ผลของเจนนิสตีนต่อการเปลี่ยนแปลงของชั้นอินทิมาในหลอดเลือดแดงคาโรติดภายหลังการทำให้บาดเจ็บด้วยบอลลูนในหนูแรทที่ถูกตัดรังไข่

นางสาวจิรวรรณ มาลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาสรีรวิทยา (สหสาขาวิชา)

บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

EFFECTS OF GENISTEIN ON NEOINTIMAL CHANGES AFTER BALLOON INJURY OF CAROTID ARTERY IN OVARIECTOMIZED RATS

Miss Jirawan Mala

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	Effects of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats
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Field of Study	Physiology
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Accepted by the Faculty of Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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จิรวรรณ มาลา : ผลของเจนนิสตีนต่อการเปลี่ยนแปลงของชั้นอินทิมาในหลอดเลือดแดงคาโรติดภายหลังการทำให้บาดเจ็บด้วยบอลลูนในหนูแรทที่ถูกตัดรังไข่ . (EFFECTS OF GENISTEIN ON NEOINTIMAL CHANGES AFTER BALLOON INJURY OF CAROTID ARTERY IN OVARIECTOMIZED RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.นพ.วสันต์ อุทัยเฉลิม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.นพ.สมพล สงวนรังศิริกุล, ศ.นพ.พิเชฐ สัมปทานุกุล, 82 หน้า.

จุดประสงค์ของการศึกษาในครั้งนี้คือ ศึกษาผลของเจน นิสตีนต่อการเปลี่ยนแปลงของชั้นอินทิมาในหลอดเลือดแดงคาโรติดภายหลังการทำให้บาดเจ็บด้วยบอลลูนในหนูแรทที่ถูกตัดรังไข่ และกลไกการออกฤทธิ์ที่เกี่ยวข้อง

หนูวิสตาร์เพศเมียอายุ 12 สัปดาห์ น้ำหนัก 240-270 กรัม จำนวน 32 ตัวถูกแบ่งเป็น 4 กลุ่มกลุ่มละ 8 ตัวคือ 1) กลุ่มหนูที่ได้รับการผ่าตัดแต่ไม่ได้ทำการผ่าตัดรังไข่ได้รับไดเมธิลซัลฟอกไซด์ (ดีเอ็มเอสโอ) 100 ไมโครลิตรต่อวันฉีดทางชั้นใต้ผิวหนังทุกวัน (กลุ่มควบคุมเชิงลบ) 2) กลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้างได้รับ ดีเอ็มเอสโอ 100 ไมโครลิตรต่อวัน ฉีดทางชั้นใต้ผิวหนังทุกวัน (กลุ่มควบคุมเชิงบวก) 3) กลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง ได้รับเจนนิสตีน 0.25 มิลลิกรัมต่อกิโลกรัมต่อวัน ฉีดทางชั้นใต้ผิวหนังทุกวัน (กลุ่มทดลองด้วย เจนนิสตีน) และ 4) กลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง ได้รับ 17 เบต้า เอสตราไดออล 0.2ไมโครกรัมต่อกิโลกรัมต่อวัน ฉีดทางชั้นใต้ผิวหนังทุกวัน (กลุ่มทดลองด้วย เอสโตรเจน) เป็นเวลา 4 สัปดาห์ จากนั้นหนู 24 ตัวที่ถูกตัดรังไข่ ถูกทำให้บาดเจ็บด้วยบอลลูนในหลอดเลือดแดงคาโรติดข้างซ้าย เลี้ยงต่ออีก 2 สัปดาห์ จากนั้น หนูทั้ง 32 ตัวถูกทำ การุณยฆาต เก็บหลอดเลือดแดงคาโรติดข้างซ้ายเพื่อประเมินการเปลี่ยนแปลงของพื้นที่ในชั้นมีเดีย ชั้นอินทิมา ชั้นอินทิมาต่อชั้นมีเดีย และศึกษาอิมมูโนพยาธิวิทยา ผลการทดลอง พบว่า พื้นที่ชั้นอินทิมาและชั้นอินทิมาต่อมีเดียเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในกลุ่มควบคุมเชิงบวก เมื่อเปรียบเทียบกับกลุ่มควบคุมเชิงลบ (2,805.27 ± 168.15 ตารางไมโครเมตร 1.27 ± 0.07 และ 1,102.05 ± 29.76 ตารางไมโครเมตร 0.47 ± 0.00 ตามลำดับ) กลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง + เจนนิสตีนและกลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง + เอสโตรเจน มีพื้นที่ของชั้นอินทิมา และอินทิมาต่อชั้นมีเดียลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่ม ควบคุมเชิงบวก (1,762.37 ± 282.40 ตารางไมโครเมตร 0.76 ± 0.11 และ 1,298.00 ± 152.02 ตารางไมโครเมตร 0.58 ± 0.07 ตามลำดับ) ผลอิมมูโนพยาธิวิทยา พบว่า จำนวนเซลล์ที่แสดงอินดิวซิเบิลไนตริกออกไซด์ซินเธสและอซิมเมทตริส ไดเมททิลอาร์จินิน (แอสมา) ลดลงอย่างมีนัยสำคัญทางสถิติในกลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง + เจนนิสตีนและกลุ่มหนูที่ถูกผ่าตัดรังไข่ 2 ข้าง + เอสโตรเจน เมื่อเทียบกับกลุ่มควบคุมเชิงบวก (ร้อยละ 12.5 ± 5.2, 10 ± 3.7 และร้อยละ 25 ± 5 18.7 ± 6.3 ตามลำดับ เปรียบเทียบกับกลุ่มควบคุมเชิงบวก ร้อยละ 77.5 ± 7.9, 82.5 ± 7.9 ตามลำดับ) สรุปผลการทดลอง การทำให้บาดเจ็บด้วยบอลลูนมีผลทำให้เกิดการเปลี่ยนแปลงของชั้นอินทิมาและการให้เจนนิสตีน 0.25 มิลลิกรัมต่อกิโลกรัมต่อวันฉีดทางชั้นใต้ผิวหนังสามารถยับยั้งการเปลี่ยนแปลงของชั้นอินทิมาในหลอดเลือดแดงคาโรติดภายหลังการทำให้บาดเจ็บด้วยบอลลูนในหนูแรทที่ถูกตัดรังไข่ โดยพบความสัมพันธ์กับการลดลงของอินดิวซิเบิลไนตริกออกไซด์ซินเธสและเอนไซม์ยับยั้งเอนโดธีเลียลไนตริกออกไซด์ซินเธส ซึ่งการออกฤทธิ์คล้ายกับการออกฤทธิ์ของเอสโตรเจน

สาขาวิชา.....สรีรวิทยา..... **ลายมือชื่อนิสิต**……

ปีการศึกษา.....2554..... **ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก**……..

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……...

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……...

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KEYWORDS : GENISTEIN/ NEOINTIMAL CHANGE / BALLOON INJURY / OVARIECTOMY / ATHEROSCLEROSIS

JIRAWAN MALA : EFFECTS OF GENISTEIN ON NEOINTIMAL CHANGES AFTER BALLOON INJURY OF CAROTID ARTERY IN OVARIECTOMIZED RATS. ADVISOR: ASSOC.PROF. WASAN UDAYACHALERM, M.D., CO-ADVISOR : ASSOC.PROF. SOMPOL SANGAUNRUNGSIRIKUL, M.D., PROF. PICHET SAMPATANUKUL, M.D., 82 pp.

The study aim was to investigate the effects of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats and its related mechanism.

Female Wistar rats weighing 240-270 g aged 12 weeks old (N=32) were randomly divided into 4 groups:- i) the sham group, n=8, treated with dimethyl sulfoxide (DMSO) 100 µl/day subcutaneously (sc); (control negative group), ii) the ovariectomized rats, n=8, treated with DMSO 100 µl/day, sc (control positive group), iii) the ovariectomized rats, n=8, treated with genistein 0.25 mg/kg/day, sc (experiment group with Gen) and iv) the ovariectomized rats, n=8, treated with 17β-estradiol 0.2 µg/kg/day, sc (experiment group with E2). After four weeks of nurturing, the 24 ovariectomized rats underwent balloon injury of left carotid artery. The left carotid arteries of the 32 rates were harvested two weeks later for histological and immunohistochemical studies. The results showed that the intimal area and the ratio of intimal area to medial area were significantly increased in the control positive group compared with control negative group (2,805.27 ± 168.15 μm^2, 1.27 ± 0.07 and 1,102.05 ± 29.76 μm^2, 0.47 ± 0.00 respectively; $p<0.005$). The intimal area and the ratio of intimal area to medial area were significantly decreased in the experiment group with Gen and the experiment group with E2 as compared with the control positive group (1,762.37± 282.40 μm^2, 0.76 ± 0.11 and 1,298.00 ± 152.02 μm^2, 0.58 ± 0.07 respectively; $p<0.005$). Immunohistochemistry study disclosed that the number of cells expressing inducible nitric oxide synthase (iNOS) and asymmetric dimethylarginine (ADMA) were decreased in the experiment group with Gen and the experiment group with E2 compared with the control positive group (12.5 ± 5.2%, 10 ± 3.7% and 25 ± 5%, 18.7 ± 6.3% respectively compared with control positive, 77.5 ± 7.9%, 82.5 ± 7.9% respectively; p <0.05). In conclusion, the balloon injury significantly led to neointimal changes of carotid arteries in ovariectomized rats. The administration 0.25 mg/kg/day of genistien subcutaneously could inhibit neointimal hyperplasia in association with reduced iNOS and endogenous NOS inhibitor factors, similar to estrogenic action.

Field of Study...Physiology…….

Academic Year..2011……..

Student's Signature…….....

Advisor's Signature…….....

Co-advisor's Signature……...

Co-advisor's Signature……...

