium.

In addition to NO, endothelial cells release PGI_2 in response to shear stress, hypoxia, and several substances that also release NO (Fig 2.5). PGI_2 increases cyclic 3′,5′-adenosine monophosphate (cAMP) production in smooth muscle and platelet. Its platelet-inhibitory effects play a greater physiologic role than its contribution to that of the endothelium-dependent relaxation. NO and PGI_2 synergistically inhibit platelet aggregation, suggesting that the presence of both mediators is required for maximal inhibition of platelet activation.

In the epicardial coronary circulation, inhibitors of the L-arginine pathway do not prevent all endothelium-dependent relaxations, particularly in intramyocardial vessels.[48] Because vascular smooth muscle cells become hyperpolarized during NO-independent relaxations, the existence of endothelium-dependent hyperpolarizing factors has been proposed.[49,50] However, C-type natriuretic peptide, previously proposed as an endothelium-derived hyperpolarizing factor, does not cause endothelium-dependent hyperpolarization.[51]

Endothelium-derived contracting factors

Soon after endothelium-derived relaxing factor / NO was discovered, it became clear that endothelial cells also could mediate contraction[43] (Fig 2.5).

Endothelium-derived contracting factors include the 21-amino acid peptide endothelin-1 (ET-1), vasoconstrictor prostanoids such as thromboxane A_2 and prostaglandin H_2, and components of the rennin-angiotensin system such as angiotensin II. Three isoforms of the endothelin peptide family exist: endothelin 1, endothelin 2, and endothelin 3. Endothelial cells produce ET-1 exclusively.[52] Translation of mRNA generates preproendothelin, which is converted to big endothelin (bET-1) that is further converted by endothelin-converting enzyme (ECE) to the mature peptide ET-1. Four isoforms of this enzyme—ECE-1a, ECE-1b, ECE-1c, and ECE-2—have been cloned.[53,54] The expression of mRNA and the release of ET-1 are stimulated by thrombin, transforming growth factor β, interleukin-1, epinephrine, angiotensin II, arginine vasopressin, calcium ionophore, and phorbal ester[52,55] (Fig 2.5).

Endothelin-1 causes vasodilation at lower concentrations but marked and sustained contractions at higher concentrations,[52,56] in the heart, the latter eventually leads to ischemia, arrhythmias, and death. Intramyocardial vessels are more sensitive to the vasoconstrictor effects of ET-1 than are epicardial coronary arteries, suggesting that endothelin has particular importance in the regulation of flow. Very low circulating levels of ET-1 indicate that most of the peptide is formed locally in the vascular wall. This may be due to the absence of stimuli to endothelin production, the presence of potent inhibitory mechanisms, or the preferential release of endothelin abluminally toward smooth muscle cells.[57] Four inhibitory mechanisms regulating ET-1 production have been delineated: (1) a cGMP-dependent inhibition,[55] (2) a cAMP-dependent inhibition,[58] (3) an inhibitory factor produced by vascular smooth muscle cells,[59] and (4) an inhibition by estrogens via an estrogen-receptor-dependent mechanism.[60] Inhibition of the endothelial L-arginine pathway augments thrombin-induced or angiotensin-induced production of ET-1; conversely, nitrates and atrial natriuretic peptide

(which activate particulate guanylyl cyclase) prevent thrombin-induced ET-1 release via a cGMP-dependent mechanism. Endothelin-1 may also promote release of NO and PGI_2 from endothelial cells through ET_B receptors; as a negative feedback mechanism, this process reduces ET-1 production in the endothelium[55] and its vasoconstrictor action in smooth muscle. It is interesting that endothelin inhibits the expression and function of inducible NO synthase.[61]

Two distinct endothelin receptors have been indentified: the ET_A- and ET_B-receptors (Fig 2.5).[62] Both are G protein-couple receptors with seven transmembrane domains and are linked to phospholipaes C and protein kinase C. Endothelial cells express ET_B-receptors involved in the formation of NO and prostacyclin, which explains the transient vasodilator effects of endothelin when infused into intact organs or organisms. The ET_A-receptors and, to some extent, ET_B-receptors mediate contraction and proliferation in vascular smooth muscle. Several endothelin-receptor antagonists have been developed and are currently being clinically evaluated in normal subjects and patients.

The cyclooxygenase pathway also produces endothelium-derived vasoconstrictors. Particularly in vein, but also in the cerebral and ophthalmic circulation, agonists such as arachidonic acid, acetylcholine, histamine, and serotonin can evoke endothelium-dependent contractions that are mediated by thromboxane A_2 or prostaglandin H_2 (Fig 2.5). Thromboxane A_2 and prostaglandin H_2 activate the thromboxane receptors in vascular smooth muscle and platelets, thereby counteracting the effects of NO and prostacyclin in both types of cell. In addition, the cyclooxygenase pathway is a source of superoxide anions, which rapidly inactivate NO to form the potent cytotoxic oxidant peroxynitrite.

The endothelium also regulates the activity of the rennin-angiotensin system. Angiotensin-converting enzyme (ACE), which converts angiotensin to angiotensin II, is expressed on the endothelial cell membrane. ACE is identical to

kinase II, which inactivates bradykinin. Angiotensin II can activate endothelial angiotensin receptors; these receptors stimulate the production of ET-1 and other mediators such as plasminogen activator inhibitor.[63] Furthermore, superoxide anion production due to the activation of NADH / NADPH oxidase has recently been linked to angiotensin II-induced hypertension.[64]

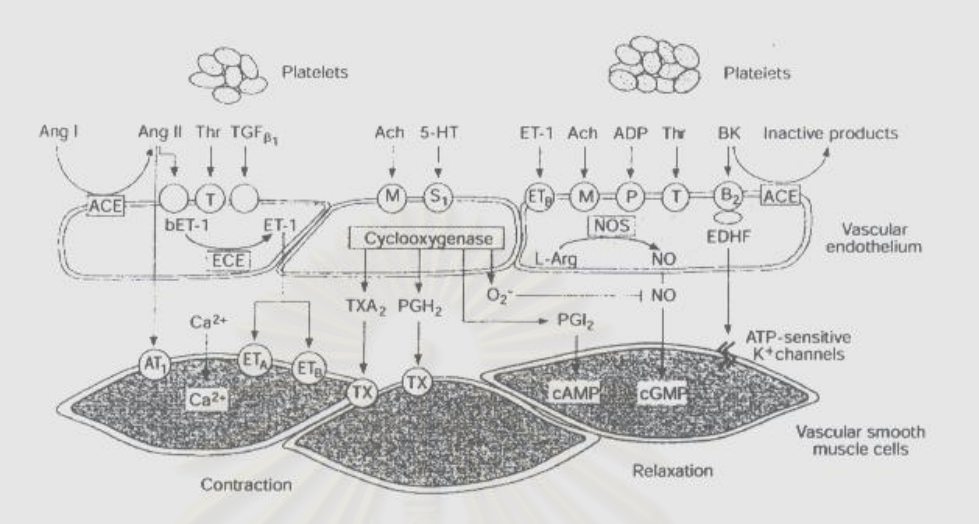


Figure 2.5 Vasoactive mediators released by the endothelium. The endothelium produces factors that promote both relaxation (right) and contraction (left). Ang = angiotensin, ACE = angotensin-converting enzyme, Ach = acetylcholine, ADP = adenosine diphosphate, ATP = adenosine triphosphate, Bk = bradykinin, cAMP / cGMP = cyclic adenosine / guanosine monophosphate, ECE = endothelin-converting enzyme, EDHF = endothelium-derived hyperpolarizing factor, ET = endothelin-1, 5HT = 5-hydroxytryptamine (serotonin), L-Arg = L-arginine, NO = nitric oxide, NOS = nitric oxide synthase, O^-_2 = superoxide, PGH_2 = prostaglandin H_2, PGI_2 = prostacyclin, TGFβ_1 = transforming growth factor β_1, Thr = thromboxane A_2. Circle represent receptors(AT = angiotenergic, B = bradykinergic, ET = endothelin receptor, M = muscarinic, P = purinergic, S = serotonergic, T = thrombin receptor, TX = thromboxane receptor). (modified from Luscher et al. 1997; 20(20 Suppl. II): II-3-II-10).[65]

ECs elaborate NO, a heterodiatomic free radical product generated through the oxidation of L-arginine to L-citrullin by NOS.

Table 2.1 Vasoregulatory substances synthesized by the endothelium

Substances	Principal effects	Other effects	Secretion	Compound	Precusor Com-pound
NO	vasodilation	Maintain basal tone of vessels; inhibits leukocyte adhesion, activation, secretion, and aggregation; inhibits smooth muscle cell migration and proliferation	Paracrine/ constitutive and induced by thrombin, ADP, bradykinin, substance P, muscarinic agonists, shear stress, cyclic strain, cytokine	Hetero diatomic free radical	L-arginine
Prostacy clin (PGI_2)	vasodilation	Retard platelet aggregation and deposition	Paracrine/ induced at sites of vascular perturbation	Eicosanoid	Arachido-nic acid
Platelet activating factor (PAF)	Vaso-constriction	Promotes leukocyte adhesion at cell surface	Juxtacrine	Phospho lipid	Arachido-nic acid

Substances	Principal effects	Other effects	Secretion	Compound	Precusor Com pound
Endothelin 1	Vasoconstriction	Mitogen for smooth muscle cells; modulates effect of numerous compounds	Paracrine/ induced by hypoxia, shear stress and ischemia	21 amino acid peptide	Preproendothelin-1 (203 amino acid)

(modified from Clines et al. 1998; 3527-3561)[66]

Moreover, endothelium-derived NO inhibits leukocyte adhesion to the endothelium.[67,68] and inhibits smooth muscle cell migration[69] and proliferation.[70] These latter effects serve to limit neointimal proliferation that occurs after vascular injury and combine with its stimulatory effect on EC migration and proliferation, suggest that NO helps to sustain vascular reparative mechanisms.

ECs also produce a less well-characterized compound known as endothelium-derived hyperpolarizing factor (EDHF) that promotes vascular smooth muscle relaxation. Muscarinic agonists stimulate ECs to release EDHF, causing a transient hyperpolarization of the cell membrane. It has been proposed that EDHF exerts its vascular effects by activating ATP-sensitive potassium channels, smooth muscle sodium-potassium ATPase or both but its role in vascular (patho)physiology requires further study.

Prostacyclin (PGI_2) and PAF, the contribution of ECs to the regulation of vasomotor tone is even more finely regulated as evidenced by the production of additional vasoactive compounds such as PGI_2 and PAF. PGI_2 was the first endothelium-derived vascular smooth muscle relaxing factor to be identified. PGI_2, which was generated locally, and PGI_2 or its analogs, which were infused

systemically, caused vasodilation and altered regional blood flow.[71] A receptor for PGI_2 (the IP receptor) is present on vascular smooth muscle as well as on platelets, consistent with early experimental observations, indicating that PGI_2 acts principally to modulates the function of these two cell types. Although IP receptors are present in the arterial vascular wall, PGI_2 is not constitutively produced and does not appear to regulate basal systemic vascular tone. Rather, PGI_2 synthesis is induced at sites of vascular perturbation, where it may regulate vasoconstriction and platelet deposition. Because of its effects on blood flow and relevant cell-cell interactions, PGI_2 may influence local inflammatory responses as well. An important recent advance has been the identification of prostacyclin H synthase-II (PHS-II), an inducible form of a key enzyme in PGI_2 formation providing a mechanism by which the production of PGI_2 and other eicosanoids can be sustained in chronic states of inflammation and vascular injury.[66]

One isoform of NOS, eNOS or NOS_3 gene product, is constitutively active in ECs but is stimulated further by receptor-dependent agonists that increase intracellular calcium and perturb plasma membrane phospholipids asymmetry. Receptor-dependent agonists that stimulate eNOS including, thrombin, adenosine 5′-diphosphate, bradykinin, substance P and muscarinic agonists, in addition to shear stress and cyclic strain (Fig 2.6).[71] The increase in eNOS activity evoked by shear stress contribute to the phenomenon of flow-mediated vasodilation, an important autoregulatory mechanism by which blood flow increases in response to exercise.

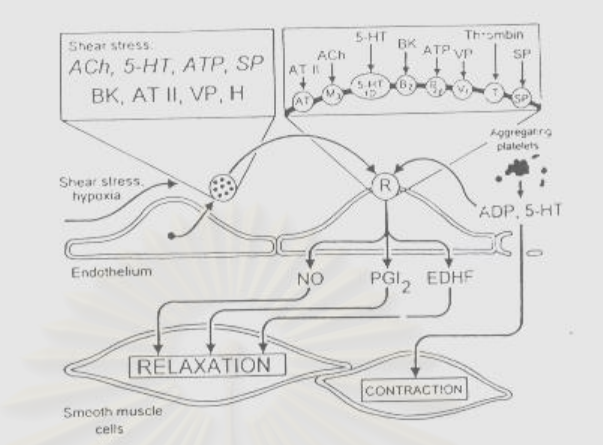


Figure 2.6 The multiple factors caused endothelium released vasoactive substances. (modified from Vanhoutte. 2000: 81 271-277).[72]

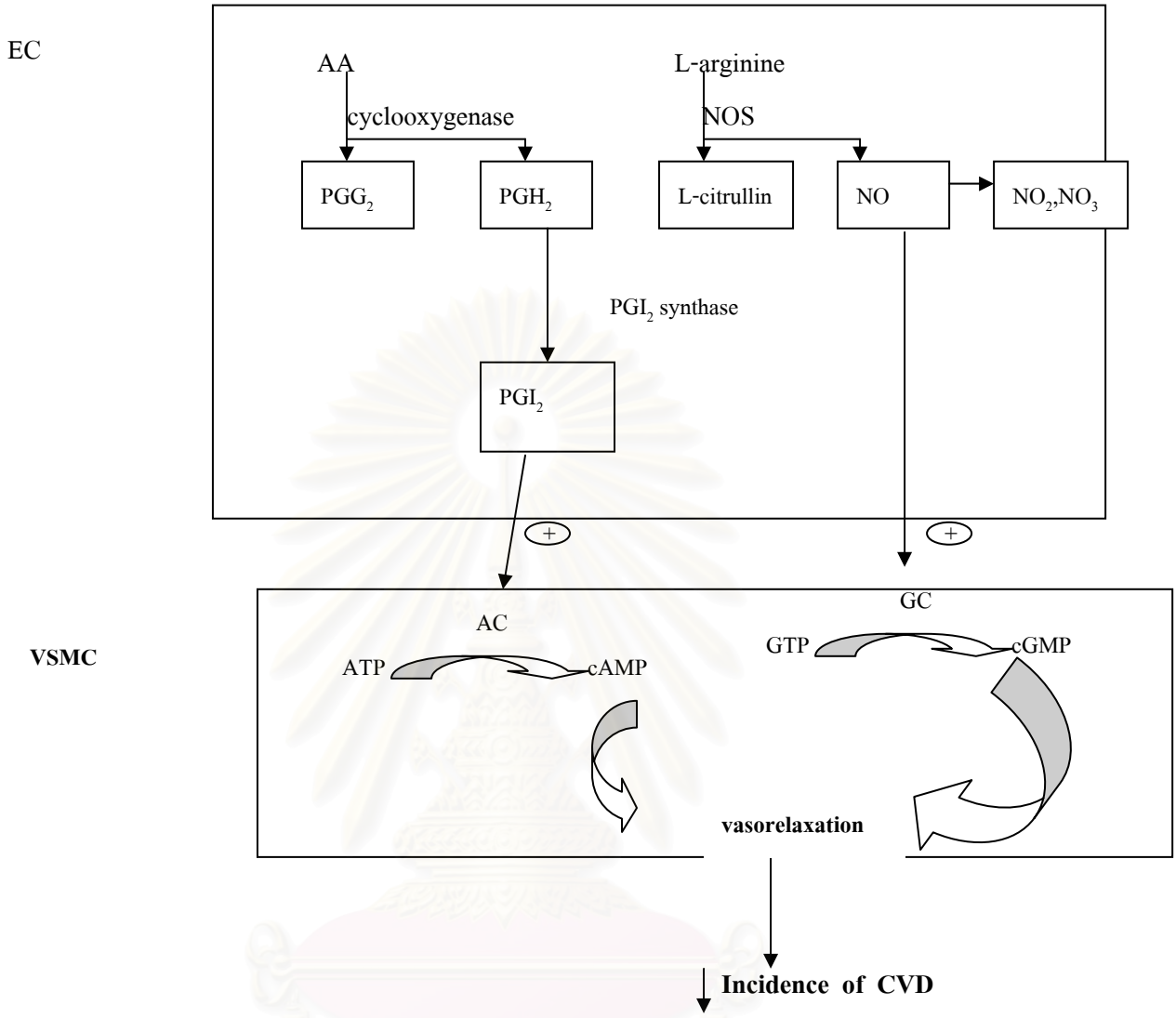


Figure 2.7 Schematic of endothelial function.
(modified from Becker. 2000)[1]

Endothelium and vascular structure

Removal of endothelial cells by balloon injury invariably leads immediately to deposition of platelets and white blood cells at the site of injury; intimal hyperplasia occurs within days to weeks. This observation suggests that endothelium regulates vascular structure and that it protects the vessels wall from activation of vascular smooth muscle cells (Fig 2.8). Endothelial dysfunction is

therefore an important factor in atherosclerosis, restenosis, and hypertensive vascular disease. Vascular structure is determined mainly by vascular smooth muscle cell growth. Endothelial cells may affect vascular structure directly and indirectly. Nitric oxide and prostacyclin inhibit platelet adhesion.[73] Endothelial dysfunction and / or denudation, however, allow platelets to adhere to the vessel wall, where they may cause contraction through the release of thromboxane A_2 and serotonin and may stimulate proliferation of vascular smooth muscle cells via release of platelet-derived growth factor.[74]

Endothelial cells produce growth promoters and growth inhibitors. Under physiologic conditions, the effects of growth inhibitors appear to outweigh those of growth promoters, which may explain why the blood vessel wall is normally quiescent with no proliferation of smooth muscle cells. Heparan sulfate, NO, and TGFβ_1 are potent inhibitors of vascular smooth muscle cell migration and proliferation.[75] In contrast, endothelial cells under certain conditions may produce various growth factors, particularly platelet-derived growth factor, epidermal growth factor, and angiotensin II (Fig 2.8). These factors may become important in disease states in which the endothelium remains morphologically intact but dysfunctional and may thereby contribute to smooth muscle cell proliferation.