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CONTENTS

PAGE

CHAPTER PAGE

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

LIST OF ABBREVIATIONS

µl	Microlitre
µM	Micromolar
ADP	Adenosine diphosphate
ADMA	Asymmetric (N^G, N^G) dimethylarginine
ARC	Arcuate nucleus
atm	Atmosphere
BW	Body weight
˚C	Degree Celsius
Ca^{2+}	Calcium ion
CAAs	Cationic amino acids
CATs	Cationic amino acid transporters
CCA	Common carotid artery
CHD	Coronary heart disease
Chol	Cholesterol
cm	Centimetre
CLRD	Chronic lower respiratory disease
COX	Cyclooxygenases
CVD	Cardiovascular disease
DDAH	N^G, N^G-dimethylarginine dimethylaminohydrolase
DMSO	Dimethyl sulfoxide
E2	17 beta-estradiol
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
EDHF	Endothelium derived hyperpolarizing factor
e.g.	exempli gratia (for example)

ERs	Estrogen receptors
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
ESRD	End stage renal disease
ET-1	Endothelin
Fig	Figure
FSH	Follicle stimulating hormone
g	Gram
GC	Guanylate cyclase
Gen	Genistein
HDL	High-Density Lipoprotein Cholesterol
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1beta
IP_3	Inositol 1, 4, 5-trisphosphate
IgG	Immunoglobulin G
L-arg	L-arginine
L-cit	L-citrulline
LCA	Left common carotid artery
LDL	Low density lipoproteins
L-NMMA	N^G monomethyl-L-arginine
LH	Luteinzing hormone
MAPK	Mitogen-activated protein kinase
mm	Millimetre
mg	Milligram
mmHg	millimetre of mercury

nm	Nanometre
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrites
OVX	Ovariectomy
oxLDL	Oxidized low density lipoprotein
PAP	The Papanicolaou test
PBS	Phosphate buffered saline
PIP_2	Phoshatidylinositol-4, 5-bisphosphate
PIP_3	Phosphatidylinositol 3, 4, 5-trisphosphate
PI_3-K	Phosphatidylinositol 3-kinase
pg	Picogram
PGI_2	Prostacyclin
POMC	Pro-opiomelanocortin
PRMT	Protein arginine methyltransferase
Sc.	Subcutaneous
SHR	Spontaneously hypertensive rats
TG	Triglyceride
TNF-α	Tumor necrosis factor alpha
US	United States
VMH	Ventromedial hypothalamus
VSM	Vascular smooth muscle

VCAM	Vascular cell adhesion molecules
VLDL	Very low density lipoprotein
WHI	Women's Health Initiative

CHAPTER I

INTRODUCTION

Atherosclerosis, thickening of arterial intima, is a chronic inflammatory disease leading to various manifestations of cardiovascular diseases (CVD). It has become a major health problem in the United States and in many developing nations. According to statistics, mortality rate of CVD accounted for 32.8% in 2008, which is one third of mortality in the US. Averagely, 2,200 Americans die of CVD each day. Meanwhile, the estimated cost of medicare is rising (1-2). Therefore, the prevention of initial atherosclerosis is worthwhile.

. Many theories about atherogenesis have been proposed during the past decades, but none can explain the entire process. Multiple risk factors play roles, and the conditions begin since childhood which slowly progress throughout the lifetime. The factors may suppress atheroprotective hormones e.g. estrogen. Usually, advanced stage of atherosclerosis is found in the elderly when the levels of these hormones have declined. This is consistent to the fact that the incidence of CVD is reduced in postmenopausal women using hormone replacement therapy (3-5). However, the randomized controlled trial study from Women's Health Initiative (WHI) did not recommend use of Conjugated Equine Estrogen (CEE) since it increased the risk of stroke and did not reduce coronary heart disease (CHD) incidence in postmenopausal women with prior hysterectomy (6). Thus, the effect of hormone replacement therapy on CVD is still controversial (7).

Interestingly, prevalence of CVD in Asia is lower than in Western nations, environment factors such as kinds and constituents of Asian foods are attributed as protection. In Asia, people have high consumptions of isoflavones. The substances may prevent chronic diseases such as atherosclerosis (2, 8-10). Many studies showed that purified isoflavones yielded more positive effects rather than crude extracts. Genistein is one of the major isoflavones and is found in soybeans. It was first isolated in 1899 with chemical structure similar to endogenous estrogen, is characterized by a phenolic ring with hydroxyl groups that is essential for binding to estrogen receptors to exert estrogenic effects, however the action is weak. Evidences

also suggest that genistein exhibits anti-inflammatory, antioxidant and antiatherogenic properties. In the anti-inflammatory aspect, genistein inhibited endothelial and monocyte activations and also suppressed tumor necrosis factor α (TNF-α); the event in turn induced proliferation of human aortic smooth muscle cells (11). Regarding antioxidant aspect, genistein decreased superoxide dismutase and glutathione peroxidase activities in endothelial cells (12). At last, the effect of genistein on antiatherogenic aspect was to improve endothelial functions (13-15). However, the mechanism(s) of genistein-related effects is still a question.

In animal models, estrogen deficiency and atherosclerosis can be constructed. The former is from ovariectomy. The atherosclerosis is induced in form of intimal hyperplasia (neointima) by balloon injury of rat carotid artery. The mechanical events of atherosclerosis consist of endothelial dysfunction, reduced NO bioavailability and increased endogenous NOS inhibitor from endothelial cells following decreased vascular smooth muscle cell (VSM) relaxation (16-17). Biochemically, synthesis and expression of hepatic low density lipoprotein (LDL) receptors are diminished resulting in accumulations of LDL and enhancing conversions of LDL into very low density lipoprotein (VLDL). For neointimal formations, the cells are from two sources - monocytes from blood stream rolling, adhering and transmigrating to subendothelial intimal area are one attribute, vascular smooth muscle cells proliferating and differentiating into constituent cells are the other (18-19). Previous studies showed that estrogen administration in different doses in ovariectomized rats could inhibit neointimal formation after vascular injury in rats using balloon injury model, but high dose of estrogen administration exhibited adverse effects such as uterine hyperplasia and dyslipidemia. The authors speculated that the prevention might be from estrogen effect on reducing iNOS and ADMA - endogenous NOS inhibitors (20-21). Since genistein can exert low estrogenic effect, we are interested that it might imitate the mechanism of estrogen and might be demonstrated in the balloon injury of carotid arteries of ovariectomized rats model.

The aim of this study is to investigate **the effect of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats and related mechanism (s).**

Research question

1. Can genistein inhibit neointimal changes after balloon injury of carotid artery in ovariectomized rats?
2. To which mechanism that genistein use to inhibit neointimal changes after balloon injury of carotid artery in ovariectomized rats?

Research hypothesis

Genistein can prevent neointimal changes after balloon injury of ovariectomized rat carotid artery in via reducing iNOS and endogenous NOS inhibitor expression.

Research design

Animal experimental research.

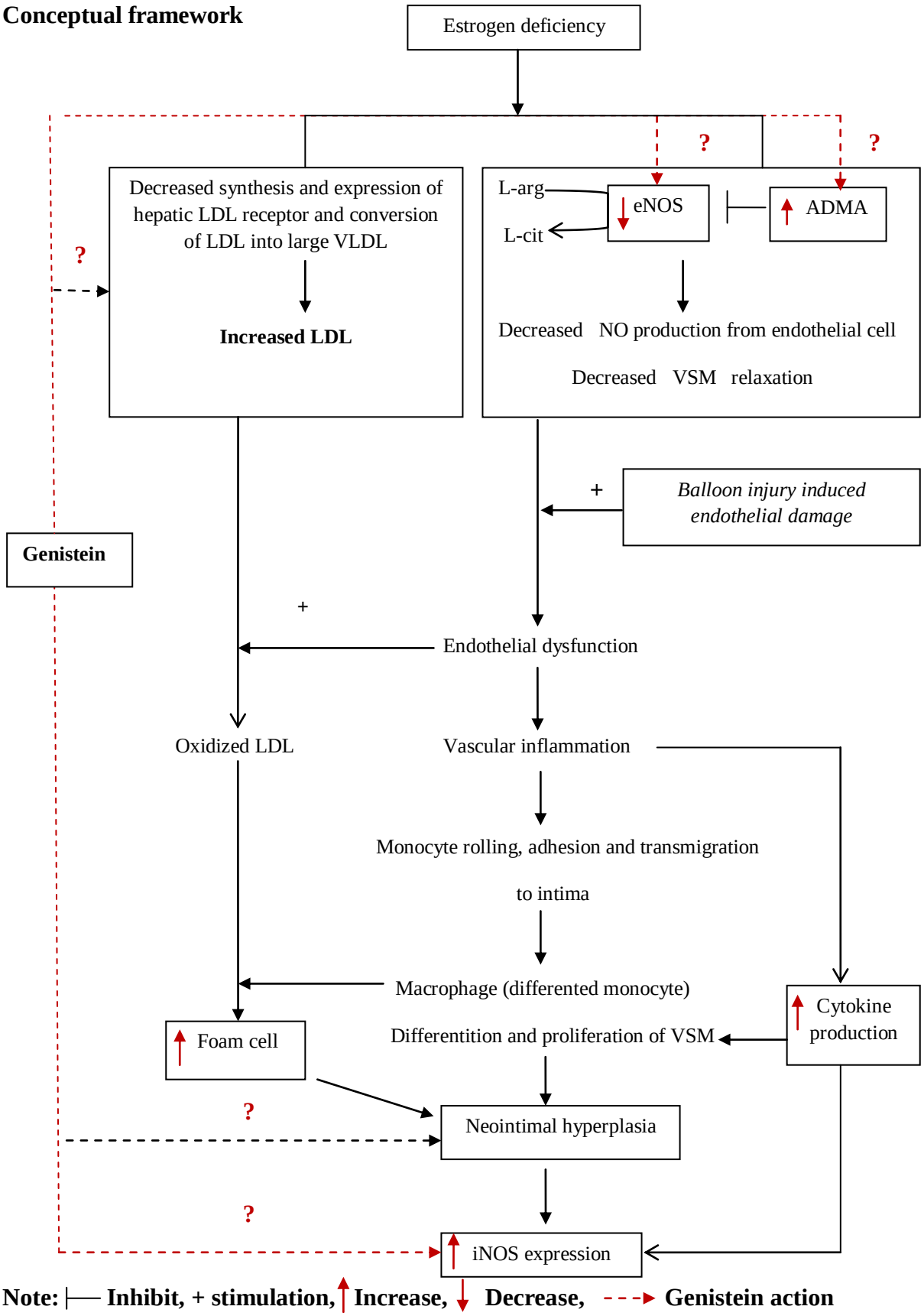
Assumption

All animals are not different.

Expected benefits and application

To support the use of genistein in clinical practice.

Conceptual framework



Note: ⊢ Inhibit, + stimulation, ↑ Increase, ↓ Decrease, - - → Genistein action

CHAPTER II

REVIEW LITERATURE

Anatomy of Arterial wall

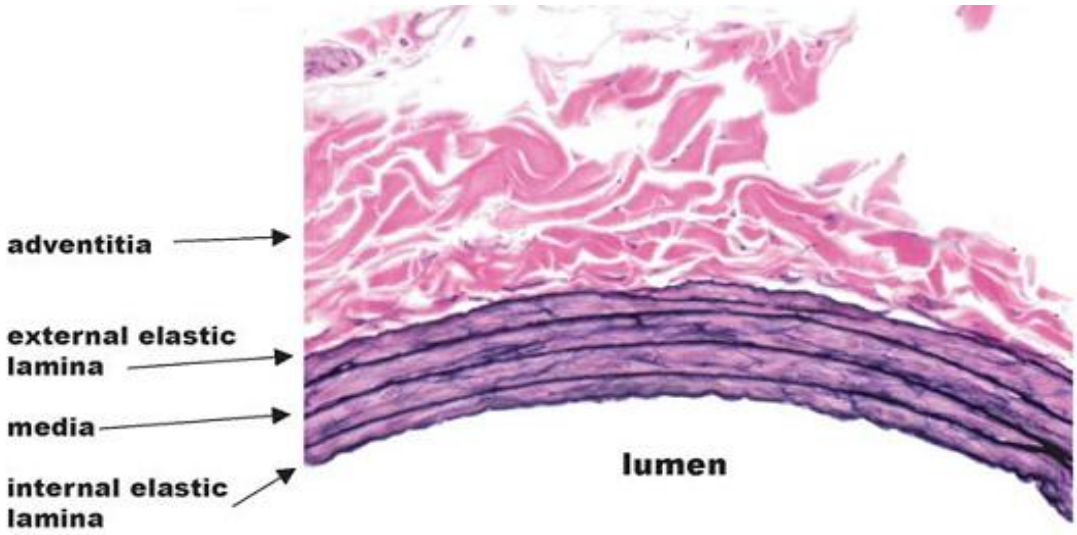


Figure 2.1 Histology of arterial wall (18).

The arterial wall consists of catalogued connective tissue composed of cells and matrix fibers placed in three tunicae: the intima, the media, and the adventitia.

The **intima,** an inner layer, containing a layer of endothelial cells and some subendothelial connective tissue. The **media**, a middle layer of smooth muscle and the **adventitia**, an outer layer of connective tissue. The walls of the aorta and the large arteries include abundant elastic tissue, much of it concentrated in the **internal elastic lamina,** a prominent band between the intima and the media, and another band, the **external elastic lamina,** between the media and the adventitia (**Fig. 2.1**). The vessels are stretched by the force of cardiac ejection during systole, and the elastic tissue permits them to recoil during diastole. This maintains diastolic pressure and aids the forward motion of the blood. The muscle is extensively innervated by noradrenergic nerve fibers, which function as constrictors. In some instances, there is a cholinergic innervation, which is vasodilator. The arteries and the arterioles offer considerable resistance to the flow of blood and are known as the **resistance vessels** (19).

Endothelial cells

The vascular endothelial cells (ECs) are a continuous layer of flat polygonal ECs that comes into direct contact with blood flow. The endothelium participates in both physiologic and pathologic processes and reacts to physical forces, chemical signaling, and immunologic mediators. Endothelium is able to interact with its cellular and a cellular environments and serves as an important regulator of vasomotor tone, hemostatic balance, inflammation, permeability, cell proliferation, survival, and immunity. The luminal surface of ECs is smooth and regular. The cells are hided by a glycoprotein coat, a glycocalix, that is accountable for the anti-thrombogenic activities of the endothelial surface. The glycocalix reduces friction from blood flow and serves as a barrier against fluid loss through the vessel wall. Its thickness varies across the vascular trees. During **inflammation, the glycocalix is sheared off, which permits attachment of leukocytes and transport of water from microvessels across the endothelium and intiates the development of atherosclerotic lesions.** These glycoprotein include von Willebrand factor (a component of the coagulation factor VIII complex), surface molecules that are involved in platelet adhesion, coagulation-regulating factors such as thrombomodulin, and fibrinolysis-regulating factors such as plasminogen activators (tissue and urokinase type) and their inhibitor (plasminogen activator inhibitor 1). Several of these factors are collected in specialized intracellular organelles, Weibel-Palade bodies, that empty their content to the endothelial cell surface when the cell is activated by thrombin or some other mediators (19).

The vascular endothelial cell plays an important role in mediating the gender-related and the estrogen-induced vasodilation (20). Physiological levels of E2 potentiate endothelium-dependent flow-mediated vasodilation in postmenopausal women (21). In the same way, endothelium-dependent relaxation of isolated aorta is greater in female than in male spontaneously hypertensive rats (SHR) (20, 22). Similar to estrogen, progesterone may promote endothelium-dependent vasodilation in porcine coronary artery (23). The vascular endothelium is known to release relaxing factors such as NO, prostacyclin (PGI2), and endothelium-derived

hyperpolarizing factor (EDHF), as well as contracting factors such as endothelin (ET-1) and thromboxane A2.

Nitric oxide

Nitric oxide (NO) liberated as an intercellular messenger from the vascular endothelium plays an important role in the modulation of microvascular tone and regional blood flow. In addition, NO inhibits platelet aggregation and also leukocyte adhesion as well, leads to prevention of thrombosis and atherosclerosis.

Synthesis, degradation and mechanisms of action of nitric oxide

NO produced when L-arginine is transformed to L-citrulline via catalysis by NOS in the presence of oxygen and cofactors. Ca^{2+} is required for the activation of nNOS and endothelial NOS (eNOS) but not inducible NOS (iNOS). nNOS is constitutively expressed in the brain, peripheral nerves (24), and heart. eNOS is constitutively expressed mainly in endothelial cells (25). Inducible NOS (iNOS) is induced mainly in macrophages by bacterial lipopolysaccharide and cytokines.

Asymmetric (N^G, N^G) dimethylarginine (ADMA)

Asymmetric (N^G, N^G) dimethylarginine (ADMA) was isolated in 1987 **(Fig 2.5**). It is metabolized by N^G, N^G-dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine (26). Arginine moieties on proteins are subject to asymmetric dimethylation by class 1 isoforms of protein arginine methyltransferase (PRMT). After protein hydrolysis, ADMA is released within cells where it is a potent inhibitor of nitric oxide synthase (NOS) (27) (**Fig 2.6**). ADMA is both exported from its site of origin, and imported from the plasma at distant sites by cationic amino acid transporters (CATs) in exchange for arginine and other cationic amino acids (CAAs) (28-29). Plasma concentrations of ADMA is predicted mortality in patients with end-stage renal disease (ESRD) (30) and cardiovascular events (31).

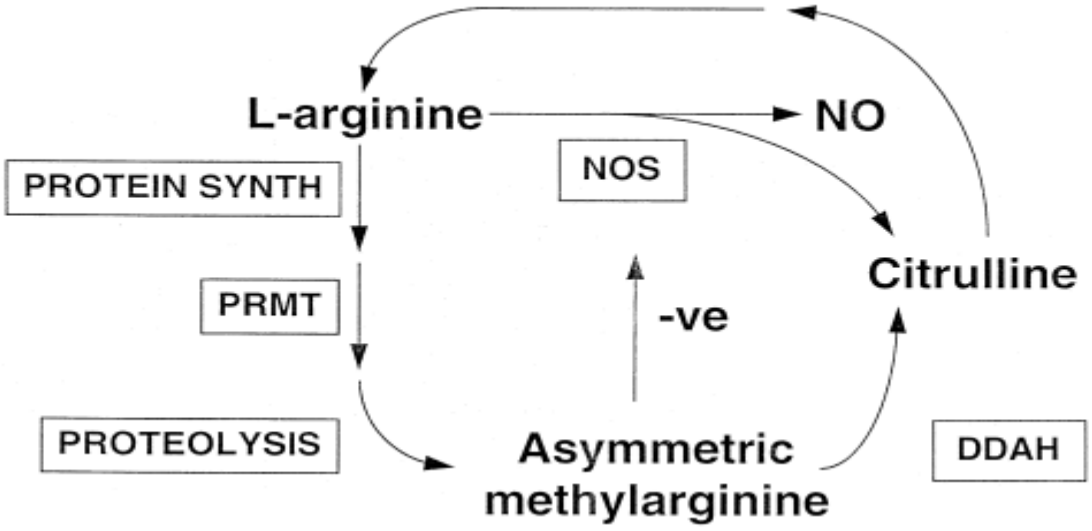


Figure 2.2 Proposed model for the regulation of intracellular asymmetric methylarginine and NO synthesis. Free arginine is metabolised to nitric oxide (NO) and citrulline by nitric oxide synthase (NOS). Arginine combined into proteins during protein synthesis can be methylated by PRMT enzymes to generate asymmetric methylarginines (L-NMMA and ADMA) which are subsequently released into the cytoplasm following proteolysis. Free intracellular L-NMMA and ADMA inhibit the production of NO catalysed by NOS. Asymmetric methylarginine is metabolised by DDAH to citrulline. Citrulline can be converted back to arginine in a two-step reaction catalysed by enzymes of the urea cycle (32).

Asymmetric imethylarginine (ADMA) and estrogen

ADMA level is lower in premenopausal women than in men of the same age and enlarged in the postmenopausal period indicates an effect of estrogens in ADMA metabolism *in vivo* (33). Estrogens can reduce the collection of ADMA in endothelia cells cultures (34), and ovariectomized rats (17). Previous studies, the supplementation of estrogens in postmenopausal women who receiving hormone alternative treatment reduced circulating ADMA (35-36). Although this may reflect increased activity of DDAH (37).