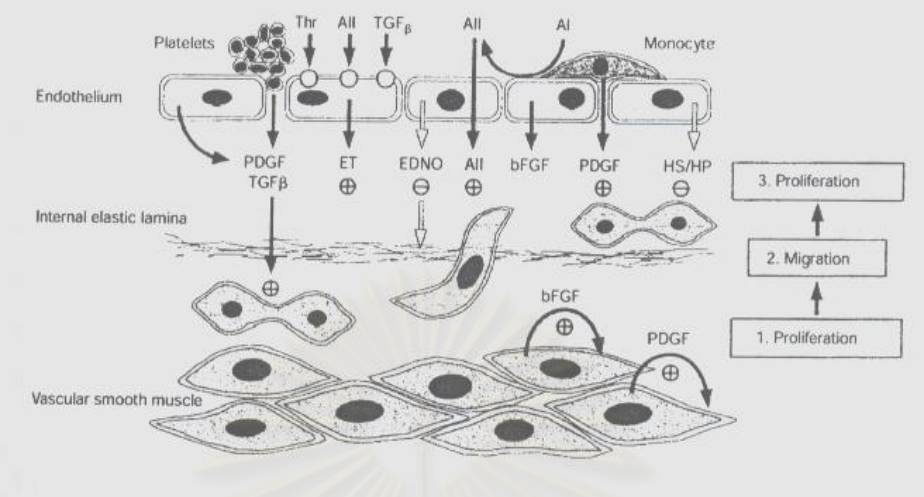


Figure 2.8 The endothelium and control of vascular structure. Under normal conditions, the endothelium does not stimulate migration and proliferation of vascular smooth muscle cells. With onset of endothelial dysfunction, platelets and monocytes adhere to the vessel wall, and growth factors are released from these cells as well as from the endothelium. AII = angiotensin II, bFGF = basic fibroblast growth factor, EDNO = endothelium-derived nitric oxide, HP / HS = heparin sulfates, PDGF = platelet-derived growth factor. (modified from Luscher et al. 1997; 20(20 Suppl. II): II-3-II-10).[65]

Endothelial Dysfunction

The term endothelial dysfunction is mostly used to denote impairment of endothelium-dependent vasodilation but probably encompasses those conditions leading to endothelial activation with abnormalities in endothelial interactions with leukocytes, platelets and regulatory substances (Table 2.2).[76] Moreover, it characterizes by an imbalance of endothelium-derived relaxing and contracting factors. It may be the cause or consequence of vascular disease and is a hallmark of known cardiovascular risk factors. It is interesting that endothelial dysfunction precedes structural vascular alterations, indicating a protective role of

the functionally intact endothelium. While some vessels are particularly prone to developing endothelial dysfunction and atherosclerosis, others appear to be protected. This difference may relate to selective alterations due to pulse pressure and / or alterations in endothelial cell function in different areas of the vascular tree. Endothelial cell denudation, however, occurs only in very late stages of atherosclerosis and plaque rupture. These changes in endothelial cell morphology are almost invariably associated with functional alterations and intimal thickening, with accumulation of white blood cells, vascular smooth muscle cells, and fibroblast and matrix deposition.

Table 2.2 Conditions associated with impaired endothelium-dependent vasodilation.

Atherosclerosis	Type I and type II diabetes mellitus
Hypercholesterolemia	hyperglycemia
Low HDL-c	Acute postprandial hypertriglyceridemia
High Lp(a)	Active and passive cigarette smoking
Small LDL particles	Dilated cardiomyopathy
Susceptibility of LDL to oxidation	Changes disease
Hypertension	Heart failure --- any cause
Hyperhomocysteinemia	Family history of coronary disease
Aging	Postmenopausal states
Vasculitic conditions	Post-Kawasaki's disease
Transplantation, atherosclerosis	Pregnancy-induced hypertension/pre-eclampsia

(modified from Todd et al. 1999. 34(3): 631-638).[76]

Risk Factors for Endothelial Dysfunction

We now recognize that the endothelium-mediated vasodilation observed by Furchgott and Zawadzki is largely due to endothelium-derived nitric oxide (NO), a single molecule with profound effects on cardiovascular physiology. Impairment of endothelial vasodilator function is now established as a major contributor to cardiovascular disease and accumulating evidence indicates that strategies for restoring endothelial function can have important therapeutic effects.

Endothelial dysfunction is recognized as the initial step in the atherosclerotic process. There are numerous risk factors that can cause endothelial damage: hypertension, hypercholesterolemia, cigarette smoking, sedentary lifestyle, menopause and diabetes mellitus.[77]

Cardiovascular risk factors and endothelial dysfunction

Hypercholesterolemia: hypercholesterolemia per se, without atherosclerotic vascular changes, inhibits endothelium-dependent relaxations, which are further reduced in atherosclerosis.[78] It appears that low-density lipoprotein (LDL) is a major determinant of this phenomenon (Fig 2.9). Indeed, incubation of isolated coronary arteries with oxidized but not native LDL selectively inhibits endothelium-dependent relaxations to serotonin, aggregation platelets, and thrombin, whereas the response to bradykinin is not affected.[79] A similar diminution of the response can be achieved by pertussis toxin or an inhibitor of NO formation, suggesting defective activation of the L-arginine pathway by G_i protein-coupled receptors.[79,80] Exogenous L-arginine improves or restores reduced endothelium-dependent relaxation in the presence of oxidized LDL, which suggests that oxidized LDL impairs the activity of NOS. The active component of LDL appears to be lysolecithine, which mimics most of the effects of LDL. *In vitro* experiments in the coronary arteries of hypercholesterolemic pigs have

demonstrated selective dysfunction of endothelium-dependent relaxation in response to serotonin and aggregating platelets and thrombin. Endothelial dysfunction is more extensive in more advanced stages of atherosclerosis. Experiment in the aorta of hypercholesterolemic rabbits suggests that the overall production of NO is not reduced but rather augmented, however, increased production of NO is inactivated by superoxide radicals produced within the endothelium[81] (Fig 2.9). Similar observations have been made in rabbits with fully developed atherosclerosis. Under the condition of both hypercholesterolemic and atherosclerosis, biologically active NO is markedly reduced, a fact also supported by bioassay experiments with coronary arteries of hypercholesterolemic pigs.[82]

Endothelin is activated in atherosclerotic vascular disease. In hyperlipidemia and atherosclerosis, endothelial cell production of endothelin is increased[83] (Fig 2.9), while the expression of endothelin receptors is downregulated.[84] A likely stimulus for the increased endothelin production is LDL, which increases endothelin gene expression and endothelin release from pocrine human aortic endothelial cells[85] (Fig 2.9). Vascular smooth muscle cells, particularly those that migrate into the intima during the atherosclerotic process, also produce endothelin. In culture vascular smooth muscle cells, endothelin can be released by growth factors such as platelet-derived growth factor and transforming growth factor β_1 and by vasoconstrictors such as arginine vasopressin.[86] Hence, several mediators involved in atherosclerosis stimulate vascular endothelin production, perhaps explaining why plasma endothelin levels are increased and correlated positively with the extent of atherosclerotic lesion formation.[83] Furthermore, unstable lesion removed from coronary arteries by arterectomy exhibits marked staining for ET-1. Thus, local vascular endothelin may contribute to both abnormal coronary vasomotion in patients with unstable angina, which may be stimulated by

ischemia or thrombin, and to vasoconstriction and the proliferation of vascular smooth muscle cells observed in atherosclerosis.

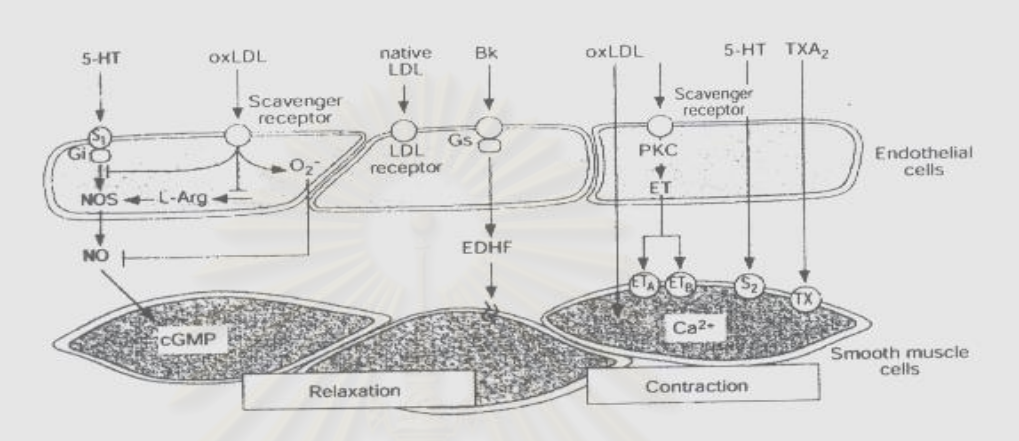


Figure 2.9 Endothelial dysfunction in hyperlipidemia and atherosclerosis. The major contributor is oxidized LDL (oxLDL), which, byactivating scavenger receptors, impairs the activity of L-arginine-NO pathway. The mechanism may involve inactivation of G_i proteins (G_i), decreased intracellular availability of L-arginine (L-Arg), and increased breakdown of NO by superoxide (O_2^-). OxLDL further activates endothelin (ET) gene expression and production via protein kinase C (PKC).
(modified from Luscher et al. 1997. 20(20 Suppl. II): II-3-II-10).[65]

Hypertension: endothelial dysfunction in hypertension may contribute to an increase in peripheral resistance (in small arteries) or to vascular complication of the disease (in large and medium-sized conduit arteries). In most models of hypertension, high blood pressure is associated with reduced endothelium-dependent relaxation. Endothelial dysfunction is more prominent in some blood vessels than in others and appears to occur as blood pressure rises; thus, endothelial dysfunction is a consequence rather than a cause of hypertension. In hypertensive subjects, acetylcholine causes paradoxical vasoconstriction of

epicardial coronary arteries. The mechanism of endothelial dysfunction differs in various models of hypertension. In the spontaneously hypertensive rat model of genetic hypertension, the activity of the enzyme NO synthase is markedly increased but inefficacious, probably due to increase inactivation of NO by superoxide anion[87] (Fig 2.10). In addition, the endothelium of spontaneously hypertensive rats and ren-2 transgenic rats produces increased amounts of protaglandin H_2 , which offset the effects of NO in vascular smooth muscle and platelets. Whether or not this occurs in humans is uncertain; however, in the forearm circulation of patients with essential hypertension, infusion of a indomethacin (INDO; cyclooxygenase inhibitor) enhances vasodilation in response to acetylcholine.[88] In contrast, salt-induced hypertension is associated with a marked impairment of endothelial NO synthase activity[89] (Fig 2.10). Plasma levels of endothelin remain normal in most patients with hypertension except in the presence of renal failure or atherosclerosis. Increased local vascular production of endothelin, however, is likely; because most of the peptide is released abluminally, plasma levels of endothelin do not necessarily reflect local tissue levels. Vascular endothelin production is reduced in spontaneously hypertensive rat but increased in angiotensin II-induced hypertension in Wistar Kyoto rats. In the latter model, functional ECE activity is also increased.[90] However, endothelin by itself does not appear to cause hypertension.[91]

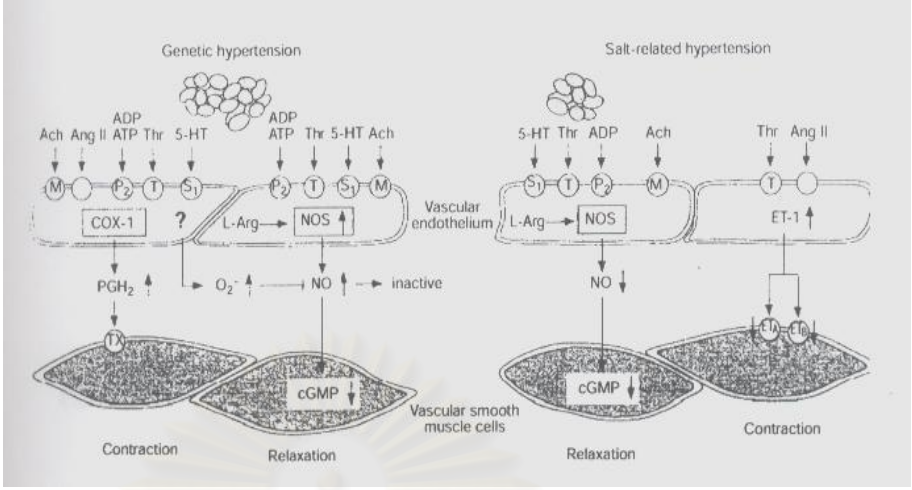


Figure 2.10 Endothelial function and hypertension.
(modified from Luscher et al. 1997. 20(20 Suppl. II): II-3-II-10).[65]

Estrogen deficiency: estrogen is an important modulator of vascular function. Estrogen replacement therapy is associated with a decreased risk of cardiovascular morbidity and mortality in postmenopausal women.[92] Accordingly, male gender is considered an independent risk factor for coronary artery disease. Estrogen modulates NO synthase activity and the formation of NO *in vitro* and *in vivo*. Estrogen deficiency is associated with endothelial dysfunction[93] and increased circulating levels of endothelin.[94] Endothelin production can be inhibited by estrogen *in vitro* and *in vivo*.[94]

Assessment of Endothelial Dysfunction

A large number of studies have assessed arterial endothelial function in health and disease over the past two decades. The ability of normal endothelium to release the vasorelaxing factor NO in response to physiological or pharmacological stimuli was tested in most of these studies. Although this is only

one of many endothelial functions, NO release is particularly important because of its actions on platelets, monocytes and smooth muscle cells.