The effects of endogenous estrogen on vascular cell, namely endothelium and smooth muscle, are divided to 2 arms, nongenomic effects and genomic effects. In nongenomic effect, estrogen can cause short-term vasodilation by estrogen binds to endothelial surface membrane estrogen receptors (ERs), which are coupled to increased Ca^{2+} release from the endoplasmic reticulum and stimulation of MAPK/Akt pathway, leads to activation of eNOS and increased nitric oxide (NO) production. NO diffuses into the VSM cells, and binds to guanylate cyclase (GC), which then increases cGMP. cGMP causes VSM relaxation by decreasing $[Ca^{2}]i$ and the myofilament sensitivity to Ca^{2+}. ER may also inhibit the production of NADPH, thereby prevent inactivation of NO and formation of peroxynitrites ($ONOO^{-}$). Endothelial estrogen receptor may also activate cyclooxygenases (COX) and increase PGI2 production. PGI2 activates prostacyclin receptors in VSM, activates adenylate cyclase (AC), and increases cAMP formation. cAMP causes VSM relaxation by mechanisms similar to those activated by cGMP. ER may also increase the production of endothelium-derived hyperpolarizing factor (EDHF), which activates K channels and causes hyperpolarization and inhibition of Ca^{2+} influx via Ca^{2+}channels that lead to VSM relaxation. On another hand, genomic effect of estrogen begins as estrogen binds to endothelial cytosolic/nuclear ERs, and receptor/estrogen complex then activates mitogen-activated protein kinase (MAPK), increased gene transcription, endothelial cell proliferation, and increased endothelial nitric oxide synthase (eNOS) production (38) **Fig.2.3**.

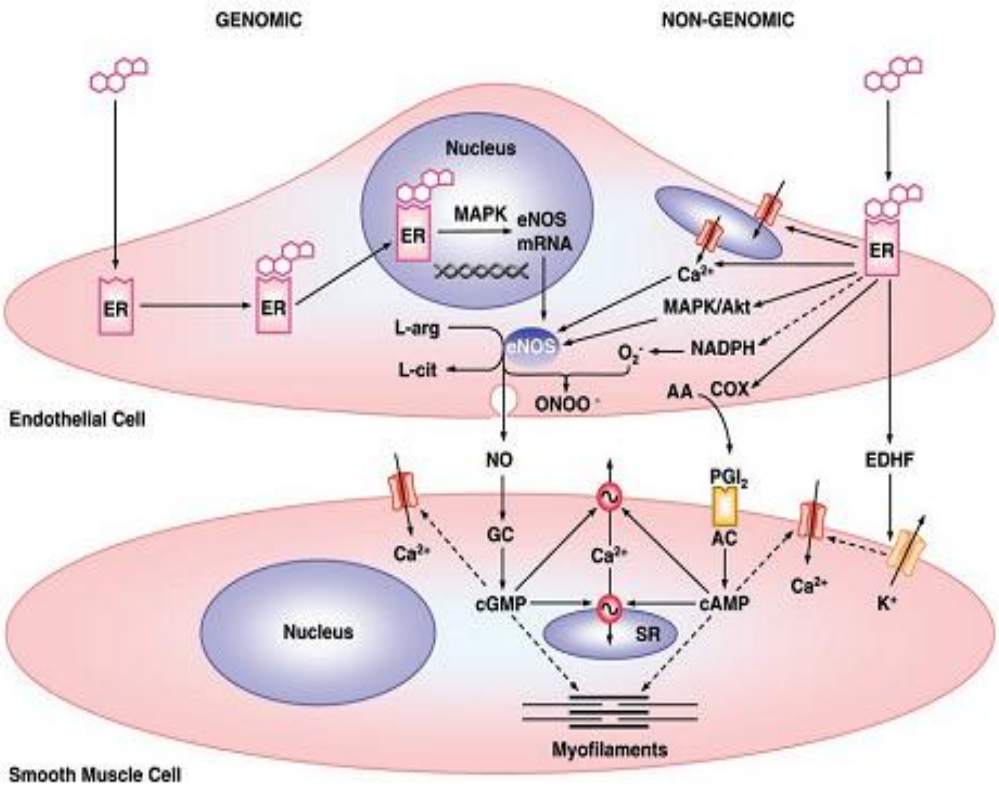


Figure 2.3 Estrogen-stimulated endothelium-dependent mechanisms of vascular smooth muscle (VSM) relaxation (38). Interrupted arrows indicate inhibition. L-arg, L-arginine; L-cit, L-citrulline; AA, arachidonic acid, SR, sacroplasmic reticulum

Estrogen receptors in blood vessels

There are two types of estrogen receptors, estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β), both of which are members of the superfamily of steroid hormone receptors (39). Estrogen receptor α and estrogen receptor β have considerable homology and similar to all steroid hormone receptors, they are transcription factors that alter gene expression when they are activated (39). Estrogen receptors are activated by estrogen binding and can also be activated by growth factors in the absence of estrogen as well (40). The latter mechanism of activation may occur when local concentration of growth factors are high or when serum estrogen concentration is low (as in men or postmenopausal women).

Estrogen receptors are present in all elements of the blood vessel wall: endothelium, smooth muscle and adventitia. Estrogen receptor-β is the greatest in a media compared with either the intima or adventia and is also localized in areas of arterial calcification in arteries from males and females (41-42).

Many studies suggest that ER-α promotes the protective effects of estrogen in response to vascular injury (43). However, ER-β is more widely shared in the body than ER-α and also ER-β is the receptor form that is predominantly expressed in human vascular smooth cell, particularly in women (44). Induction of ER-β mRNA expression has also been demonstrated after balloon vascular injury to the aorta of the male rat. Furthermore, experiments on transfected HeLa cells have shown that in response to 17β-estradiol (E2), ER-α is a stronger transactivator than ER-β at low receptor concentrations. However, at higher receptor concentrations, ER-α activity self-squelches, and ER-β becomes the stronger transactivator. These data support a role for ER-β in the direct vascular effects of estrogen and in the regulation of vascular function (45).

Many studies indicate that in rat carotid artery (46) and aorta (47), mRNA of both estrogen receptors were constitutively expressed at low levels in the smooth muscle cells, **whereas after denudation the expression of the estrogen receptor β mRNA increase while that of ER-α remains low. Treatment of ovariectomized female rats with the isoflavone phytoestrogen genistein, which shows 20 time higher binding affinity to ER-β than to ER- α** (48) or with 17β – estradiol, which does not differentiate between the two receptor, provides a similar dose- dependent vasculoprotective effect; however, only 17β-estradiol, but not genistein, induces a dose- dependent uterotrophic effect (46). As genistein with the dosages used is unlikely to exert its effect via tyrosine kinase inhibition (46), **the result suggests that ER- β rather than ER- α mediates the vasculoprotective effect of estrogen**.

Tissue-Specific patterns of ER-α and ER-β mRNA Expression in the rat

Tissue	**Receptor*ER-α**	**Receptor ER-β**
Epididymis	+++	+
Prostate	+	+++
Testis	+++	+
Pituitary	++	+
Ovary	+++	+++
Uterus	+++	++
Bladder	+	++
Lung	0	+
Liver	+	0
Kidney	++	0
Thymus	+	+
Adrenal	++	0
Olfactory lobe	0	+
Cerebellum	0	+
Brain stem	0	+
Spinal cord	0	+
Heart	+	0

Table 2.1 Relative levels of expression are indicated by the number of plus signs: 0, not detected; +, low; ++, medium; +++, high (49).

Binding affinity of various ligands for ER-α and ER-β relative to binding affinity of Estrogens

	Relative binding affinity	
Ligand	ER-α	ER-β
17β - estradiol	100	100
Diethylstilbestrol	468	295
Hexestrol	302	234
Dienestrol	223	404
Estrone	60	37
17α-Estradiol	58	11
Moxestrol	43	5
Estriol	14	21
4-OH-Estradiol	13	7
2-OH-Estradiol	7	11
Estrone-3-sulfate	<1	<1
4-OH-Tamoxifen	178	339
ICI-164384	85	166
Nafoxidine	44	16
Clomifene	25	12
Tamoxifen	7	6
Coumestrol	94	185
Genistein	**5**	**36**

Bisphenol A	0.05	0.33
Methoxychlor	0.01	0.13

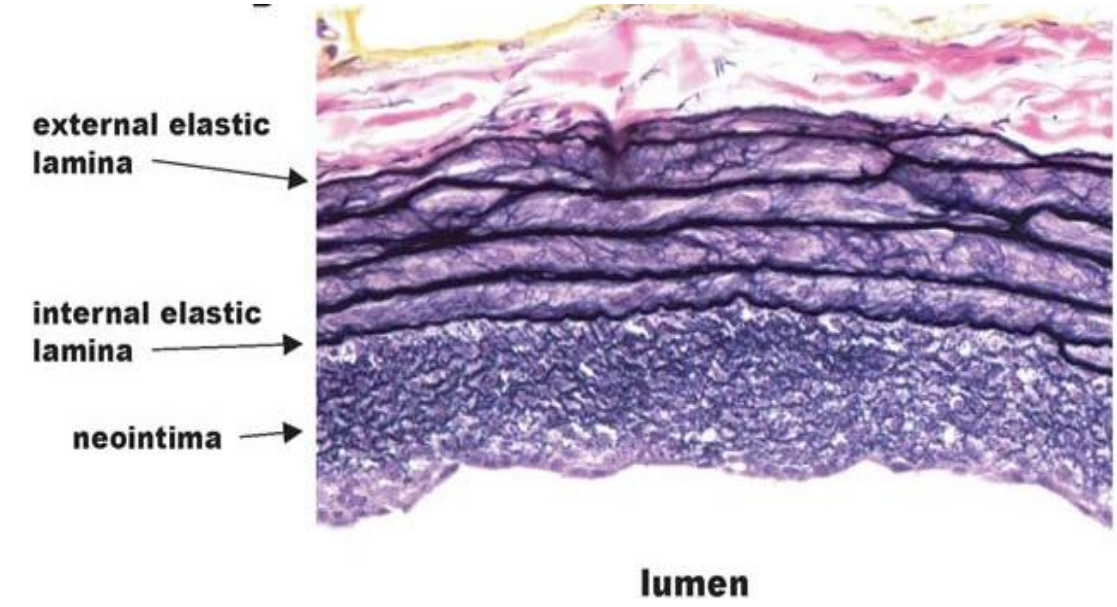
Table 2.2 *Relative binding affinity is the ratio of concentrations of E2 and competitor required to displace 50% of specific radioligand binding. Relative binding affinity was set to 100% for estrogen (49).

Balloon injury model study

Balloon injury of the arteries of animal (including rabbit, fowl, swine, and primate) has been extensively used as **an experimental model for the early injury phase of atherosclerosis.** In this model, inflation of the balloon catheter denudes vascular endothelium leads to neointimal hyperplasia. Previous study, showed that balloon injury induced migration/proliferation of adventitial fibroblasts as target cell for activation and migration into media and neointima after endothelial injury (50). In addition, some studies reported the extensive inflammatory cell infiltration of the adventitia and perivascular tissue of balloon injured carotid arteries of ovariectomized rats within 24 hours of the insult (51-54). An early inflammatory reaction whereby leukocytes enter the arterial wall in large numbers from the periadventitial tissues may play an important role in intiating the vascular injury response and ultimately create the extent of neointima formation **(Fig. 2.4 A)** (55).

Dongqi Xing and his colleagues (17), found that the balloon injury of ateries has been shown to elicit aggregation of leukocyte (granulocyte/neutrophils, monocyte/macrophages, and T lymphocytes) in the adventitia surrounding the injury site within hours after the event. The view of inflammatory cells is associated with increased expression of adhesion molecules and leukocyte-specific cytokines in adventitial tissues. Moreover, this study showed that leukocytes migrated balloon injured arteries in large number via the adventitial route within 24 hours of balloon injury. Leukocytes were more abundant in the adventitial and periadventitial region than in the media or neointima of injured vessels throughout the period of observation and virtually disappeared from the injured artery by 7 days after injury **(Fig. 2.3 B).**

A)



B)

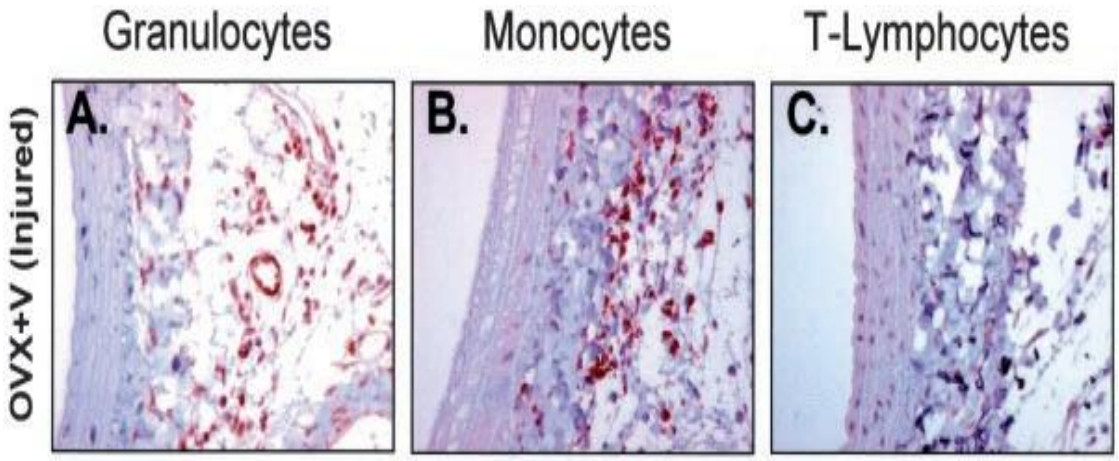


Figure 2.4 Representative Balloon-injured carotid artery sections demonstrating a matrix-rich intimal hyperplasia (18). Granulocyte A), monocytes/ macrophage B) and T lymphocyte C) specific antibody-stained sections (magnification x 100) of injured common carotid arteries of OVX rats (B) (55).

Inducible NOS and vascular injury

In 1995, Marks and colleagues (56) showed that a single treatment with a protein adduct of NO that possessed a prolonged biologic half-life prevented intimal hyperplasia in response to rabbit femoral arteries injury. The injured vascular was exposed to the NO protein adduct for 15 min. Single treatment with S-nitroso-serum albumin inhibited development of intimal hyperplasia as compared to control groups at 14 days. Therefore, NO could prevent vascular destruction. Groves and other (57) showed that 3 weeks of treatment with an oral NO donor molsidomine resulted in 32% reduction of intimal thickness following angioplasty in porcine carotid arteries. Lee and team (58) showed that chronic inhalation of NO following balloon-induced arterial injury in the rat carotid artery resulted in 43% decrease in the intima /media ratio at 2 weeks. The results from these studies suggested the role of NO in intimal hyperplasia formation.

First study on the role of endogenous iNOS in the injured vascular response was investigated by Hansson and other. in 1994 (59). They found that iNOS mRNA was upregulated in the medial smooth muscle cells after balloon-induced endothelial damage from 1–14 days. In another study, Yan and colleagues (60) demonstrated that did not only medial SMCs show upregulation of iNOS but that the neointimal cells showed a greater expression of iNOS base on both Western blot and Northern blot analysis. Moreover, the upregulation of iNOS promoter on the neointimal cells as exhibited by transfection studies using a reporter gene under the control of the iNOS promoter. However, the neointimal cells were less responsive to the antiproliferative effects of NO than the medial smooth muscle cells as measured by the inhibition of cellular proliferation. As with the other protective actions of iNOS after vascular injury, the expression of iNOS possibly functions to resolve the injury response and limit excessive SMC proliferation. One mechanism was via the induction of a G0/G1 cell cycle arrest, preventing cells from entering the synthesis phase of the cell cycle and undergoing proliferation (61).

The negative role of iNOS was confirmed by Koglin and other. (62). They transplanted allogeneic hearts into iNOS -/- and iNOS +/+ mice and evaluated the coronary arteries for signs of transplant vasculopathy. Interestingly, the iNOS -/-

recipients showed significantly more neointimal thickening compared to the iNOS +/+ recipients. From these study, it appeared that iNOS may play a protective role in the short term development of transplant arteriosclerosis, suppressing neointimal smooth muscle cell aggregation.

Regulation of iNOS activity and expression

The bioavailability of L-arginine is an important regulator of iNOS activity *in vivo*. Recently studies, indicate that this may involve the catabolism of L-arginine to ornithine and urea by arginase, a metalloenzyme, which expression has been documented in several cell types of the vasculature including smooth muscle cells (63). Increased expression of arginase during vascular injury may shunt L-arginine metabolism to ornithine and polyamine synthesis, inhibit iNOS-derived NO production, and promote cell proliferation (63). In order hand, cytokines such as IL-1β activate NF-κB in many cell types including VSMC and activation of NF-κB is a requirement for iNOS expression (64-65).

Regenerated endothelial cells

The regenerated endothelium expressed von Willebrand factor and eNOS. However, rings with such regenerated endothelium displayed a major impairment of the endothelium-dependent relaxation to aggregating platelets, serotonin, ergonovin or thrombin, and a greater propensity to exhibit endothelium-dependent contractions and the remaining relaxation was no longer inhibited by pertussis toxin. This indicated that Gi-protein coupling is defective in regenerated endothelial cells. By contrast, the Gq-coupling to eNOS seemed intact as relaxation to bradykinin were not damage. Likewise, endothelium-dependent relaxations elicited by the calcium ionophore A23187 were comparable to those acquired in rings of the same arteries covered with native endothelial cells. Taken in conjunction with the normal response to bradykinin, this demonstrated that the intrinsic ability of the regenerated endothelium to produce NO was not affected. This series of experiments prompted the apparent conclusion that there is a selective loss of the Gi-dependent coupling to eNOS in regenerated endothelium (66).

Estrogen and vascular injury

Estrogen (E2) has a potent inhibitory effect on leukocyte migration into the periadventitial and adventitial domains of arteries within the first day after vascular injury. Furthermore, E2 reduced the granulocyte and monocyte/macrophage populations of 24-hour injured vessels by 50% but seemed to have a stimulatory effect on the smaller population of T lymphocytes (55). In another study, suggested that E2 regulated granulocyte function under inflammatory conditions (67). *In vitro* evidence has demonstrated an ER-dependent mechanism for inhibition of neutrophil chemotaxis (68). Moreover, E2 has been shown to induce endothelial NOS (eNOS) and cyclooxygenase activity in the vessel wall increase nitric oxide production and prostacyclin, which inhibit leukocyte adhesion and infiltration (69). On the other hand, E2 has been shown to modulate monocyte chemoattractant protein-1 (MCP-1) expression in murine macrophages in vitro through an ER-dependent mechanism. Furthermore, E2 has been shown to inhibit human monocyte chemotaxis in response to MCP-1 in a modified Boyden chamber (70).

Ishibahshi and his colleagues (71) , found that the balloon injury caused intimal hyperplasia, which was accompanied by the impaired endothelium-dependent relaxation and cyclic GMP production, and accumulation of asymmetric dimethylarginine (ADMA) as an endogenous NOS inhibitor. Bilateral ovariectomy accelerated the intimal hyperplasia. The acceleration was accompanied by the enhanced impairment of NO production, attenuated reendothelialization, and enhanced accumulation of ADMA. Administration of 1.5 mg/kg/day 17β-estradiol improved the accelerated intimal hyperplasia with concomitant improvement of the impaired NO production and accumulated asymmetric dimethylarginine.

The initial phase of Atherosclerosis

Vascular inflammation has been recognized as an initial phase in the pathogenesis of atherosclerosis (72). Natïve endothelial cells do not stimulate monocyte adhering to the vascular bed. Early vascular inflammation upregulation of key vascular endothelial cell adhesion molecules such as E- and P-selectins (referred to as CD62E and CD62P, respectively). Irritated vascular endothelium expressing CD62E and CD62P results in rolling of monocytes on endothelial cells. Followed by activation of circulating monocytes. The next step is stiff adhesion. Activation of circulating monocytes could be mediated by a number of pro-inflammatory cytokines, oxidized low density lipoprotein (oxLDL), and oxLDL/anti-oxLDL IgG immune complexes. Firm adhesion is regulated by vascular cell adhesion molecule-1 (VCAM-1, CD106) and intercellular adhesion molecule-1 (ICAM-1, CD54). Recent studies, showed that deletion of both CD62E and CD62P reduced the progression of atherosclerosis in apoE-/-mice (73-75).

Four early cellular steps provided to the initiation of inflammatory processes associated with atherosclerosis :-i) endothelial cell activation resulting in enhanced adhesive property of vascular endothelial cells, ii) activation of monocytes leading to the firm adhesion of circulating inflammatory cells, iii) transmigration of monocytes to the intima where these cells transform to become macrophages, and, finally, iv) uptake of modified LDL, such as an oxLDL, by macrophages climax in transformation of macrophages to lipid-laden macrophages, also known as foam cells (76). Reportedly, these studies have demonstrated the causal relationship between adhesion of monocytes to endothelial cells as controlling the initial events of atherogenesis. process associated with atherogenesis are only beginning to appear. Pro-inflammatory cytokines, TNF-α and IL-1β have been shown to induce activation of endothelial cells resulting in the coordinated up-regulation of CD54, CD62E and CD106 expression (77-79).