Study of coronary artery

In vivo assessment of coronary endothelial function was first reported in the mid-1980s. Since Ludmer et al.[95] were among the first to demonstrate that acetylcholine (up to 10^{-6}M) could be safely infused selectively into the coronary circulation to assess conduit vessel vasomotion. This has served as the gold standard for endothelial function testing for the last decade. Acetylcholine-induced vasoconstriction is one of the earliest manifestations of endothelial dysfunction,[96]occurring before abnormalities with other endothelium-dependent stimuli [cold presser testing, flow-mediated vasodilation (FMD)]. N^G-monomethyl L-arginine (L-NMMA), nitric oxide synthase inhibitor, can be selectively infused to assess basal NO activity in the coronary circulation. Resistance vessel function in the coronary circulation can now be readily assessed by measuring coronary blood flow with intracoronary Doppler wires.[97] In normal arteries, acetylcholine stimulated the endothelial release of NO, resulting in vasodilation. In subjects with endothelial dysfunction, vasoconstriction was observed due to its direct smooth muscle constrictor effect. This response contrasted with the response to nitroglycerine, an exogenous source of NO and therefore an endothelium-independent vasodilator.

Study of peripheral artery

Non-invasive detection of endothelial dysfunction in the brachial and femoral arteries was first described in 1992.[98] In this technique, arterial diameter is measured in response to an increase in shear stress, which causes endothelium-dependent dilation and in response to sublingual nitroglycerine, an endothelium-independent dilator. This technique has been shown to be reproducible[99] and to correlate well with invasive testing of coronary endothelial dysfunction.[100]

Endothelial function has also been investigated in the forearm microcirculation by intra-arterial infusion of endothelium-dependent and -independent vasodilator substances, followed by measurement of forearm blood flow using plethysmographic technique.[101,102] These techniques have provided important insights into the risk factors for atherogenesis in children and in young adults and are being used in various studies of endothelial dysfunction in asymptomatic subjects.

Reversibility of Endothelial Dysfunction

Studies over the past decade have demonstrated that endothelial dysfunction can be attenuated by a variety of therapeutic interventions. To date, most interventions attempting to improve endothelial dysfunction have targeted one or more of the numerous risk factors that can cause endothelial damage such as hypertension, hypercholesterolemia, cigarette smoking, sedentary lifestyle, menopause and diabetes mellitus. Several pharmacological agents have been suggested to achieve vascular protection through various mechanisms. Beneficial changes to endothelium might result from promotion of vascular relaxation, inhibition of vasoconstriction, reduction in the production of free radicals, or other mechanism that protect the endothelium from injury.[103]

In 1990, various studies have demonstrated that endothelial dysfunction in both humans and animal models can be attenuated by a variety of intervention (Table 2.3).[76]

Table 2.3 Treatment associated with improvement of endothelial dysfunction in humans.

Acute	Chronic
LDL lowering with pheresis	LDL lowering with statins, resins
ACE inhibition	ACE inhibition
Antioxidants (vitamin C and E+C)	Antioxidants (probucal with lovastatin)
Estrogen	Estrogen
L-arginine, D-arginine	Estrogen + progesterone
Tetrahydrobiopterine, methyltetrahydrofolate	L-arginine
Deferoxamine	Exercise

(modified from Todd et al. 1999; 34(3):631-638)[76]

Menopause

At the end of the fifth decade of life, women begin to show the rising LDL – cholesterol and triglycerides that characterized by Western men who are approximately 10 years younger. During this period, which coincides with ovarian failure, one can also detect acceleration in the incidence of coronary heart disease (CHD). Ultimately, almost as many women die of CHD and more die of stroke than men. Great emphasis has been placed on the potential benefits of hormone replacement therapy (HRT), because it seemed evident that the menopausal rise in events indicated the loss of estrogenic benefits. This was apparently confirmed by a series of community – based studies, which formed that women using HRT had many fewer myocardial infarctions and less CHD death than those without therapy. Less emphasis is often given to define the lipoproteins as one of the

risk factors in middle – aged and older women, and as a result therapy with proven benefit may not begin.

Menopause, whether natural or surgically induced, was strongly associated with an increased risk of atherosclerosis----- that is, detection of calcium deposit in the abdominal aorta---- in a study comprising more than 600 women. The risk of atherosclerosis showed an increased trend with number of postmenopausal years.[77,78]

The Nurses' Health Study cohort provided valuable data on some of the issues involving menopause and cardiovascular risk. Women found at highest risk of coronary heart disease were those who had undergone bilateral oophorectomy without receiving estrogen replacement therapy; those given estrogen replacement after oophorectomy demonstrated no excess risk, nor did women who had undergone natural menopause.[104]

Recently, the Women's Health Initiative (WHI) study, a trial that was designed to give an answer regarding the possible cardioprotective effects of ERT and HRT, has been stopped because of an increased incidence of breast cancer.[38]

Hormone Replacement Therapy

The finding that estrogen receptors are localized on endothelial and vascular smooth muscle cells of several mammalian species has suggested that the hormone may directly influence vascular function.[105] Estrogen receptor expression has also been demonstrated in human vascular tissue.[106] Estrogen therapy has been shown to have a beneficial effect on endothelial function in postmenopausal women with atherosclerotic coronary arteries.[107]

Current studies of genistein supplementation

In 2001, the cross-sectional study examined the association between usual dietary isoflavone intake and cardiovascular (CVD) risk factors, including lipids and lipoproteins, body mass index (BMI) and fat distribution, blood pressure, glucose and insulin was study by Goodman-Gruen et al.[108] They demonstrated that women with high genistein intake (≥ 1.0 mg / d) had a significantly lower BMI, waist circumference and fasting insulin than those with no daily genistein consumption. They concluded that these data showed a protective role for dietary soy intake against CVD in postmenopausal women.

Karamsetty et al (2001) studied the effects of genistein on the impairment in pulmonary arteries isolated from rats exposed to chronic hypoxia. As the results, the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (100 μM) completely blocked the genistein, daidzein and 17β-estradiol-induced restoration of the relaxation response to carbachol, whereas the estrogen receptor antagonist ICI 182,780 (10 μM) had no effect on the relaxation responses to carbachol. These results suggested that phytoestrogen genistein acted like estrogen in restoring nitric oxide-mediated relaxation in chronically hypoxic rat pulmonary arteries and this effect did not appear to be mediated by inhibition of tyrosine kinases or by known estrogen receptors[109] which was consistent the study by Squadrito.[16]

Altavilla et al (2001) found that OVX rats showed a reduced calcium-dependent NO synthase (cNOS) activity. Treatment with α-zearalenol or with 17β-estradiol reverted the endothelial dysfunction and increased cNOS activity in lung homogenates. These effects were abolished by the pure estrogen receptor antagonist ICI182,780. These results suggested that α-zearalenol improved endothelium-dependent relaxation in OVX rats through an estrogen receptor-mediated effect[110] which was consistent with the study by Nevala[111] and Figtree.[15]

Nevala and his colleage (2001) demonstrated that genistein- and daidzein-induced relaxation were inhibited by the antagonists of large conductance Ca^{2+}-activated K^{+}-channels (K_{Ca}). Moreover, they found that 17β-estradiol-induced relaxation was reduced by the antagonist as the above and the antagonist of small conductance Ca^{2+}-activated K^{+}-channels. They concluded that in the noradrenaline preconstricted rat mesenteric arteries, the relaxations caused by 17β-estradiol, genistein and daidzein were antagonized by large and small conductance K_{Ca}-channel inhibitors, suggesting the role of these channels as one of the relaxation mechanisms.[111]

Walker and his group (2001) showed that genistein produced increased blood flow in premenopausal women. In addition, genistein and 17β-estradiol were inhibited to the same degree by the NO synthase inhibitor N^{G}-monomethyl-L-arginine. These results indicated that genistein caused L-arginine / NO-dependent vasodilation in forearm vasculature of human subjects with similar potency to 17β-estradiol and potentiates endothelium-dependent vasodilation to acetylcholine.[112] These results were consistent with the study by Squadrito.[16]

Simons et al (2000) demonstrated that there were no significant effects of phytoestrogens supplementation (80 mg / d) on blood pressure and plasma lipid or lipoprotein concentration in healthy postmenopausal women. These results suggested that it did not affect on lipid and lipoprotein or endothelial function in healthy postmenopausal women.[37]

Current studies of estrogen deficiency induced endothelial dysfunction

In 2001, Wassmann and his co-worker studied in spontaneous hypertensive rats (SHR) and found that estrogen deficiency led to an enhanced vasoconstriction by angiotensin II and increased vascular free radical production via increased vascular AT_1 receptor expression, resulting in endothelial dysfunction. Estrogen

replacement therapy and AT_1 receptor antagonist prevent these pathological changes. Therefore, estrogen deficiency-induced AT_1 receptor overexpression and oxidation stress may play an important role in cardiovascular diseases associated with menopause.[113]

In 2000, Hernandez and his group demonstrated that estrogen could decrease blood pressure and increase vascular conductance in ovariectomized rats. This effect might be due to an increase in NO synthase and / or preventing oxidative stress then improving endothelial function.[114] This study was consistent with the study by Binko.[115]

Moreover, it revealed that physiological doses of estrogen immediately stimulated NO released from human endothelial cells through activation of a cell-surface estrogen receptor that was coupled to increase in intracellular calcium.[116] There was also some current studies clarified another mechanisms, for example non genomic pathway. Recently, Simoncini et al (2002) found that non genomic ER signaling trigger by a SERMs led to a rapid activation of NO synthesis in human endothelial cells. The ability of raloxifene to facilitate ERα-PI_{3k} interaction may provide an additional insight into the structure-function relationship of specific SERMs, which promotes the non-transcriptional effects of ER.[117]

CHAPTER III

MATERIALS AND METHODS

Animals Preparation

Female mature, non-pregnant, Wistar rats 10 weeks of age (BW; 220-280 g) were used for this study. These animals were obtained from National Laboratory Animal Center of Salaya Campus, Mahidol University, and were maintained on normal rat food and tap water ad libitum under controlled environmental conditions of 12-hr light/dark period. Before starting the experiment, solid food were withdrawn from the animal's diet for 12 hours, with no limitation on water supply.

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
Indomethacin	Sigma, USA.
N^{ω}-L-arginine methyl ester (L-NAME)	Sigma, USA.
Normal saline	Thai Nakorn Patana Co.,Ltd., Thailand
Pentobarbiturate sodium (Nembutal R)	Sanofi, Thailand
Sodium nitroprusside	Sigma,USA

Chemical	Company
Acetylcholine	Sigma, USA
Norepinephrine	Sigma, USA
Krebs-Ringer solution	
Sodium chloride (NaCl)	Merck, USA
Potassium chloride (KCl)	Riedel-de Hach, Germany
Calcium chloride ($CaCl_2$)	Riedel-de Hach, Germany
Sodium bicarbonate ($NaHCO_3$)	Riedel-de Hach, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Riedel-de Hach, Germany
Magnesium sulphate ($MgSO_4$)	Riedel-de Hach, Germany

The compositions of Krebs-Ringer solution were given 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

Methods

In order to study the preventive and therapeutic effects of genistein on endothelium-dependent and endothelium-independent vasorelaxation of mesenteric arterioles, we designed the experimental study as shown in figure 3.1.

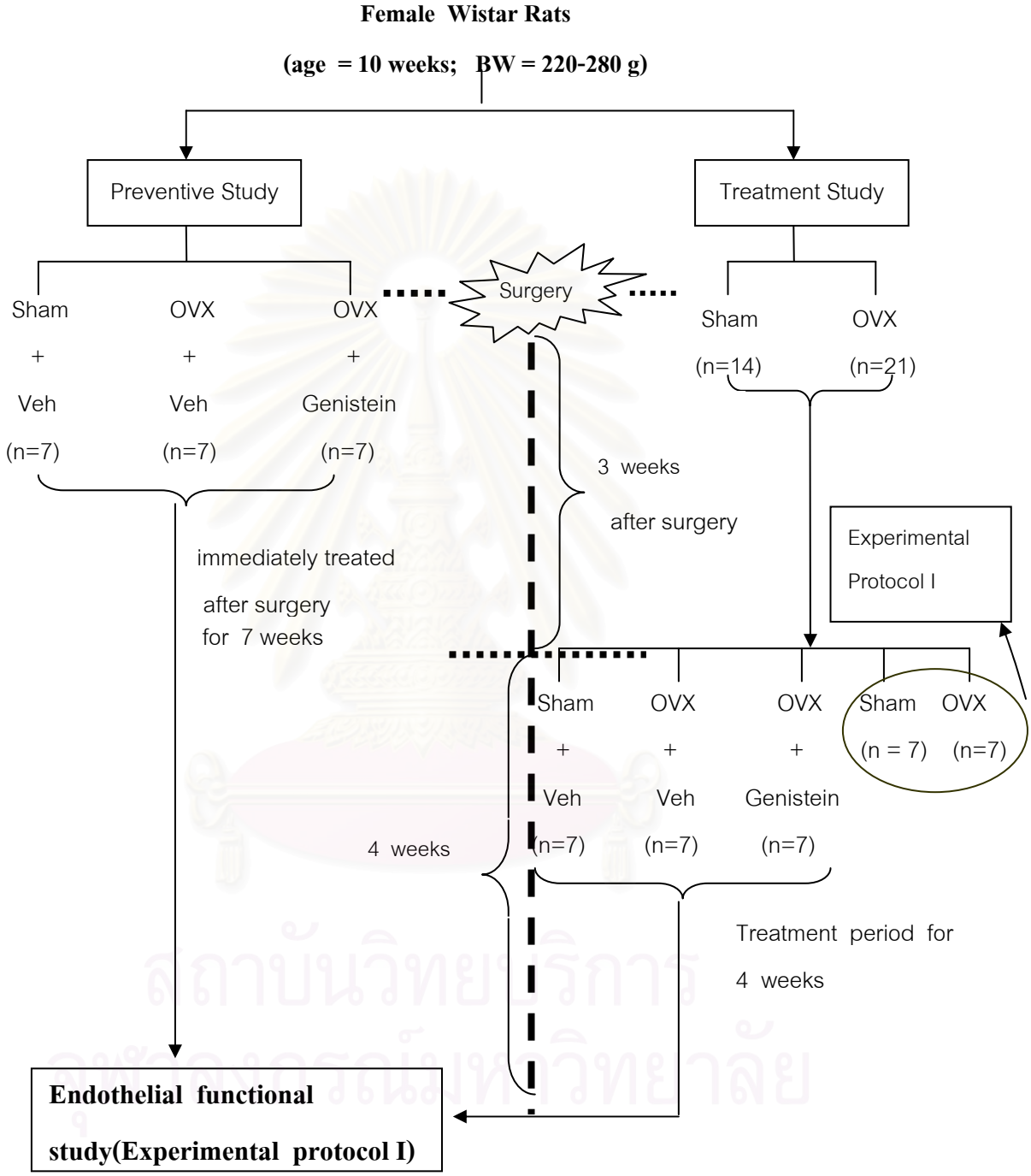


Figure 3.1 Experimental design to study the preventive and treatment effects of genistein on endothelial function.