Phytoestrogens

Epidemiological data from Eastern countries suggest that regular dietary consume of soy products may decrease cardiovascular risk in postmenopausal women (80-81).

The majority of phytoestrogens found in typical human diets can be classified into two primary classes, isoflavones and lignans. phytoestrogens may have a role in modulating hormone - related disease based on their structural similarity to 17β-estradiol (**Fig.2.5**). Isoflavones make up the most common di-phenolic structure that equivalent the architecture of the potent synthetic estrogens di-ethylstilbestrol and hexestrol. Two of the major isoflavones found in humans are genistein and daidzein. Genistein and daizein are parent compounds, which are metabolized from their plant precursors, biochanin A and formononetin, respectively. In plants, isoflavones are inactive when present in the bound form as glycosides, but when the sugar residue is removed, these compounds become activate.

Many studies showed that a diet high in isoflavones improved endothelium-dependent vasodilation in ovariectomized rats (82) and enhanced the vascular response to acetylcholine in atherosclerotic female (but not in male) rhesus monkeys (83). The long-term effects of a diet enriched in isoflavones in rats showed that after ten months, the amount of eNOS was increased in aorta and endothelium-dependent vasodilation was improved. Interestingly, eNOS expression and vascular reactivity of animals on a standard diet could be improved to a similar extent within six (but not two) months after switched to a soy-enriched diet (84). By contrast, a study indicated that isoflavones supplemention did not have any benefic on the eNOS system on cerebral arteries of hypercholesterolaemic rabbits (85).

In the study of Walker and other, genistein enhanced eNOS activity and endothelium-dependent vasodilation in both postmenopausal women as well as in men (86). Genistein proved to be as effective equimolar concentrations of 17beta-estradiol (87). Many studies, genistein evoked vasodilation was insensitive to eNOS inhibition (88). After several days treatment periods, genistein induced eNOS activity in endothelial cells in culture (89). However, the study of Liu and other disclosed that genistein also acutely activated eNOS *in vitro* via activation of protein kinase A and

phosphorylation at eNOS–Ser (90). However, the mechanism of genistein related is still question for investigation.

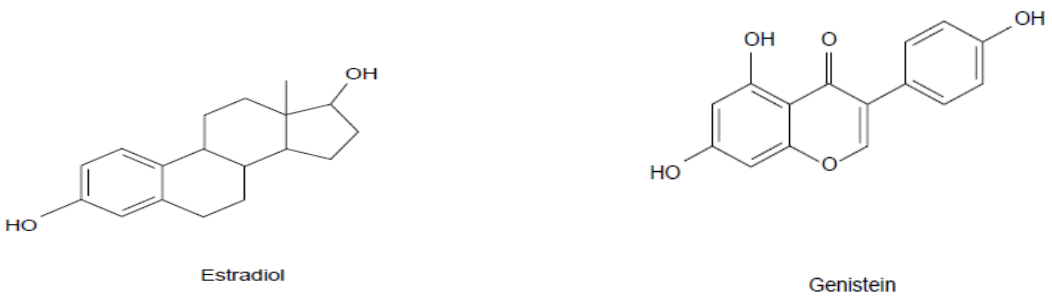


Figure 2.5 Chemical structures of the estradiol and genistein (91).

Genistein

Genistein has been a potent inhibitor of tyrosine kinase for more than 20 years (92). The protein tyrosine kinase is an important drug target for anticancer treatment because several growth factor receptors and oncogenes are controlled by tyrosine phosphorylation (93). The inhibitor of tyrosine kinase may explain the antiproliferative effect of genistein. In addition, genistein has agonistic activity on both ER-α and ER-β, but its affinity on ER-β is considerably greater; genistein's affinities for ER-β and ER- α were determined to be 145 nM and 8.4 nM, respectively. Physiological concentrations of free genistein activate ER-β. Genistein acts via an ER-dependent pathway involving extracellular signal- regulated kinase ½ activation, and at higher concentration (>25 µM) its effects are attributed to its tyrosine kinase inhibitor activity (92).

Genistein would exert a protective effect on vascular endothelium via its regulatory action on EC proliferation, apoptosis and leucocyte adhesion, events that play a critical role in vascular diseases. This modulatory action was detected either under physiological or inflammatory conditions. The molecular mechanism displayed by the phytoestrogen involves the participation of ER and the activation of NO pathway (94). Serum concentration of genistein in human consuming dietary soy supplementation varies between 0·74 –2·5 mM in Asian and 14–20 nM in Western population (95). The concentration of genistein employed in the present study (0·1–

100 nM) is close to human plasma levels. The low concentration of genistein stimulated endothelial cell proliferation. Genistein exhibited an anti-mitogenic effect. Moreover, genistein exerts both estrogenic and non-estrogenic actions that depend on the isoflavone concentration.

Soy diet inhibits activation and subsequent adhesion of inflammatory cells

In vitro studies using human monocytic cell lines showed that oxLDL treatment of monocytic cells increases their adhesion to endothelial cells. Furthermore, pre-exposure of monocytes to soy isoflavones (1 μM final concentration with equimolarmixture of genistein, daidzein and equol) as well as to sera from animals fed soy diet inhibited oxLDL-induced monocyte adhesion to endothelial cells (96). These studies suggested that there is a direct effect of soy isoflavones on monocyte activation and subsequent adhesion, although detailed mechanism (s) is not known. With these findings, it is reasonable to hypothesize that the soy-dependent atheroprotection is mediated through the regulation of monocyte activation.

Soy isoflavones have no uterotrophic activity

Physiological concentrations of soy isoflavones can also be expected to be safe for the uterine endometrium, as the uterotrophic effect of estrogens appear to be mediated solely by ER-α. This has been demonstrated gracefully in ER-α- knockout mice, in which estrogens failed to exert a uterotrophic effect (97). As expected, soy isoflavones have shown no impact on endometrial proliferation in clinical studies. Although endometrium expresses both alpha and beta receptors, specific synthetic agonists for ER-β did not decrease uterine weight in rats or prevent the proliferative in response to a concurrently administered ER-α specific agonist (98). In women, soy isoflavones do not suppress the endometrial proliferative response to estrogen. Based on these observations, ingestion of genistein within the nutritional range would not be expected to either increase or decrease endometrial cancer risk.

CHAPTER III

MATERIALS AND METHODS

I Animals

Adult female Wistar rats, weighing 240-270 g and aged 12 weeks, were used in this study (N=32). The animals were obtained from National Laboratory Animal Center of Salaya Campus, Mahidol University, and were maintained on standard laboratory rat food and tap water *ad libitum* under controlled environmental conditions of 12-hr light/dark period. This study protocol was approved by Faculty of Medicine, Chulalongkorn University under ethical principles and guidelines for the use of animals for scientific purposes and policies of Chulalongkorn University. (Protocol number 17/54)

II Experimental protocol (Fig.3.1)

Twenty- four rats underwent bilateral ovariectomy (OVX). The sham group underwent sham operation and served as a control group (n=8). Body weights were recorded weekly vaginal cytology was performed at the beginning and at four- week post ovariectomy to ensure the estrogen deficiency. Animals were allowed to recover from surgery for four weeks, then they were divided into 4 groups:-

i) Sham group rats were injected subcutaneously with 10% dimethyl sulfoxide (DMSO) 100 µl/day (Sigma Chemical Co., USA) (n=8; Sham+DMSO),

ii) Ovariectomized rats were injected subcutaneously with 10% DMSO 100 µl/day (n=8; OVX+DMSO).

iii) Ovariectomized rats were injected subcutaneously with genistein 0.25 mg/kg/day (Sigma Chemical Co., USA) (n=8; OVX+Gen).

iv) Ovariectomized rats were injected subcutaneously with 17β-estradiol 0.2 µg/kg/day (Sigma Chemical Co., USA) (n=8; OVX+E2).

The balloon injury at the left carotid artery was performed in ovariectomized rats after 4 weeks treatment.

Three groups of ovariectomized rats underwent balloon injury at left carotid artery after those treatment daily for 4 weeks and another two week post surgery. Blood sampling was performed at sacrifice by cardiac puncture, after 8 hours fasting, to measure serum levels of total cholesterol, high- density lipoproteins (HDL), triglyceride, low-density lipoproteins (LDL) by a chemical analyzer enzymatic, colorimetric method (CHOD/PAP) (Department of laboratory, ISO 15189 Accreditation number 4006/47, Chulalongkorn hospital) and serum estradiol was determined by Chemiluminescent microparticle immunoassay (CMIA) (BRiA laboratory). Then, The animals were perfusion-and pressure –fixed at 100 mmHg using phosphate buffered saline (PBS) following 10% neutral buffer formalin (99-100) to wash the left carotid artery from remain blood component and retain its as near to dwell state. The balloon-injured left carotid artery was yielded for histological analysis and immunohistochemical studies for von Willebrand factor, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and asymmetric dimethylarginine (ADMA) as endogenous NOS inhibitor studies. Uterus was removed and weighed to determining the effects of genistein and estradiol on reproductive organ. All surgeries were performed under anesthesia and aseptic technique.