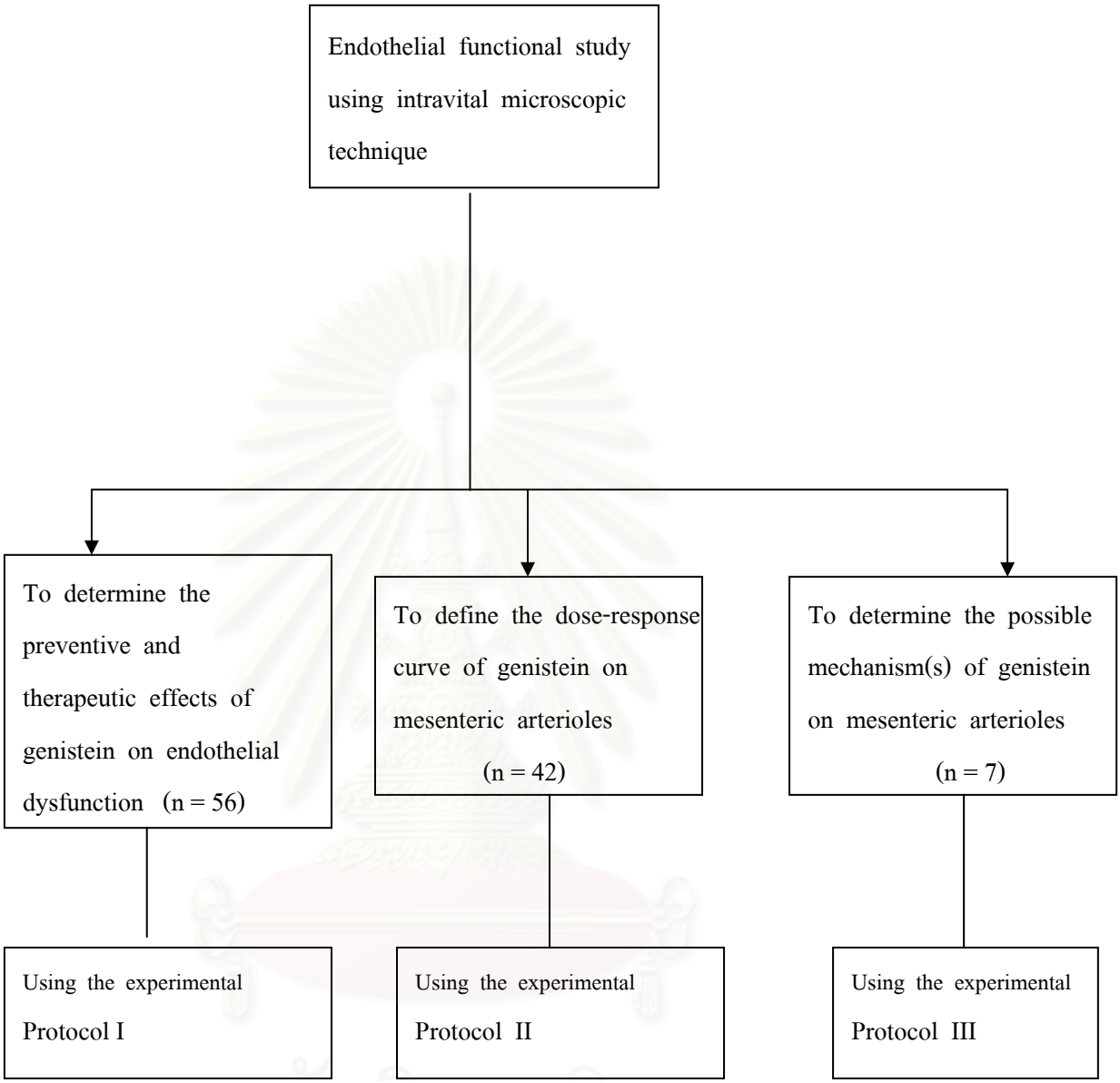


Figure 3.2 Endothelial functional study protocols.

Research Operation

1 Animal preparation for studying the preventive and therapeutic effects of genistein on endothelial cells dysfunction

For ovariectomized procedure, rats were anesthetized by intraperitoneally (i.p) injection of sodium pentobarbital at a dose of 45 mg/kg body weight. They

were subjected to a bilateral ovariectomy (OVX rats). Sham operated animals were used as controls. These animals were divided into two major groups, the prevention and the treatment ones.

The prevention group, designed to study the preventive effects of genistein on endothelial dysfunction, was subdivided into three subgroups; sham with vehicle ($Sham_{veh}$), ovx with genistein (OVX_{gen}), and ovx with vehicle (OVX_{veh}). The $Sham_{veh}$ received dimethyl sulfoxide (DMSO), the vehicle for genistein, in the dosage of 100 μl subcutaneously everyday. The OVX_{gen} was given daily subcutaneous injection of genistein 0.25 mg/Kg body weight in 100 μl of DMSO.[136] And the OVX_{veh} received 100 μl of DMSO subcutaneously daily. The further protocol (protocol I) was performed after 7 weeks of treatment.

The treatment group, designed to study the treatment effects of genistein on endothelial dysfunction, was divided into two major groups; $animals_{3\text{-}week}$ ($OVX_{3\text{-}week}$,$Sham_{3\text{-}week}$), $animals_{treat}$ (OVX_{veh}, $Sham_{veh}$, OVX_{gen}). Animals were subjected to a bilateral ovariectomy (ovx group). Sham-operated animals were used as controls. After 3 weeks of washout period, the experimental procedure was performed on samples of both two groups ($OVX_{3\text{-}week}$ and $Sham_{3\text{-}week}$) in order to confirm that the lack of estrogens has already induced the endothelium to become dysfunction so that we can use this endothelial dysfunction model for further studying of genstein actions. After that the three subgroups (OVX_{veh}, $Sham_{veh}$, and OVX_{gen}) were treated in the same manner as the preventive group, apart from that all the treatments were given after 3 weeks of washout period.[137] Then, after 4 weeks of treatment,[16] the further protocol (protocol I) was performed.

2 Direct visualization of mesenteric microcirculation

Video image of the mesenteric microcirculation of the distal ileum was obtained by epi-illumination fluorescent microscopy using fluorescene isothiocyanate-labeled 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.[138,139,140] After FITC – dextran – 250 reached the mesenteric arterioles, images of vessels could be observed by epi-illumination fluorescene 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 fluorescene microscopy using a × 20 objective lens and a 10 × eyepiece (CFI Plan Fluor) which was used to observe mesenteric arteriolar diameter. Video images of micro vessels were stored on videotape (Sony, SLV-X 311) connected with the video timer. During the experiment, micro vessel images could be printed by using video graphic printer (Sony, UP – 890 CE). Diameter of mesenteric microvessel images were measured from the fluorescene video image of FITC – labeled dextran on the video monitor using digital image processor system (Fig. 3.3)[141] with the software "Global Image". The arteriolar diameter was calculated as the mean of triple measurements from three video frames by using the same reference point as a marker for measuring each vessel in each frame (Fig 3.4). Vasodilation responses were expressed as the percentage of maximal relaxation after preconstriction with norepinephrine (NE ; 10^{-5} M).[142]

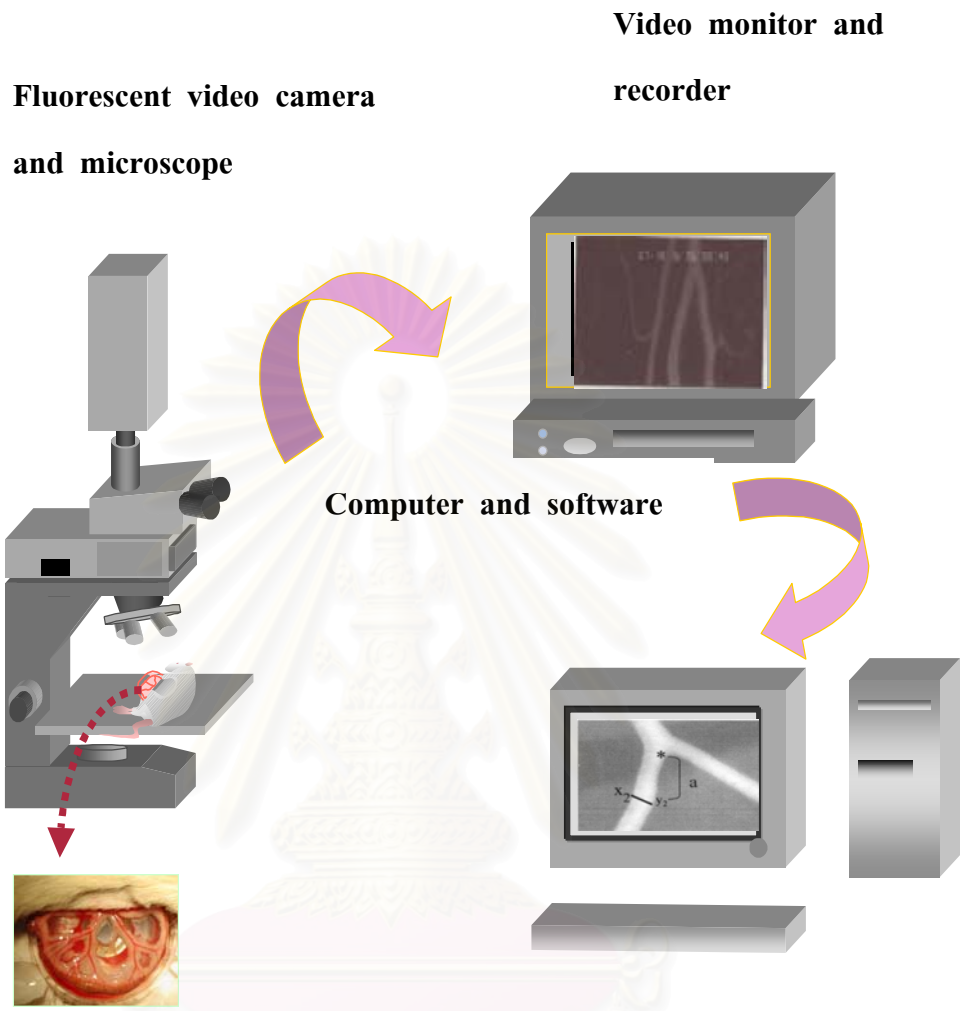
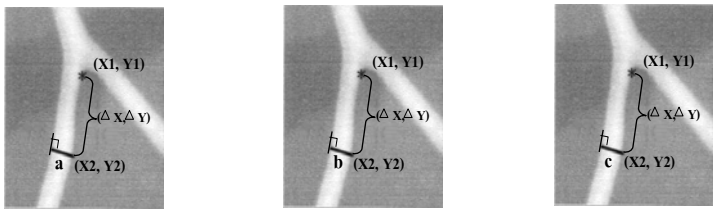


Figure 3.3 Endothelial functional study using intravital microscopic technique.

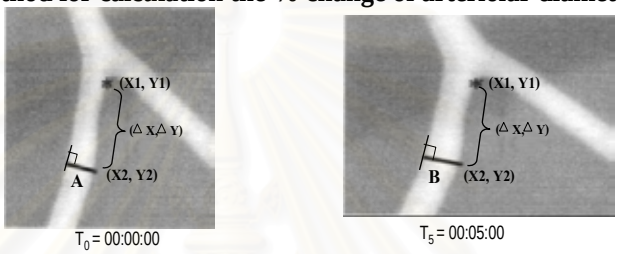
Method for measurement of arteriolar diameter



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

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

Method for calculation the % change of arteriolar diameter



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

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

Figure 3.4 Methods for calculation the percentage of arteriolar diameter change.

3 Plasma E_2 determination

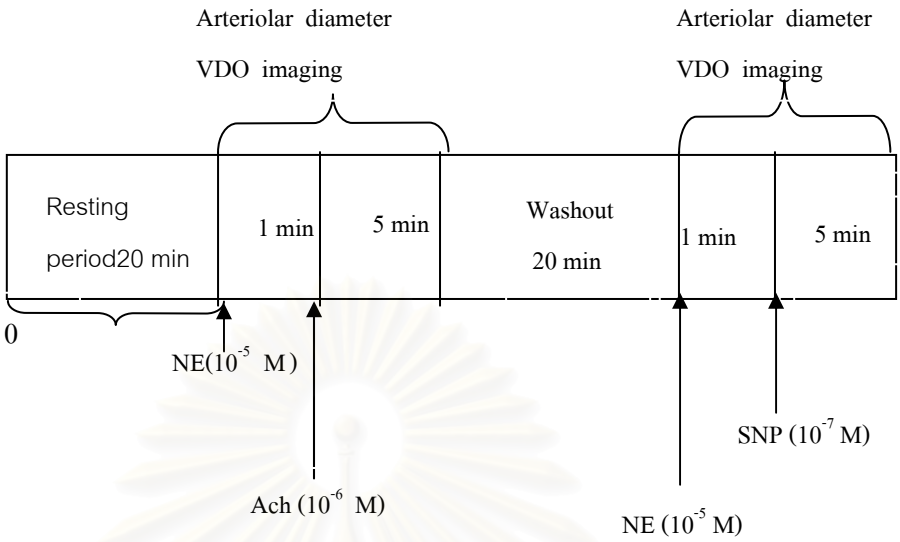
Blood samples were collected during anestrous period at the end of the experiment. In order to evaluate plasma E_2 levels, blood samples (3 ml) were collected in polyprophylene tubes containing heparin (50,000 IU) and after centrifugation at 3500 × g at 4°C for 10 minutes, each sample was stored at -70°C until analysis. Plasma E_2 levels were determine by electrochemiluminescence immunoassay (ECLIT) with a commercial available kit. Functional sensitivity assay is 44 pmol / l (12 pg / ml) and reproducible measured with an inter-assay coefficient of variation of $\leq$ 20 %.

Protocol I: To determine the preventive and treatment effects of genistein

On the day of the experiment, rats were anesthetized with intraperitoneally injection (i.p) of 45 mg/Kg body weight 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. Blood pressure was measured via a canular inserted into common carotid artery by using polygraph system (NIHON KODEN, Japan). Heart rate was calculated based on blood pressure tracing.

The abdomen was opened and the small intestine was displaced to expose a segment of the mesentery. The distal of ileum was exteriorized.[143,144,145] 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 $CaCl_2 .2H_2O$, 7.14 $NaHCO_3$, 1.18 KH_2PO_4, 1.64 $MgSO_4.7H_2O$ and maintained at 37°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 jugular vein. The arteriolar diameter was observed before and after the application of Ach (10^{-6} 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^{-7}M),[146] 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 Ach procedure.



At the end of the experiment, blood sample was drawn from common carotid artery. Uterus was weighed.

Protocol II: To determine the dose-response curve of arteriolar diameter to genistein: EC_{50} of genistein

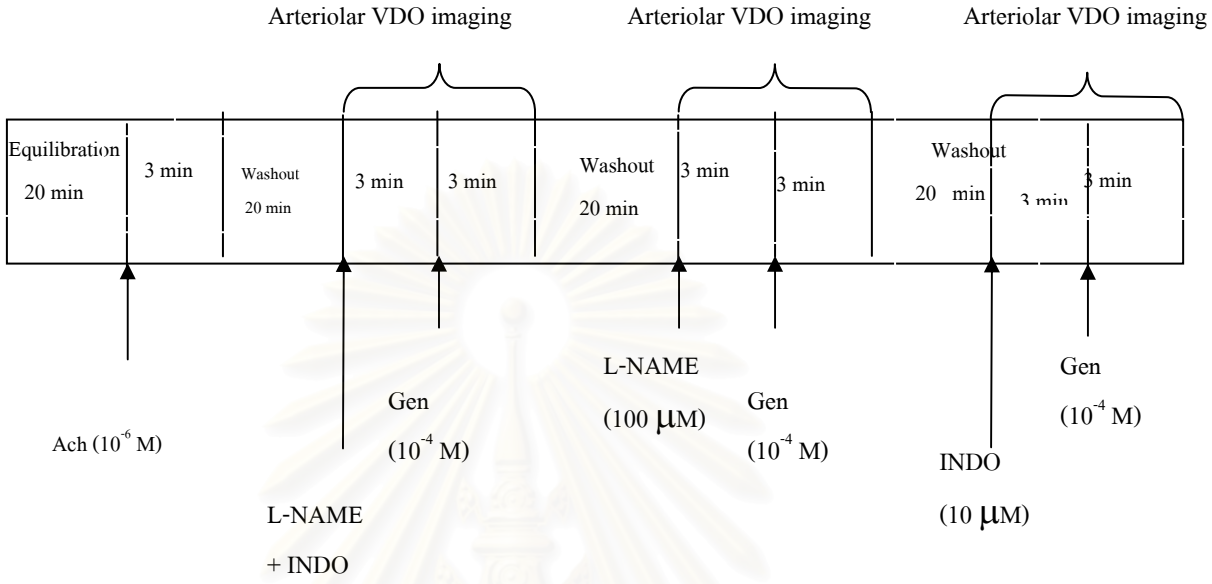
By using normal female Wistar rat (age:10 weeks; BW :220-280g), the preparation of mesenteric arteriolar study was performed. The vessels were labeled with 5 mg % of FITC – dextran 250 (Sigma, USA) injected into jugular vein. The vessels were preconstricted with NE (10^{-5} M) and followed by the various doses of genistein ($10^{-2} – 10^{-7}$.M).[14] The direct visualization of mesenteric arteriole was performed. The relaxation was expressed as a percentage of the norepinephrine – induced constriction diameter. Data were analyzed with nonlinear regression of sigmoidal dose – response curves which was used to calculate the EC_{50}.

At the end of the experiment, uterus was weighed.

Protocol III: To define the mechanism(s) of genistein.

After defining the mesenteric arterioles using 0.2 ml of 5 % FITC-dextran 250, they were equilibrated for 20 minutes and were allowed to reach

a steady – state level of baseline diameter. The functional integrity of endothelium was accessed by a relaxation response to acetylcholine (Ach; 10^{-6}M).[16] Then the vessels were washed three times with Krebs-Ringer solution and equilibrated for 20 minutes. L – NAME plus INDO (NOS inhibitor and cyclooxygenase inhibitor, respectively) were applied topically for 3 minutes[147] prior to testing genistein. The mesenteric arteriolar diameter was measured before and after adding these two substances by a computer – assisted image analyzer. Genistein (10^{-4} M) was applied topically and mesenteric arteriolar diameter was recorded and measured. After that, the vessels were washed with Krebs-Ringer solution three times and a 20 minutes washout period was used to allow the vessels to return to their base – line diameter. N^{ω} - L – arginine methyl ester (L – NAME; 100 μM), the inhibitor of nitric oxide synthase (NOS inhibitor), was applied topically on the mesenteric arterioles for 3 minutes[147] before testing genistein. The mesenteric arteriolar diameter was measured before and after applying L – NAME by a computer – assisted image analyzer. At steady state, genistein (10^{-4} M) was applied topically. Then, the mesenteric arteriolar diameter was measured by a computer-assisted image analyzer. The vessels were washed with Krebs-Ringer solution three times and a 20 minutes washout period was used to allow the vessels to return to their base –line diameter. Indomethacin (INDO; 10 μM), cyclooxygenase inhibitor, was applied topically for 3 minutes[147] before genistein applying. The mesenteric arteriolar diameter was measured before and after applying INDO by a computer – assisted image analyzer. Genistein (10^{-4} M) was applied topically in the same manner as L-NAME application. After finishing the experiment, rats were terminated with overdose of sodium pentobarbital.



Data Analysis

Results were shown as mean ± SEM. One – way ANOVA was used to determine the difference of means. Post Hoc test was used for multiple comparison among groups. Student's t-test was used to compare the difference of means between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$ and the mechanism study section. The statistical differences were considered at the probability level (p-Value) of lower than 0.05.

CHAPTER IV

RESULTS

According to the previous experimental protocols, this chapter is composed of four major parts of results. They are the results of treatment group, prevention group. dose-response of genistein on mesenteric arterioles and the mechanism(s) of action of genistein.

1) The treatment effects of genistein on endothelial dysfunction

The treatment group was divided into five subgroups; $OVX_{3\text{-week}}$, $Sham_{3\text{-week}}$, OVX_{veh}, OVX_{gen} and $Sham_{veh}$. Before starting the experiment, female Wistar rats (age; 10 weeks) were weighed and randomly assigned to receive genistein (0.25 mg / Kg.BW, sc.; OVX_{gen}) or vehicle (DMSO 100 μl, sc.; OVX_{veh}) daily for four weeks except $OVX_{3\text{-week}}$ group. $OVX_{3\text{-week}}$ was assigned to confirm that three weeks after surgery, the vascular endothelium became dysfunction. $Sham_{3\text{-week}}$ and $Sham_{veh}$ were served as control of their OVX group. Other parameters, for example, blood pressure, heart rate and uterine weight were performed during / and at the end of the experiment, respectively. Mean arterial pressure (MAP) was calculated based on systolic and diastolic blood pressure.

1.1 The treatment effects of genistein on body weight

After three weeks of washout period, the percentage of change of the body weight was significantly different between $Sham_{3\text{-week}}$ and $OVX_{3\text{-week}}$. After 4 weeks of treatment period, the percent change of BW was significantly increased in OVX_{veh} as compared to $Sham_{veh}$ and OVX_{gen} (Table 4.1).

1.2 The treatment effects of genistein on mean arterial pressure (MAP), and heart rate (HR)

Mean arterial pressure (MAP) was significantly increased in $OVX_{3\text{-week}}$ as compared to its control as well as the comparison between OVX_{veh} and $Sham_{veh}$. There was not significantly different between $OVX_{3\text{-week}}$ and OVX_{veh} groups as well as $Sham_{veh}$ and OVX_{gen} groups (Table 4.1). Heart rate (HR) was not significantly different among these groups (Table 4.1).

At the end of the experiment, uterus was collected and weighed in order to confirm the decrement effects of 17β-estradiol. As the results, uterine weight was significantly decreased in OVX groups as compared to their Sham groups (Table 4.1).

Table 4.1 Mean±SEM of percent change of body weight (BW), mean arterial pressure (MAP), heart rate (HR), uterine weight and plasma E_2 in treatment group (n = 7 in each group).

group	% change of BW	MAP (mmHg)	HR (beat/min)	Uterine weight/BW	Plasma E_2 (pg / ml)
$OVX_{3\text{-week}}$	25.27±3.59[a]	141.43± 4.71[a]	351.43± 41.40	0.009±0.005[a]	13.57± 1.68[a]
$Sham_{3\text{-week}}$	7.48±3.88	120.95± 3.06	358.57± 12.04	0.023±0.004	38.63± 2.11
$Sham_{veh}$	7.89±3.13	118.10± 6.90	377.14± 29.28	0.017±0.001	37.11± 1.85
OVX_{gen}	12.98±3.84	123.33± 8.61[d]	368.57± 22.68[NS]	0.003±0.001[b]	15.88± 3.16[b]
OVX_{veh}	48.55±3.68[c]	139.99± 8.82[c]	368.57± 22.68[NS]	0.007±0.004[c]	17.56± 0.12[c]

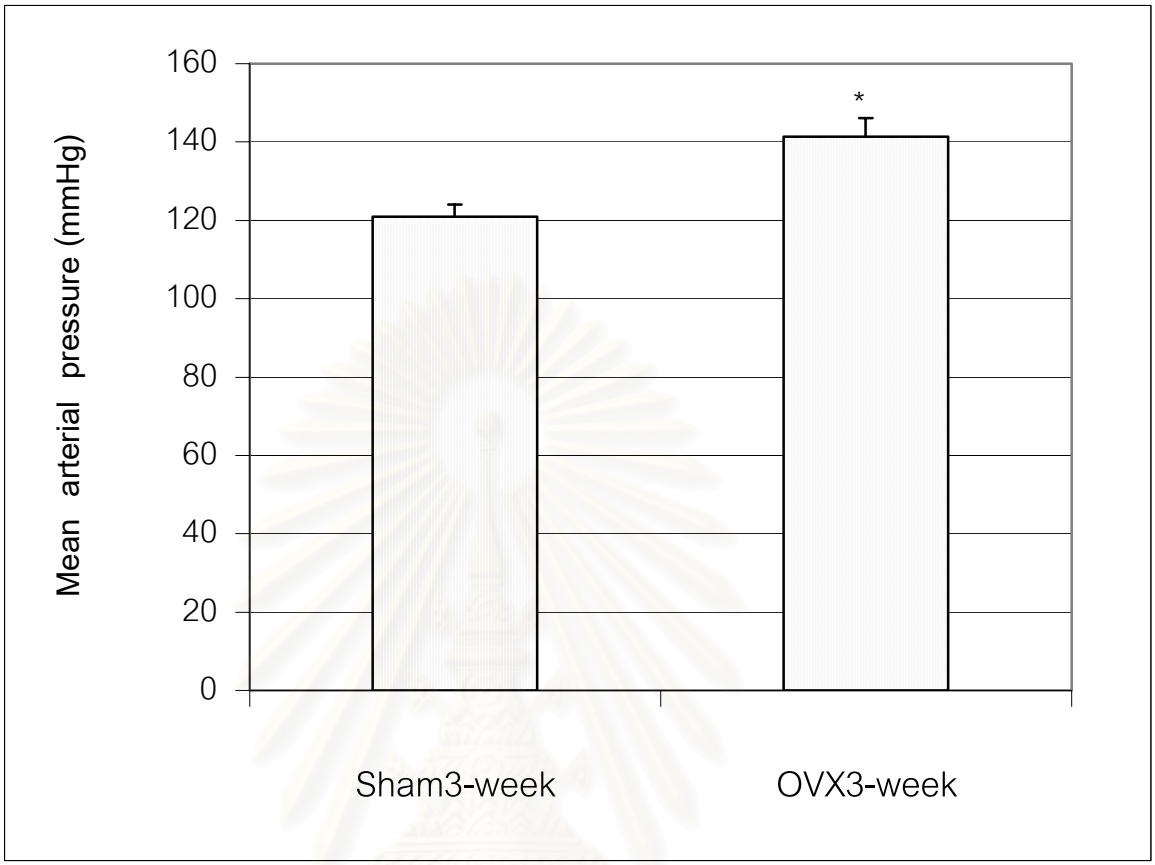
[a] $p < 0.05$ significantly different as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$

[b] $p < 0.05$ significantly different as compared between OVX_{gen} and $Sham_{veh}$

[c] $p < 0.05$ significantly different as compared between OVX_{veh} and $Sham_{veh}$

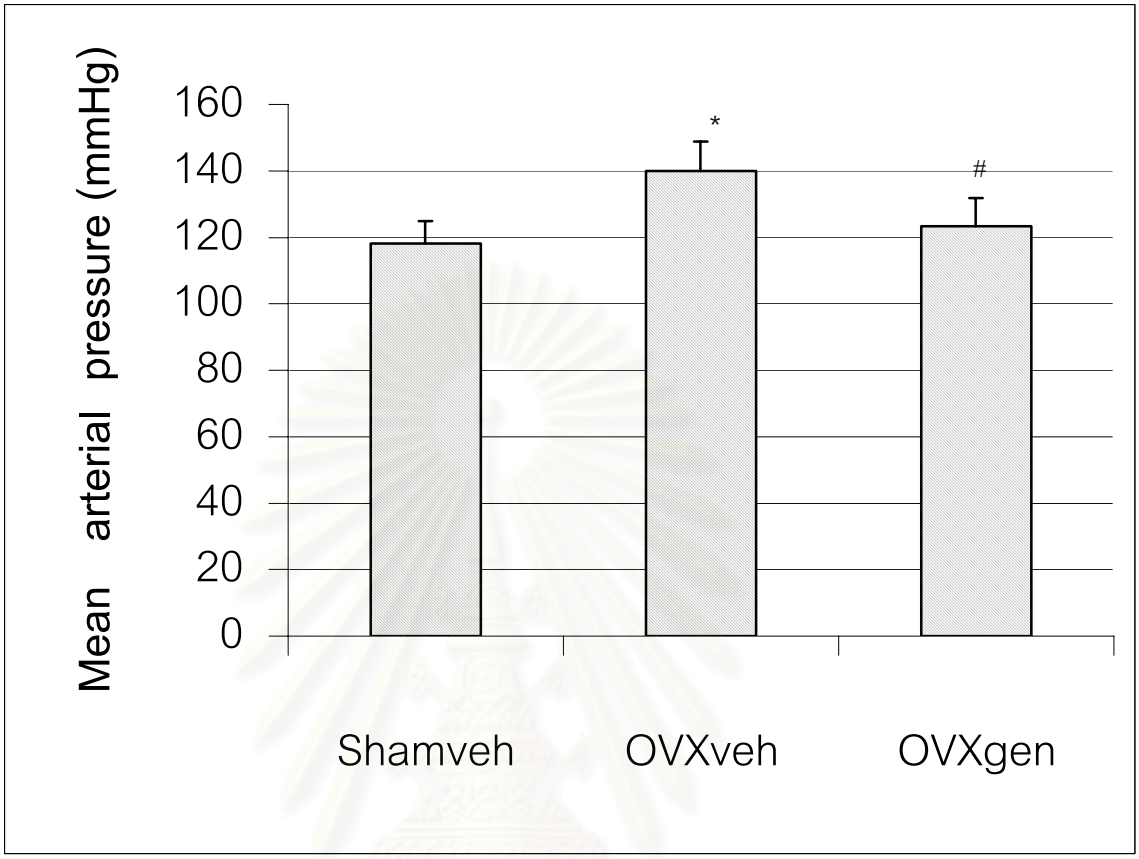
[d] $p < 0.05$ significantly different as compared between OVX_{gen} and OVX_{veh}

NS nonsignificantly different as compared OVX_{gen} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}



*p < 0.05 significantly different as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$

Figure 4.1 A The effect of estrogen deprivation on MAP as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$. MAP was monitored after surgery for 3 weeks (3 weeks of washout period). n = 7 in each group



* $p < 0.001$ significantly different as compared between OVX_{veh} and $Sham_{veh}$

# $p < 0.05$ significantly different as compared between OVX_{gen} and OVX_{veh}

Figure 4.1 B The effect of genistein in treatment group on MAP as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}. MAP was monitored after treatment with genistein or vehicle daily treatment for 4 weeks. (n = 7 in each group)

1.3 The comparison of plasma E_2 levels between ovx and sham groups

It is accepted that estrogen deficiency induced endothelial dysfunction. In this part, we would like to confirm that after 3 weeks of washout period, estrogen deprivation caused vascular endothelial dysfunction. After bilateral ovariectomy for three weeks, blood sample was collected and determined for E_2 levels. We found that plasma E_2 was significantly decreased in OVX groups as compared to their Sham groups, while it was not significantly different between OVX_{veh} and OVX_{gen} groups (Table 4.1).

1.4 The treatment effects of genistein on vascular response

In order to confirm that after 3 weeks of washout period, the endothelium became dysfunction, rats were divided into two groups: the bilateral ovariectomy ($OVX_{3\text{-week}}$) and the surgery without bilateral ovariectomy ($Sham_{3\text{-week}}$). Both of them passed washout period for three weeks. After that vascular endothelial function was studied and recorded for analysis. As the results, the response to acetylcholine was significantly decreased in $OVX_{3\text{-week}}$. In addition, the mesenteric arteriolar diameter was significantly decreased in $OVX_{3\text{-week}}$ (Table 4.2, Fig 4.2 A). Conversely, it was not significantly different in SNP testing between these two groups. Therefore, three weeks of washout period led to endothelium becoming dysfunction.

Table 4.2 The response to Ach and SNP on mesenteric arteriolar diameter compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$ at the same age after three weeks of washout period (n = 7 in each group).

group	% change of mesenteric arteriolar diameter	
	Ach testing	**SNP testing**
$OVX_{3\text{-week}}$	$2.76 \pm 2.98^{*}$	30.84 ± 5.46^{NS}
$Sham_{3\text{-week}}$	33.69 ± 1.05	33.47 ± 2.37

$^{*}p < 0.001$ significantly different as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$

NS nonsignificantly different as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$

After confirming the endothelial dysfunction after 3 weeks of washout period, the treatment protocol was started. Acetylcholine (Ach 10^{-6} M) and sodium nitrprusside (SNP 10^{-7} M) were applied for studying endothelium-dependent and -independent vasorelaxation, respectively. As the results, the response to acetylcholine on mesenteric arterioles was significantly decreased in OVX_{veh} as compared to $Sham_{veh}$ groups ($p < 0.001$). In the same way as comparison between OVX_{veh} and OVX_{gen} group, we found that the response to acetylcholine was significantly decreased in OVX_{veh} group compared to OVX_{gen} ($p < 0.001$). While, it was not significantly different between $Sham_{veh}$ and OVX_{gen} group (Table 4.3, Fig 4.2 B, Fig 4.3).

Table 4.3 The effects of Ach and SNP on mesenteric arterioles in treatment group (n = 7 in each group).