The experimental protocol

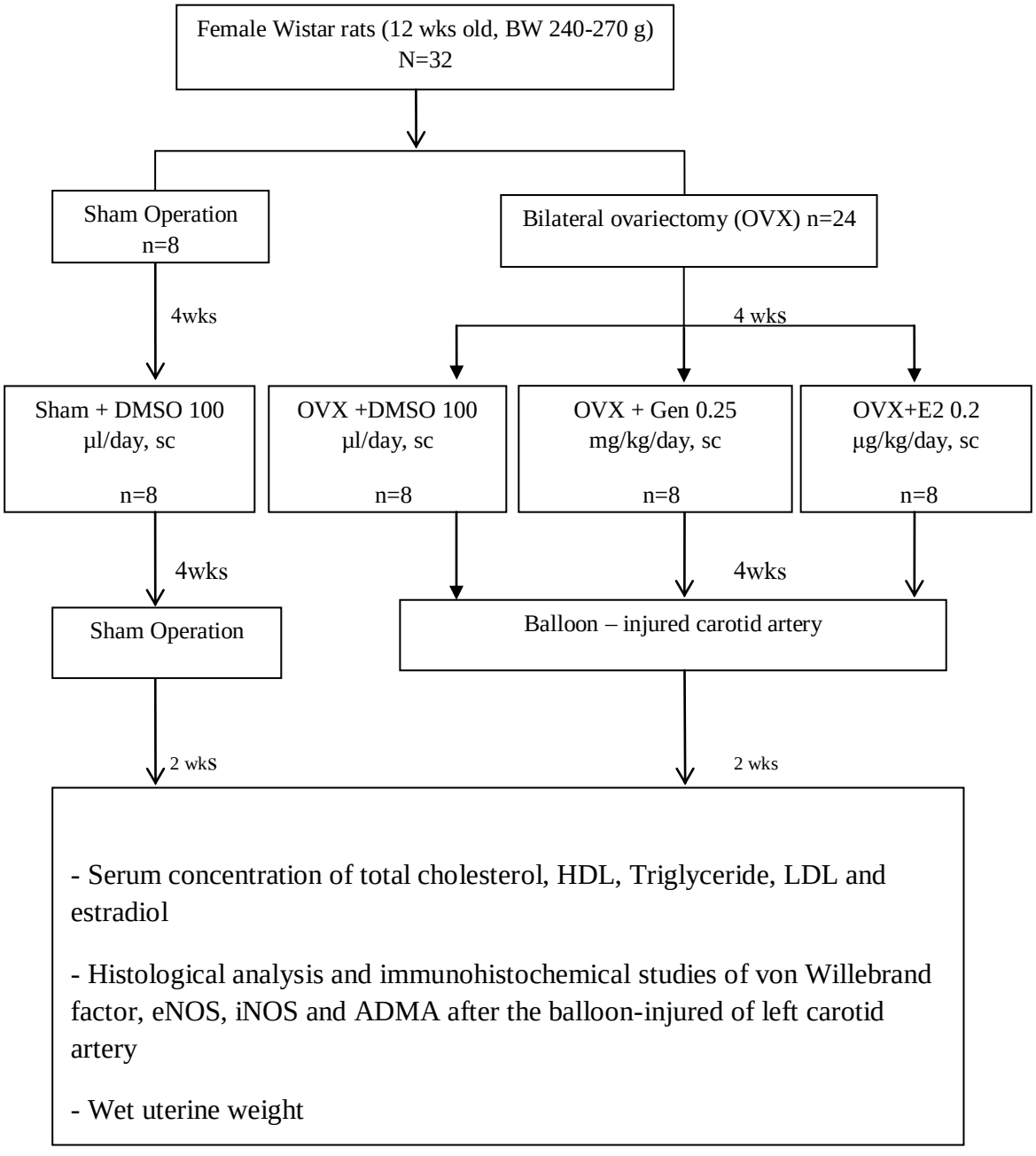


Figure 3.1 The simple diagram showing the experimental protocol.

III The bilateral ovariectomy (OVX)

The bilateral ovariectomy was done under general anesthesia with pentobarbiturate sodium (45 mg/kg/ BW) injected intraperitoneally. Once the animal were sedated to a surgical level of anesthesia (confirmed by toe or tail pinch, and pattern of breathing and heart rate). Bilateral lumbar incision were made approximately ½ inch below the ends of the ribs. The ovary was gently extracted by grasping the periovarian fat. The cranial portion of the uterus and associated uterine vessels were clamped and the remaining tissue was tied off with Ethicon 4-0 monocryl sterile absorbable sutures. The ovary were then removed. After suturing the muscle layer and skin layer were closed with Ethicon 4-0 monocryl sterile sutures (101).

IV Vaginal cytology assay

Vaginal cytology assay in the beginning and four weeks post ovariectomy was examined to ensure the estrogen deficiency state. Vaginal smear samples were taken between 9: 00 am -10: 00 am. Using a plastic pipet filled with 10 µl of normal saline. The plastic pipet was inserted and flushed into the vagina, then smeared on a glass slide, and the smear was fixed with ethanol. Smears were stained with the usual Papanicolaou method and observed with light microscope (102).

V Rat Carotid Artery Balloon Injury Model

The balloon injury of left carotid artery was performed according to the method described previously (18). In brief, three groups of ovariectomized rats after those treatment daily for 4 weeks were anesthetized with pentobarbiturate sodium (55 mg/kg body weight) injected intraperitoneally. The ventral neck region from the chin down to just above the sternum was shaved and placed the animal supine, head toward the surgeon. The cervical area was swabed with a providine solution following 70% alcohol (**Fig.3.2 B**). A straight incision was operated below the chin toward the tail all the way to the top of the sternum just above the rib cage and separated the central muscle from parallel neck muscles over the left carotid artery unit completely dissection and retraction of muscle. The left common carotid artery (LCA), the

bifurcation of the common carotid into external and internal carotid artery was exposed and added lidocain hydrochloride 1-2 drops on top of the common carotid carotid artery to dilate the blood vessel and its branches. Next step, the vagus nerve (a thin white sheath lying adjacent to the left common carotid artery) was isolated from the common carotid artery (CCA) **(Fig. 3.4).**

(A) (B)

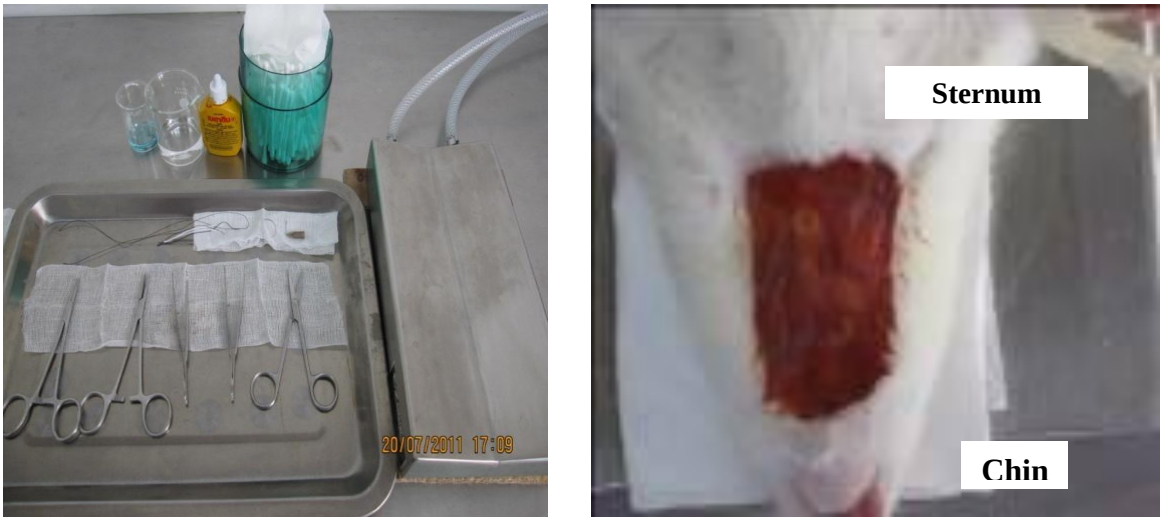


Figure 3.2 Photograph of the sterilizing surgical instrument, antiseptic solution, operating platform and surgical area (A), the photograph of the surgical site on the ventral neck region from the chin down to just above the sternum(B).

(C) (D)

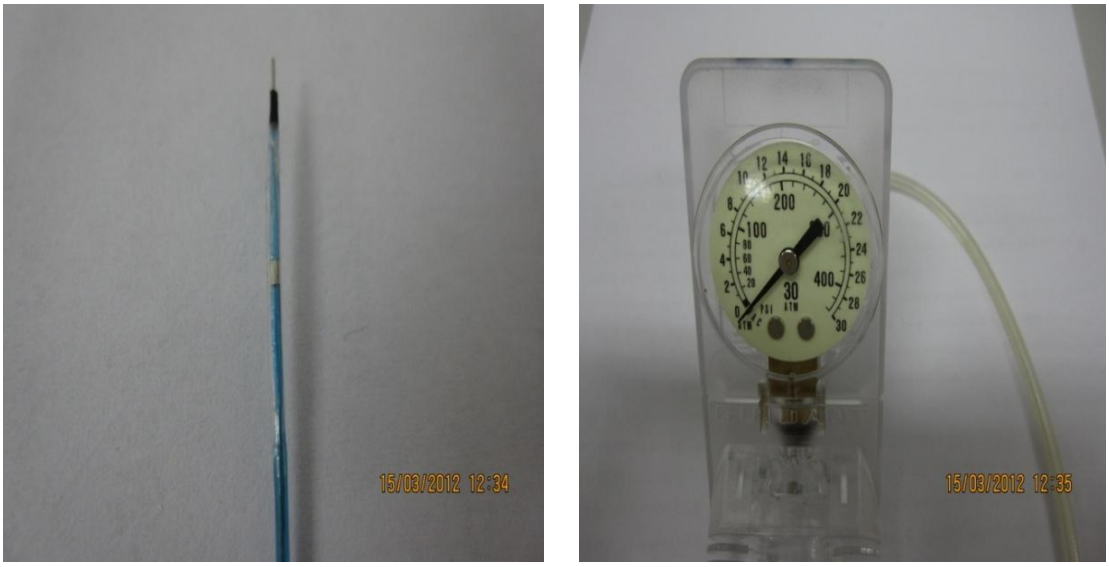


Figure 3.3 Photograph of an arterial balloon embolectomy catheter size 1 mm (C) and an automated balloon inflation device (D).