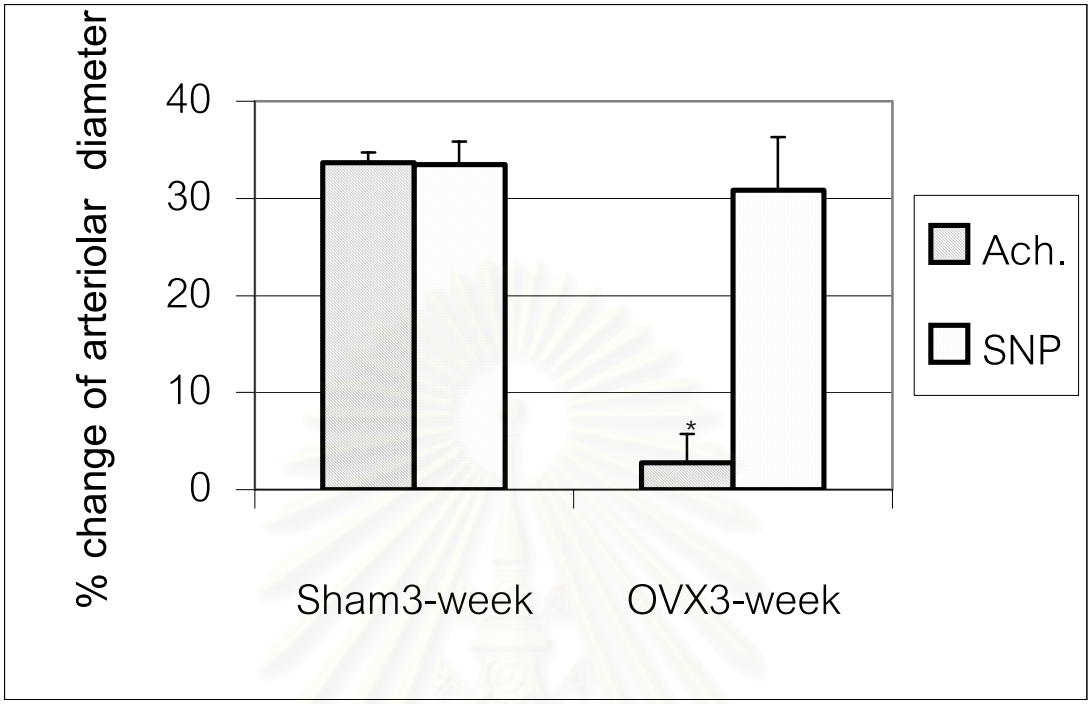
group	% change of mesenteric arteriolar diameter	
	Ach. testing	**SNP testing**
$Sham_{veh}$	45.46 ±3.59	34.69±5.01
OVX_{veh}	3.03 ±3.99*	28.22±2.82NS
OVX_{gen}	33.52 ±3.25#	30.09±2.81NS

*$p < 0.001$ significantly different as compared OVX_{veh} VS $Sham_{veh}$

#$p < 0.001$ significantly different as compared OVX_{gen} VS OVX_{veh}

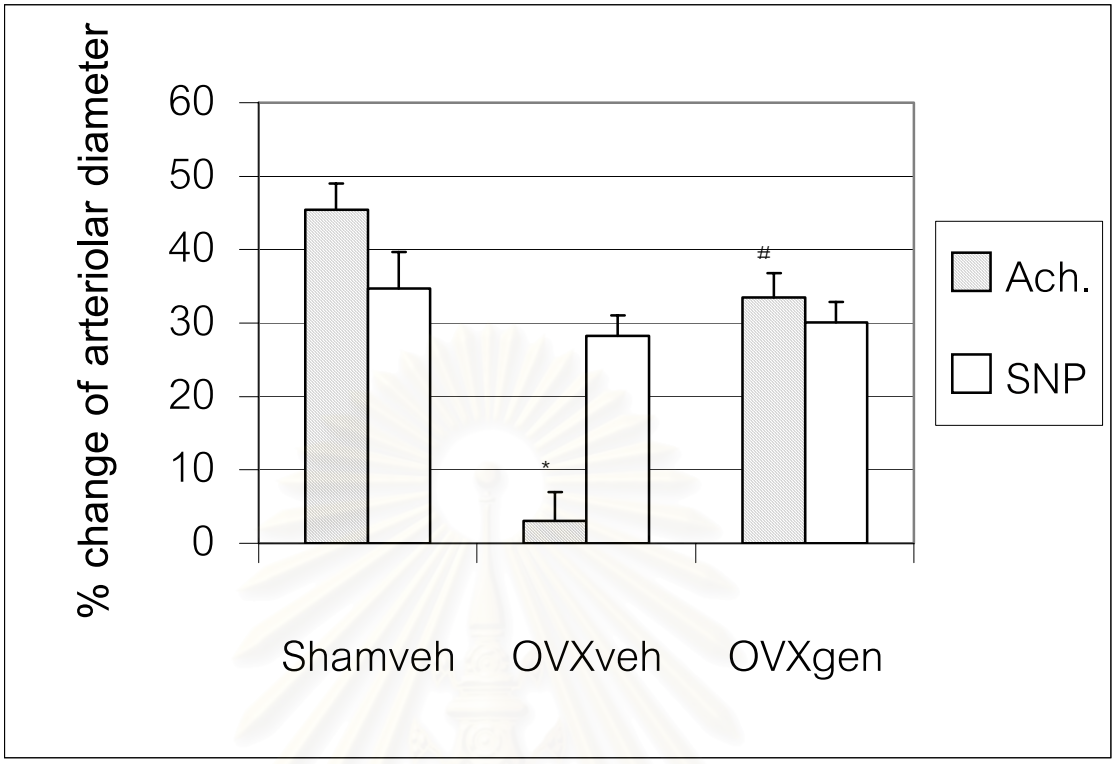
NS nonsignificantly different as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}

Besides testing the response to acetylcholine, we also studied the response to sodium nitroprusside (SNP 10^{-7} M) on mesenteric arterioles (endothelium-independent vasorelaxation). As the results, it was not significantly different among these groups (Table 4.3, Fig 4.2 B).



* $p < 0.001$ significantly different as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$

Figure 4.2 A The effects of estrogen deprivation on Ach- and SNP-induced vasorelaxation as compared between $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$. Ach- and SNP-induced vasorelaxation were expressed by mean±SEM of the percentage of change of the arteriolar diameter which monitored after surgery for 3 weeks (3 weeks of washout period). n = 7 in each group



# $p < 0.001$ significantly different as compared between OVX_{gen} and OVX_{veh}

* $p < 0.001$ significantly different as compared between OVX_{veh} and $Sham_{veh}$

Figure 4.2 B The effects of genistein in treatment group on Ach- and SNP-induced vasorelaxation as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}. Ach- and SNP-induced vasorelaxation were expressed by mean±SEM of the percentage of change of the arteriolar diameter which monitored after daily treatment with genistein or vehicle for 4 weeks. (n = 7 in each group)

A. B. C.

Figure 4.3 Videomicroscopic images to demonstrate vascular responses in OVX_{gen} (A = before applying NE; B = after applying NE for 1 minute; C = after applying Ach for 3 minutes).

2) The preventive effects of genistein on endothelial function

The preventive group was divided into three subgroups; $Sham_{veh}$, OVX_{veh} and OVX_{gen} groups. All of them were immediately treated with genistein or vehicle after surgery daily for seven weeks. Before starting the experiment, these animals were weighed. After seven weeks of treatment period, they were weighed again. During the experiment, vascular response both to acetylcholine and sodium nitroprusside were studied.

2.1 The preventive effects of genistein on body weight and uterine weight

We found that the percentage of change of the body weight (BW was significantly increased in OVX_{veh} group when compared to $Sham_{veh}$ (Table 4.4). At the end of the experiment, uterus was weighed. As the results, uterine weight of OVX_{gen} and OVX_{veh} were significantly less than $Sham_{veh}$, while it was not significantly different between OVX_{gen} and OVX_{veh} (Table 4.4).

2.2 The preventive effects of genistein on mean arterial pressure (MAP) and heart rate (HR)

Neither mean arterial pressure (MAP) nor heart rate (HR) were significantly different among these groups (Table 4.4, Fig 4.4).

Table 4.4 Mean±SEM of percent change of body weight (BW), mean arterial pressure (MAP), heart rate (HR), uterine weight and plasma E_2 of preventive group (n = 7 in each group).

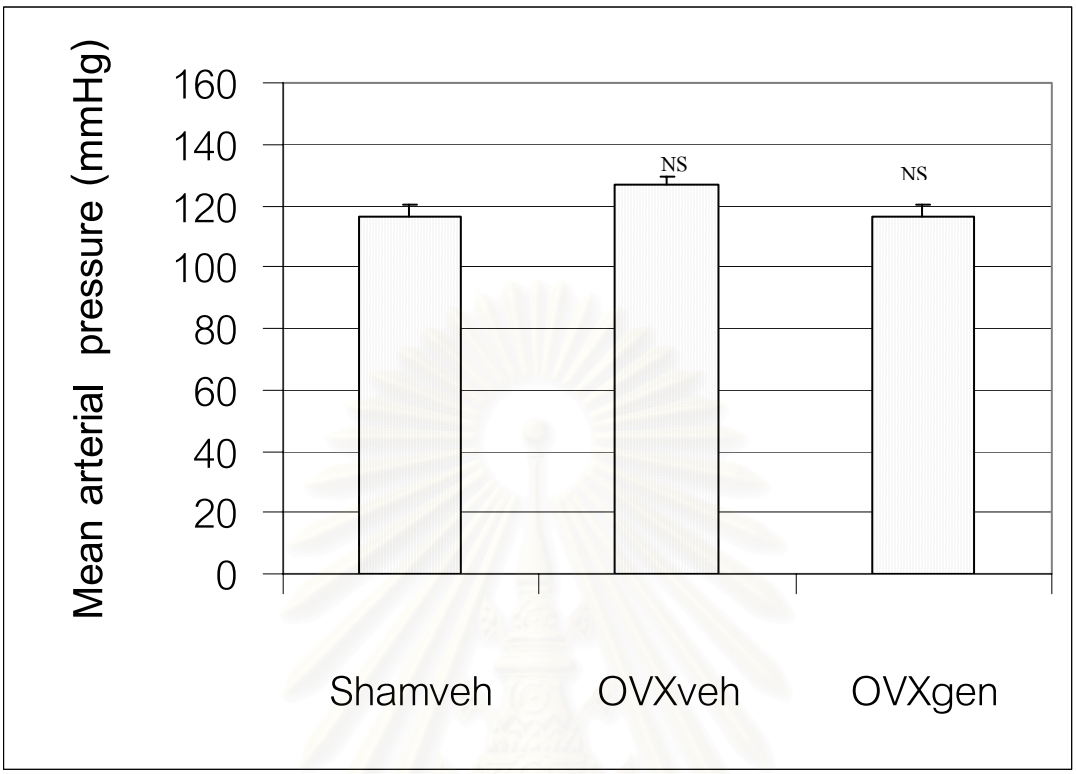
group	% change of BW	MAP (mmHg)	HR (beat/min)	Uterine weight/BW	Plasma E_2 (pg / ml)
$Sham_{veh}$	9.23±3.28	116.19± 4.01	342.86±11.07	0.015± 0.003	36.97± 0.91
OVX_{gen}	25.27±3.34	116.19± 4.39^{NS}	$342±11.07^{NS}$	0.003± 0.001^{*}	17.88± 1.44^{*}
OVX_{veh}	$29.94±2.43^{**}$	126.67± 3.01^{NS}	$353.33±6.67^{NS}$	0.005± 0.001^{**}	16.93± $+0.53^{**}$

** $p < 0.001$ significantly different as compared OVX_{veh} VS $Sham_{veh}$

* $p < 0.05$ significantly different as compared OVX_{gen} VS $Sham_{veh}$

NS nonsignificantly different as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}

NS



NS nonsignificantly different as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}

Figure 4.4 The effect of genistein in preventive group on MAP as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}. MAP was monitored after treatment with genistein or vehicle everyday for 7 weeks. Values were expressed as mean±SEM of MAP. (n = 7 in each subgroup)

2.3 The preventive effects of genistein on vascular response

The vascular response to acetylcholine was significantly decreased in OVX_{veh} as compared to $Sham_{veh}$ group ($p < 0.001$). Moreover, it was significantly different between OVX_{gen} and OVX_{veh} ($p < 0.001$). On the other hand, it was not significantly different between $Sham_{veh}$ and OVX_{gen} group (Table 4.5, Fig 4.5, Fig 4.6).

Table 4.5 The effects of Ach and SNP on mesenteric arterioles in preventive group ($n = 7$ in each group).

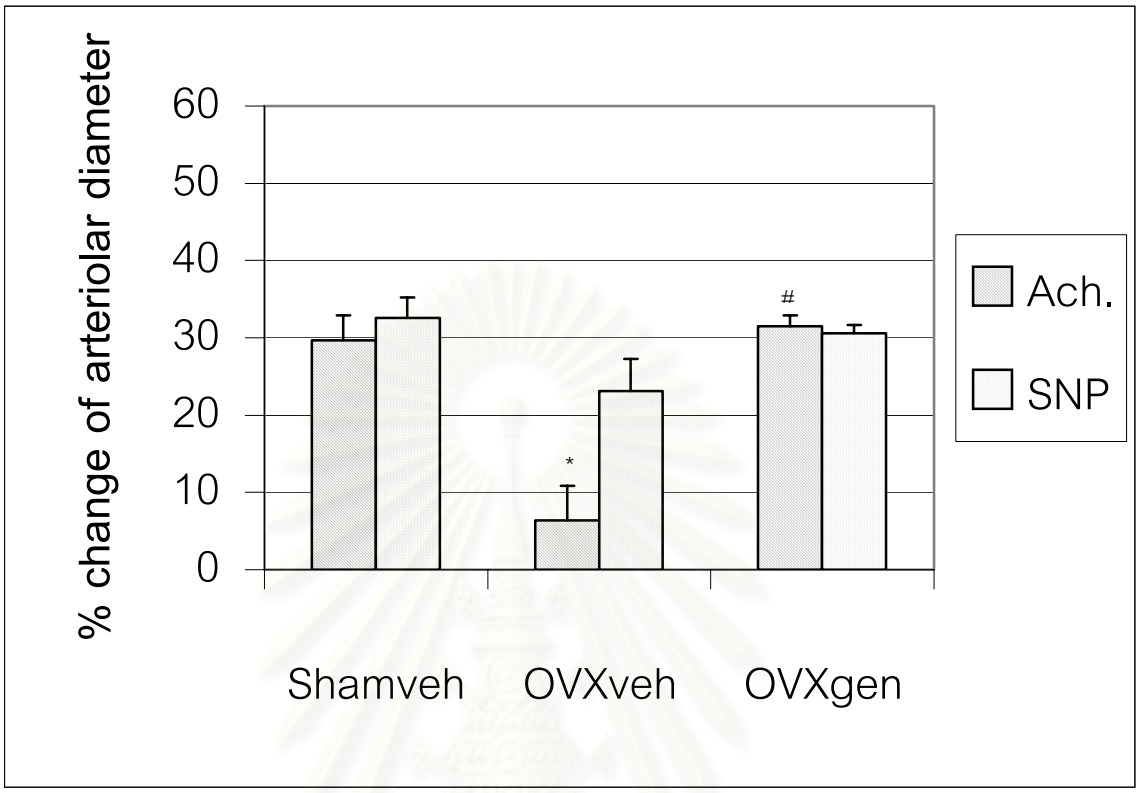
group	% change of mesenteric arteriolar diameter	
	Ach testing	**SNP testing**
$Sham_{veh}$	29.66 ±3.20	32.57±2.69
OVX_{veh}	6.40 ±4.48*	23.16±4.11^{NS}
OVX_{gen}	31.50 ± 1.40$^{\#}$	30.57±1.05^{NS}

$^{*}p < 0.001$ significantly different as compared OVX_{veh} VS $Sham_{veh}$

$^{\#}p < 0.001$ significantly different as compared OVX_{gen} VS OVX_{veh}

NS nonsignificantly different as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}

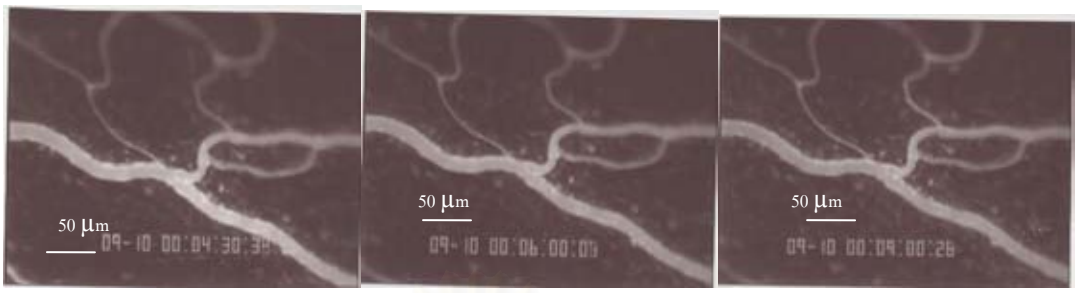
Conversely, the vascular response to sodium nitroprusside (SNP 10^{-7} M) was not significantly different among these groups (Table 4.5, Fig 4.5).



*p < 0.001 significantly different as compared between OVX_{veh} and $Sham_{veh}$

# p < 0.001 significantly different as compared between OVX_{gen} and OVX_{veh}

Figure 4.5 The effects of genistein in preventive group on Ach- and SNP-induced vasorelaxation as compared OVX_{veh} VS $Sham_{veh}$ and OVX_{gen} VS OVX_{veh}. Ach- and SNP-induced vasorelaxation were expressed by means±SEM of the percentage of the arteriolar diameter changes monitored after daily treatment with genistein or vehicle for 7 weeks. (n = 7 in each group)



A. B. C.

Figure 4.6 Videomicroscopic images to demonstrate vascular response in OVX_{gen} of preventive group (A = before applying NE; B = after applying NE for 1 minute; C = after applying Ach for 3 minutes).

3) Dose-response curve of genistein on mesenteric arterioles

In order to determine the mechanism(s) of action of genistein on endothelial cells, various doses of genistein were examined on mesenteric arterioles in mature female Wistar rats (age; 10 weeks). The response of doses (10^{-2} - 10^{-7} M) were observed for thirty minutes and recorded. We found that the optimal duration of response was at three minutes after topically applying genistein. After passing the three minutes, the percentage of change of mesenteric arteriolar diameter was gradually declined and diameter then returned to the baseline diameter. The optimal dose response was genistein at 10^{-4} M (Fig 4.7).

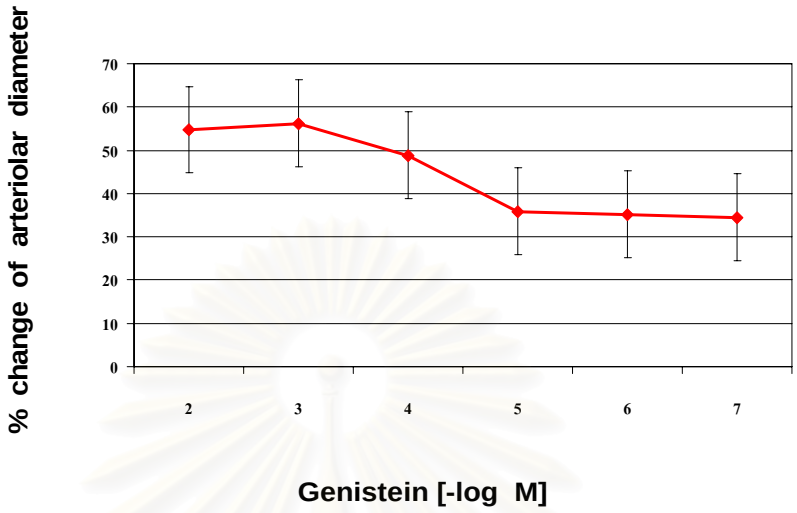


Figure 4.7 The percentage of mesenteric arteriolar diameter changes after topical applying genistein in various doses (10^{-2} to 10^{-7} M; n = 7 in each dose).

4) The mechanism of action of genistein on mesenteric arterioles

In order to test the functional integrity of endothelium, acetylcholine was then topically applied prior to studying the mechanism(s) of action. The preconstricted vessels with norepinephrine (NE) were significantly reduced by acetylcholine (10^{-6} M).

Before starting the protocols, vessels were rinsed with Krebs-Ringer solution and equilibrated for 20 minutes. In order to block the L-arginine / NO pathway and cyclooxygenase pathway, L-NAME plus INDO were then topically applied prior to genistein. These inhibitors could reduce vasodilation compared to steady state. Genistein did not significantly enhance vasodilation (Table 4.6, Fig 4.8).

After equilibration period, N^{ω} -L-arginine methyl ester (L-NAME), NOS inhibitor, was topically applied for 3 minutes and followed by genistein. L-NAME reduced vasodilation compared to steady state. Genistein significantly increased vasodilation when compared to the change of percentage of mesenteric arteriolar diameter with L-NAME ($p < 0.05$).

After rinsing with Krebs-Ringer solution and equilibrating for 20 minutes, indomethacin (INDO), a cyclooxygenase inhibitor, was topically applied to the mesenteric arterioles prior to genistein. INDO reduced vasodilation compared to steady state, while genistein did not reduce vasoconstriction. It was not significantly different between the change of percentage of mesenteric arteriolar diameter after applying INDO and genistein (Table 4.6, Fig 4.8).

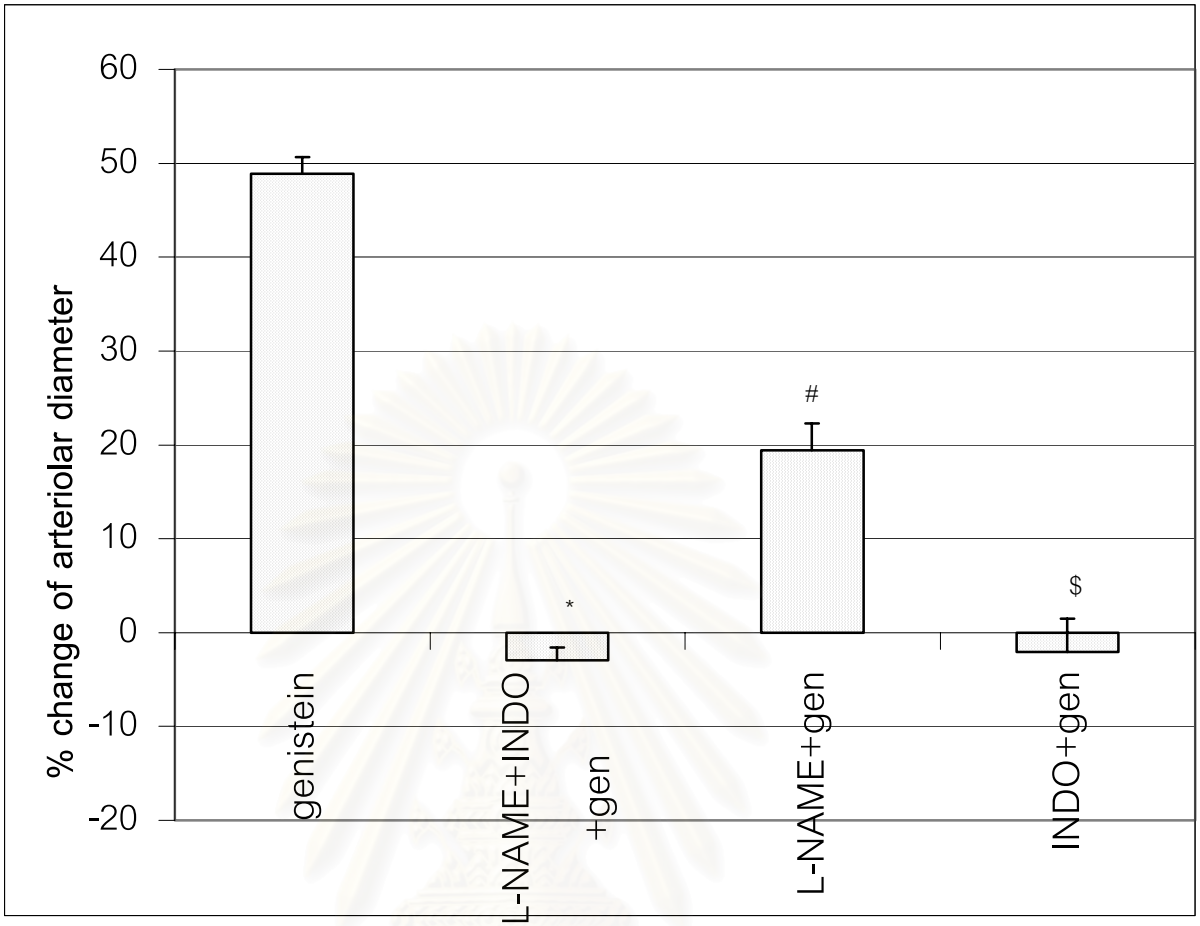
Table 4.6 Mean±SEM of the effects of genistein, L-NAME+genistein, INDO+genistein and L-NAME plus INDO+genistein on mesenteric arterioles (n = 7 in each group).

	% change of arteriolar diameter
Genistein	48.86±1.80
L-NAME + genistein	19.45 ± 2.86^{NS}
INDO + genistein	$-2.04 \pm 3.54^{*}$
L-NAME + INDO + genistein	$-2.95 \pm 1.39^{\#}$

$^{*}p < 0.001$ significantly different as compared INDO + genistein VS Genistein

$^{\#}p < 0.001$ significantly different as compared L-NAME + INDO + genistein VS Genistein

NS nonsignificantly different as compared L-NAME + genistein VS Genistein



*p <0.001 significantly different as compared between L-NAME+INDO + genistein and genistein

# p < 0.001 significantly different as compared between L-NAME + genistein and genistein

$ p <0.001 significantly different as compared between INDO + genistein and genistein

Figure 4.8 The percentage of arteriolar diameter change after topical applying genistein, L-NAME+INDO+genistein, L-NAME+genistein and INDO+genistein. Values were expressed as means±SEM (n = 7 in each experiment).

CHAPTER V

DISCUSSION

After the age of 51 years old, the average age of menopause, the incidence of cardiovascular diseases among women rises steadily, approaching that of men by age of 70.[5] There is increasing interest in the use of HRT to compensate postmenopausal women with low plasma levels of estrogen, thereby potentially reducing the risk of cardiovascular diseases. Since the Women's Health Initiative (WHI) study of health outcomes included over 16,000 postmenopausal women who were taking either estrogen plus progestrogen HRT or placebo, the study was due to run for 8.5 years but has been halted at just over 5 years because the number of cases of breast cancer had reached a pre-specified safety limit.[38] The study had not shown any benefit for cardiovascular diseases, including heart attacks and strokes, although it had shown some other benefits for hip fractures and bowel cancer. Therefore, many investigators have tried to search for alternative modality instead of ERT / HRT. Phytoestrogens, plant-derived compounds, have the similar structure as 17β-estradiol which can bind to estrogen receptor (ER).[3,23] They also exert estrogenic and anti-estrogenic activity.[23] Interestingly, epidemiological data showed that Japanese postmenopausal women have significance lowered incidence of coronary heart disease (CHD) than postmenopausal women in Western area.[7] However, it has been no evidence about their adverse effects yet. So they are ones of the interesting new alternative therapy for postmenopausal women.

In this chapter, we would like to discuss the **treatment and preventive** effects of genistein on endothelial dysfunction which demonstrated in bilateral ovariectomized rat.

1) The treatment effects of genistein on endothelial dysfunction

1.1 The effects of estrogen deprivation on body weight(BW) and uterine weight

Before starting the experiment, plasma E_2 of $OVX_{3\text{-week}}$ and $Sham_{3\text{-week}}$ were determined. It was revealed that plasma E_2 was significantly reduced in $OVX_{3\text{-week}}$ as compared to $Sham_{3\text{-week}}$. In addition to confirm the effects of E_2, the uterus was weighed (Table 4.1). In $Sham_{3\text{-week}}$, the uterus was heavier than its OVX group because most estrogen products elicited hyperplastic activity on the endometrium.[118] Therefore, $Sham_{3\text{-week}}$ showed endometrium growth more than $OVX_{3\text{-week}}$ (Table 4.1).

It was also found that the change of BW was significantly increased in $OVX_{3\text{-week}}$ and OVX_{veh} when compared to their sham groups (Table 4.1). The reason of this data might be the similar effect of genistein to 17β-estradiol in lipoprotein metabolism such as activation of LDL receptor, precipitation of LDL catabolism, increase of HDL, decrease in oxidized LDL and lipoprotein(a) and also benefit on carbohydrate metabolism These effects yield to the distribution of body fat and result in changed body weight. However, in our experiment, we did not study the effects of genistein on lipoprotein and carbohydrate metabolism.

1.2 The treatment effects of genistein on mean arterial pressure (MAP) and heart rate (HR)

Our experiment showed that genistein could reduce MAP which elevated in the estrogen deprivation rats (ovx groups) to the level as in the control groups. According to previous studies, genistein, the major composition of

phytoestrogens, has the similar structure to 17β-estradiol and also can bind to both types of ER, it might act in the similar mechanism as 17β-estradiol via genomic and non-genomic pathway, for example genomic-dependent pathway by increasing mRNA, and non-genomic pathway by activating vasoactive substances leading to normalize blood pressure as the effect of endogenous 17β-estadiol in $Sham_{veh}$. In our experiment, we did not clarified the effects of genistein on genomic expression pathway.

Besides of the decrement of plasma E_2 in OVX groups, it was found that the more decreased plasma E_2, the more reduced the response to acetylcholine (Table 4.3, Fig 4.2 A). These findings confirmed that estrogen deficiency was associated with endothelial dysfunction. The vascular protective action of estrogen is reported to be mediated indirectly by an effect on lipoprotein metabolism and by direct effect on the vessel wall itself.[119,124] Functionally competent estrogen receptors have been identified in vascular smooth muscle cells, and specific binding sites have been demonstrated in endothelium.[120,121,123] Estrogen administration promotes vasodilation both in human and animal model, in part by stimulating prostacyclin and nitric oxide synthesis.[120,121] Conversely, estrogen deficiency led to an enhanced vasoconstriction by angiotensin II.[111] In addition, estrogen exerts, *in vitro*, a direct inhibitory effect on the smooth muscle cell by inhibiting calcium influx. Furthermore, the effects of estrogen on the vessel wall has a rapid non-genomic component involving membrane phenomena, such as alteration of membrane ionic permeability and activation of membrane-bound enzymes[116] as well as the classical genomic effect involving receptor activation and gene expression.[117,120]

Besides the beneficial effects on endothelial function, 17β-estradiol was demonstrated to accelerate reendothelialization[148,149] in female ERβ knockout mice. These previous studies suggested that ERα but not ERβ mediated the

beneficial effects of E_2 on reendothelialization[122,145,150,151,152] which was disagree with the study by Iafrati.[144] Moreover, Yue et al.[152] showed that Selective Estrogen Receptor Modulator (SERM) could inhibit vascular smooth muscle cells (VSMCs) proliferation as well.

The control of blood pressure (BP) is mediated through complex, overlapping mechanisms that interact to produce appropriate responses in a wide variety of circumstances. The arterial blood pressure, although subject to large diurnal variations, is relatively tightly controlled among blood pressure (BP), cardiac output (CO) and peripheral vascular resistance (PVR); ($BP = CO \times PVR$). There are various mechanisms involving the control of BP, for example autonomic control and baroreflexes, neuronal control, stress, acute exercise, aging, menopause and so on. It was also found that endothelial dysfunction produced high BP because one popular hypothesis asserted that the increased intraluminal pressure damaged the endothelium, allowing the release of endothelin and inhibiting the release of NO.[128] However, either high BP causes endothelial dysfunction, or endothelial dysfunction promotes high BP is unclear.

Vascular endothelium itself produces various vasoactive substances both vasodilator agents (NO and PGI_2) and vasoconstrictor agents (ET).[72] This study revealed that MAP of OVX_{veh} was significantly increased as compared to $Sham_{veh}$ and OVX_{gen}. These results were consistent with the previous study which showed that 17β-estradiol increased CO and arterial flow velocity, and decreased vascular resistance and BP.[113,114,129,130] The mechanism(s) involved in the vascular effects of estrogen was(were) beginning to be elucidated. Genomic-dependent mechanisms were involved in the response of the reproductive vasculature to gonadal hormones, and estrogen receptors were found in a number of other tissues.[119,130,131,132] Recent data suggested that the acute effects of estrogen in

other circulations might be independent from the classical estrogen receptor, while genomic mechanism might be involved in chronic vascular effects.

These findings suggested that estrogen deficiency might produce high blood pressure while genistein supplementation might act similarly as 17β-estadiol to normalize it.

1.3 The treatment effects of genistein on vascular response

After 3 weeks of washout period, Ach-induced vasorelaxation (Ach 10^{-6} M) was markedly reduced in $OVX_{3\text{-week}}$ as compared to $Sham_{3\text{-week}}$. Moreover, MAP of $OVX_{3\text{-week}}$ markedly increased (Table 4.1, Fig 4.2 A). While it did not produce any significant change in the relaxant effect caused by SNP (10^{-7} M). These results demonstrated that 3 weeks of washout period, the endothelial cells have become dysfunction. It suggested that estrogen deficiency was associated with endothelial dysfunction. After applying genistein in the OVX_{gen}, we found that genistein supplementation could promote Ach-induced vasorelaxation (Table 4.3, Fig 4.2 B).

Our data suggested that estrogen deficiency was associated with endothelial dysfunction and endogenous estrogen could preserve endothelial function and also attenuated endothelial injury. According to our study, genistein supplementation, like 17β-estradiol, could improve endothelial dysfunction. However, while E_2 had produced the beneficial effects for long-term treatment of menopausal symptoms, it was reported to produce many adverse effects such as breast and endometrial cancer and thromboembolism.[38] So plant-derived estrogen, especially genistein may become new alternative treatment for menopausal women.

Endothelial dysfunction plays a pivotal role in the vascular pathogenesis because the abnormal endothelium has lost its ability to prevent abnormal vasoconstriction and to inhibit platelet aggregation and smooth muscle cell proliferation.[133] The beneficial effects of estrogen include its ability to regulate

lipoprotein metabolism.[119] This action could reduce cardiovascular events that account for 25 % to 50 %.[133] One such mechanism may involve the effects of estrogen on vascular function. Specifically, estrogen may directly enhance the activity of endothelium-derived relaxing factor nitric oxide.[116,117,153]

Ach-induced relaxation based on endothelial function resulted in an increased NO and PGI_2 production.[47,48,71,72,134,135] Previous studies showed that 17β-estradiol improved endothelial dysfunction by alteration of membrane ionic permeability and activation of membrane-bound enzyme which has called non-genomic phenomena.[119] 17β-estradiol, on the other hand, acted via genomic pathway.[119,120]

Genistein, a major composition of phytoestrogens or called plant-derived compounds, has the similar structure to 17β-estradiol[3]and can bind to ER as well.[3] Our results showed that the marked response to acetylcholine was observed in OVX_{gen} when compared to OVX_{veh} because the involved mechanism might be due to the same activity as the estrogenic ones, for example, activated NOS activity, enhanced Ca^{2+} flux into ECs to promote NOS activity[15,16,18,20] and COX activity.[143] Furthermore, it might inhibit Ca^{2+} influx to vascular smooth muscle cells. There was no previous studies demonstrated about the effects of genistein on both L-arginine / NO pathway and COX pathway, especially COX pathway and the combination of those pathway as well. We did not know whether genistein can activate reendothelialization because this model was not studied. Our experimental data indicated that genistein supplementation could improve endothelial dysfunction.

2) The preventive effects of genistein on endothelial function

2.1 The preventive effects of genistein on body weight (BW) and uterine weight

It is well recognized that cardiovascular diseases are the leading cause of death in postmenopausal women and body fat distribution is independent predictor of these diseases.[126] Our results showed that the significant difference of BW before and after 7-week treatment period was observed in ovx groups. The reason of these results was similar to that of the treatment. So $Sham_{veh}$ remained to demonstrate this activity of endogenous 17β-estradiol when compared to ovx groups. Although genistein has similar structure to 17β-estradiol and can bind to both type of ER,[22,36] it has weak estrogenic effects. Therefore, it might have slightly benefit on carbohydrate regulation and lipoprotein metabolism or another unknown involved factors which need further study**.** Additonally, the uterine weight was significantly decreased in ovx groups when compared to Sham. The reason was described previously in the treatment study.

2.2 The preventive effects of genistein on vascular response

Our results demonstrated that MAP was markedly increased in OVX_{veh} as compared to $Sham_{veh}$. This finding suggested that estrogen deprivation showed the impairment of endothelial function because endothelium, in pathology, can not regulate the physiological balance between the production of endothelium-derived relaxing factors (EDRF / NO) and endothelium-derived vasoconstrictor substances (EDCF / ET).[128] Moreover, Ach-induced vasorelaxation was significantly reduced in OVX_{veh}. Eventually, endothelial dysfunction may lead to systemic hypertension.[47,113,129,130] Our study did not clarify the morphologic change of endothelium. It may become injury after estrogen cessation. Genistein, plant-derived compounds, supplementation could preserve endothelial function which could be observed the comparability between OVX_{veh} and OVX_{gen}. It also revealed

that the response to acetylcholine was improved in OVX_{gen}. In addition, the normalization of MAP was shown as compared to $Sham_{veh}$. Owing to the similar structure to 17β-estradiol,[3] the estrogenic effects of genistein might manifest in both genomic and non-genomic manners, especially vessel wall.

The normal aging process induces a turnover and regeneration of endothelial cells resulting in abnormal function. Generally, the normal life span of an adult human endothelial cell has been estimated to be around 30 years. After this time, cells tend to disappear and are replaced by regenerated endothelium. However, these degenerated cells have lost some of their ability to release EDRF / NO, in particular in response to platelet aggregation and thrombin, resulting in the imbalance of vasoactive substances produced from vascular endothelial cells.[77]

Recently, it is accepted that the leading cause of death is atherosclerosis, a degenerative disease of the endothelium which is the inner surface of the arteries. A key feature of atherosclerosis is the formation within the arterial wall. The experimental data of treatment protocol showed that estrogen deficiency led to endothelial dysfunction as compared to sham groups. As previously described, estrogen deprivation is one of the risk factors for degenerative disease, especially CVD. One of the explanation is that the ERs have been identified both in vascular smooth muscle cells and vascular endothelial cells.[118,119] The absence of estrogen binding to its receptors occurring after menopause results in a paradoxic response of vessels to acetylcholine (vasoconstriction). Moreover, some previous studies demonstrated that E_2 could prevent vascular injury[148,152] and also could promote endothelial recovery.[150,151,152] In addition, improvement of endothelial dysfunction was demonstrated in both human[40,118,119] and animal models.[148,150,151,152]

As we know, the estrogens have both rapid and relatively slow effects on vasculature. Within a few minutes of estrogen exposure, blood vessels increased

in diameter (vasodilation), probably due to activation of an enzyme which increases concentration of nitric oxide, a well-known vessel relaxant. Within hours and days of estrogen exposure, blood vessels undergo a variety of changes which involve vascular gene and result in reducing vascular injury and atherosclerosis. The slow effects of estrogen are termed "genomic" which mediated by interaction between estrogen-receptor complexes and cellular DNA, thereby activating relevant vascular genes.

Estrogen cessation brings about a number of degenerative diseases, for example, cardiovascular diseases which are the leading cause of death among menopausal women. Based on epidemiological data, the incidence of CVD increased with the time after menopause.[1,2] Although ERT has beneficial effects for menopausal women, it is not applied for premenopausal women for the preventive purpose because of its adverse effects on the endometrium and breast.[118] Moreover, health promotion strategy is better than treatment after the pathology occurs since damaged cell is irreversible. Previous studies demonstrated that E_2 accelerated endothelial re-growth in female ERβ knockout mice with endothelial injury,[151] inhibited vascular smooth muscle cells (VSMCs) proliferation and inhibited neointimal formation *in vivo* after vascular injury.[145,152] Additionally, E_2 preserved endothelial function by activation NOS activity.[154]

Genistein, a tyrosine kinase inhibitor,[21] may prevent VSMCs proliferation which is the feature of atherosclerotic development.[21,25] It may also normalize endothelial function by preserving the response of acetylcholine. Interestingly, estrogen deficiency did not produce any abnormality of vascular smooth muscle cell function because it may take a long time to show the abnormality of the function and proliferation of VSMCs. Furthermore, genistein may regulate cell proliferation through the alteration of gene expression and synthesis of protein involved in the regulation of the cell cycle.[145,152]

These experimental data indicated that genistein supplementation could preserve endothelial function. It may have beneficial effects and become the vasculoprotective substance for menopausal women.

3) Mechanism(s) of action of genistein on endothelial cells

According to our two previous protocols (treatment and preventive studies), they were obviously demonstrated that genistein could improve and preserve endothelial dysfunction in bilateral ovariectomized rats. We observed that it could reverse the dysfunction of endothelium in ovx groups to similar response to acetylcholine as endogenous estrogen in sham groups (Table 4.5). Previous studies demonstrated that E_2 exerted the cardioprotective effects by activating both directly on blood vessels wall and indirectly on regulating lipoprotein metabolism.[105,106,107,119] After binding to its specific receptors either in vascular endothelial or vascular smooth muscle cells, it manifests both rapid effects (non-genomic) and long-term effects (genomic); increasing dilation by activating NOS activity and inhibiting the response of blood vessels to injury, respectively.[119] There have been reported that genistein, a major composition of phytoestrogen-isoflavones, has the similar structure to 17β-estradiol.[3,19,22,36] Moreover, many studies demonstrated that genistein might act via endothelium-dependent, *in vitro*.[16,109,114] So our hypothesis were that genistein probably acted in the same way *in vivo.* According to our study, we found that there was response in a few minutes after topically applying genistein on mesenteric arteries, *in vivo*. Furthermore, the predominance pathway of endothelium-dependent vasorelaxation is L-arginine / NO. Various studies have focused on potential alterations of the L-arginine / NO pathway to explain the reduced response to acetylcholine.

Under physiological condition, the tone of peripheral blood vessels is controlled by a balance between endothelium-derived vasodilator (NO, PGI_2, endothelium-derived hyperpolarizing factor [EDHF]) and vasoconstrictors (ET, thromboxane, oxygen-derived free radicals and endothelium-derived contracting factor [EDCF]).[134] NO is synthesized from the quanidine- nitrogen terminal of L-arginine by enzyme called NO synthase, which is constitutive in normal endothelial cells.[72,131,132] The activation of this NO synthase depends on the intracellular concentration of calcium ions in the endothelial cells and calmodulin. It can be inhibited competitively by L-arginine analogues such as L-NAME or L-NMMA.[131] After being synthesized, NO diffuses to the vascular smooth muscle cells and relaxes them by stimulating a cytosolic enzyme, soluble guanylate cyclase, and then leads to an increased in cyclic 3′,5′-guanosine monophosphate (cyclic GMP). The increment of cGMP increase is associated with inhibition of the contractile apparatus. The production of NO is a major contributor to endothelium-dependent relaxations in large isolated arteries such as mesenteric arteries. Its significance *in vivo* is suggested by the observation that inhibitor of NO synthase cause vasoconstriction in most vascular beds.[131] There is other rapid effects of vasorelaxation which does not pass the endothelial cells called endothelium-independent pathway. This protocol, we would like to clarify that genistein was an endothelium-dependent or -independent vasodilator agent by using the combination inhibitors (L-NAME plus INDO) to inhibit endothelium-dependent action. If genistein could reduce vasoconstriction caused by L-NAME plus INDO, it might act through EDHF pathway (endothelium-dependent vasorelaxation) or acted directly on vascular smooth muscle cells (endothelium-independent vasorelaxation). Our result demonstrated that genistein did not promote vasodilation of the L-NAME plus INDO preconstricted vessels. This result suggested that genistein might bind to ER in plasma membrane of vascular

endothelial cells and enhanced the response to acetylcholine and then brought about increasing vasodilator agents. When the vascular endothelial function was inhibited by L-NAME plus INDO, it could not produce vasorelaxation caused by genistein. It was indicated that genistein was an endothelium-dependent vasodilator agent but it did not act via EDHF pathway. However, we did not know that it acted via which mechanisms (L-arginine /NO or cyclooxygenase pathway). Therefore, the major pathway (L-arginine / NO) was first identified.

If genistein acted via L-arginine / NO pathway, it could not relax vasconstriction caused by L-arginine analogues. In order to prove the endothelium-dependent vasodilation caused by genistein, L-NAME (a NO synthase inhibitor) was then topically applied so that it could not enhance NO. Our result was revealed that genistein could not completely reduce vasoconstriction caused by L-NAME which disagreed with the study by Squadrito.[16] It was indicated that genistein did not act mainly via L-arginine / NO pathway (Table 4.6, Fig 4.8). There might be another pathway which involved the endothelium-dependent vasorelaxation of genistein.

Besides NO production, prostacyclin (PGI_2), a major production of vascular cyclooxygenase, is formed primarily in endothelial cells and acts synergistically with NO. It causes relaxation of vascular smooth muscle by activating adenylate cyclase and increasing the production of cyclic 3′,5′-adenosine monophosphate (cyclic AMP). Arachidonic acid, a precusor substrate of PGI_2, is converted to PGI_2 by cyclooxygenase enzyme.[131] Therefore, INDO (a cyclooxygenase inhibitor) was applied in order to clarify an endothelium-dependent vasorelaxation of genistein via cyclooxygenase pathway. The experimental data showed that genistein could not reduce vasoconstriction caused by INDO. It was suggested that genistein caused vasorelaxation mainly via cyclooxygenase pathway (Table 4.6, Fig 4.8).

Therefore, these experimental data indicated that genistein improved and preserved endothelial dysfunction predominantly via cyclooxygenase pathway which agreed with the study by Jun.[143] However, these findings clarified our hypothesis for only one aspect. There may be additional mechanism(s) involved the improvement and preservation of endothelial dysfunction which need further study.

CHAPTER VI

CONCLUSION

In this study, we would like to summarize the results as given below.

1. After 3 weeks of washout period, plasma E_2 and uterine weight were significantly decreased in $OVX_{3\text{-week}}$ which was similar to OVX_{veh} OVX_{gen} in both treatment and preventive groups. These results indicated that plasma E_2 level was associated with uterine weight. Genistein supplementation had no effect on the uterus.

2. After 3 weeks of washout period, mean arterial pressure (MAP) was significantly increased in $OVX_{3\text{-week}}$ while heart rate (HR) was unchanged. The results implied that estrogen deficiency had result on MAP.

3. Ach-induced vasorelaxation was markedly decreased in $OVX_{3\text{-week}}$ while SNP-induced vasorelaxation was unchanged. Theses results demonstrated that 3 weeks of washout period, estrogen deficiency led to vascular endothelial dysfunction.

4. After 4 weeks of daily treatment with genistein or vehicle, MAP was significantly increased in OVX_{veh} but was not observed in OVX_{gen}. Moreover, Ach-induced vasorelaxation was significantly decreased in OVX_{veh} when compared to $Sham_{veh}$ and OVX_{gen}. While it was not significantly different between OVX_{gen} and $Sham_{veh}$. SNP-induced vasorelaxation was unchanged among these groups. These results suggested that genistein supplementation could improve endothelial dysfunction.

5. After immediately daily treated with genistein or vehicle for 7 weeks, the results of MAP, Ach- and SNP-induced vasorelaxation were similar to the

results of treatment study. The experimental data indicated that genistein supplementation could prevent endothelial dysfunction.

6. Genistein could not reduce vasoconstriction caused by L-NAME+INDO. It slightly reduced vasoconstriction caused by L-NAME while it could not attenuate vasoconstriction caused by INDO. These results indicated that genistein was an endothelium-dependent vasodilator agent and acted mainly via cyclooxygenase pathway.

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