(E)

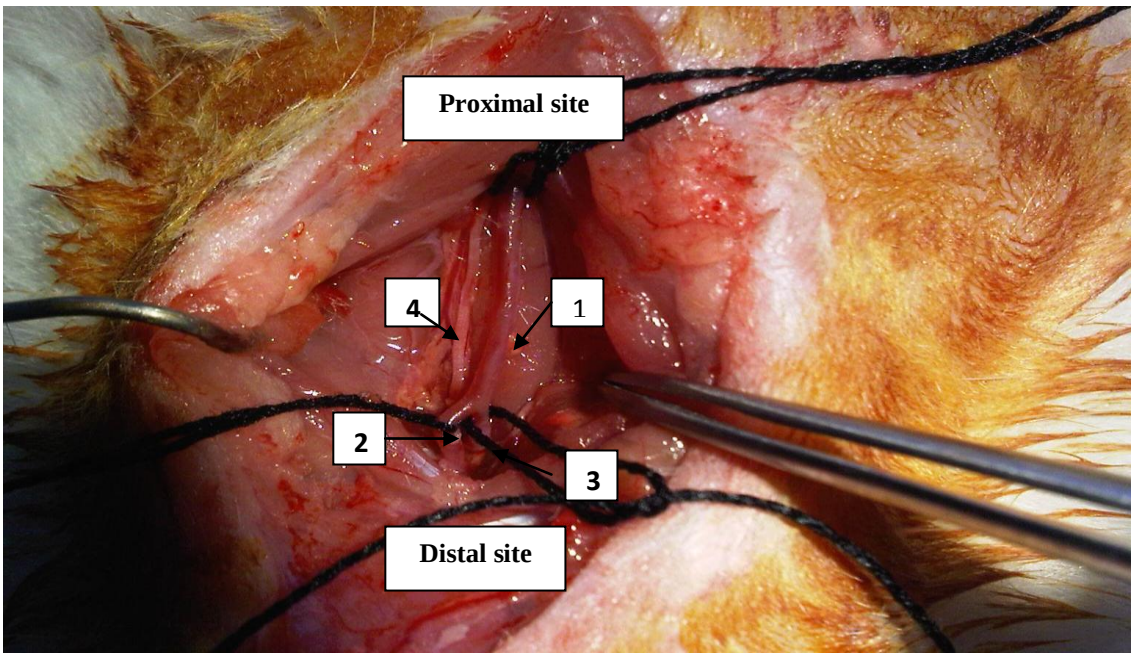


Figure 3.4 Photograph of the left common carotid artery (1), The bifurcation of the common carotid and its branches, external (2) and internal carotid artery (3), The vagus nerve (a thin white sheath lying near the left common carotid artery) (4). Proximal site as close to the sternum and distal site narrow chin.

The external carotid artery branch was looped but did not tie at distal site. An arteriotomy incision was creating in the external carotid branch and inserted the uninflated balloon into the arteriotomy hole and advance it all the way to the arterial clamp on the common carotid artery. The balloon catheter was inflated with pressure 1 atm via an automated balloon inflation device. Back-and-forth (3 times) with the pressure filled balloon with 1 atm repeated inflation and withdrawal of the catheter to induce endothelial cell damage and mural distension. The external carotid artery was ligated after removal of the catheter and the wound was closed. An anti-septic agent was swabbed on all sides of wound to reduce infection and cover sterile gauze pads. (**Fig. 3.8 I, J, and K**).

(F)

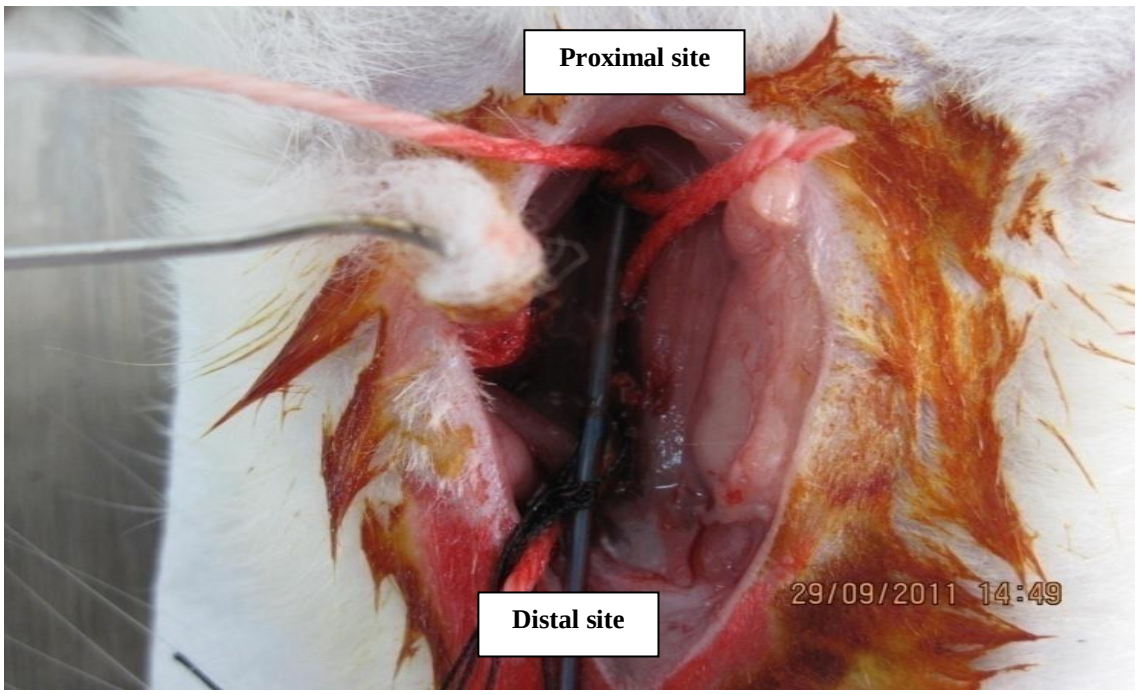


Figure 3.5 Photograph of the rat left carotid artery vasculature during insert the **uninflated** balloon into the arteriotomy.

(G)

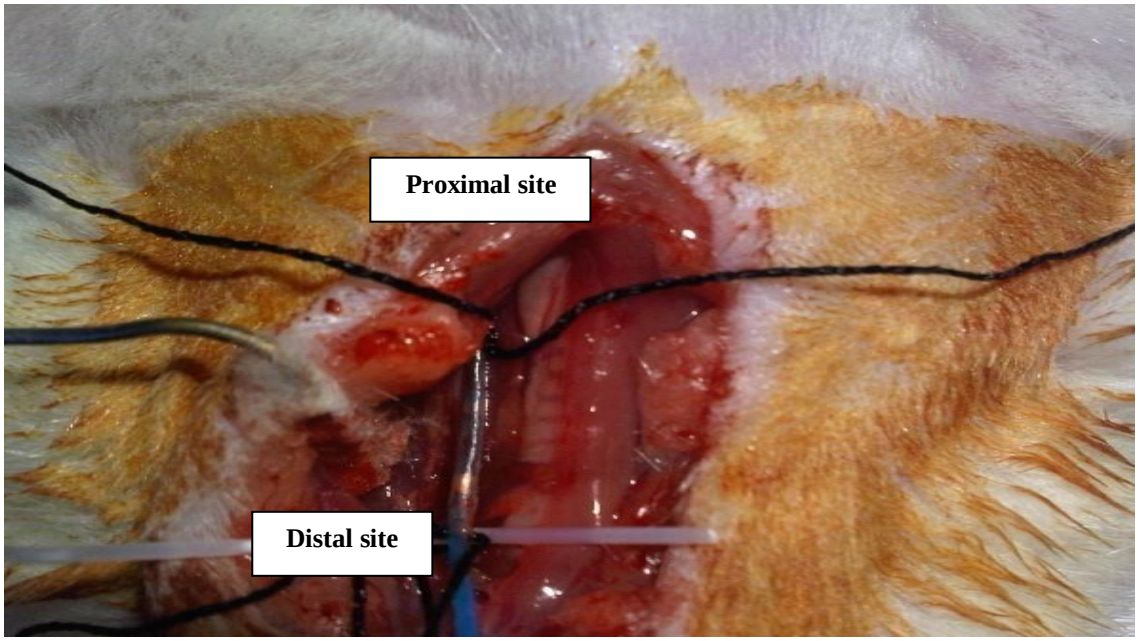


Figure 3.6 Photograph of the rat left carotid artery vasculature during insert the **inflated** balloon catheter inflated with pressure 1 atm via an automated balloon inflation device into the arteriotomy.

(H)

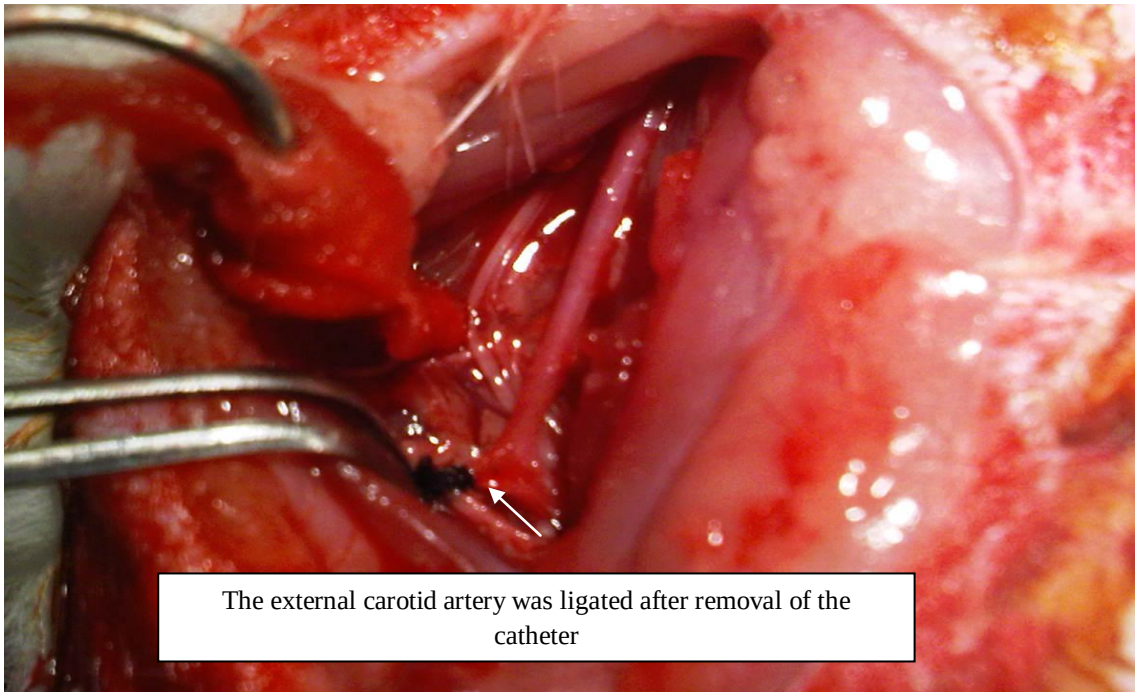


Figure 3.7 Photograph of the external carotid artery was ligated after removal of the catheter.

(I)

(J)

(K)

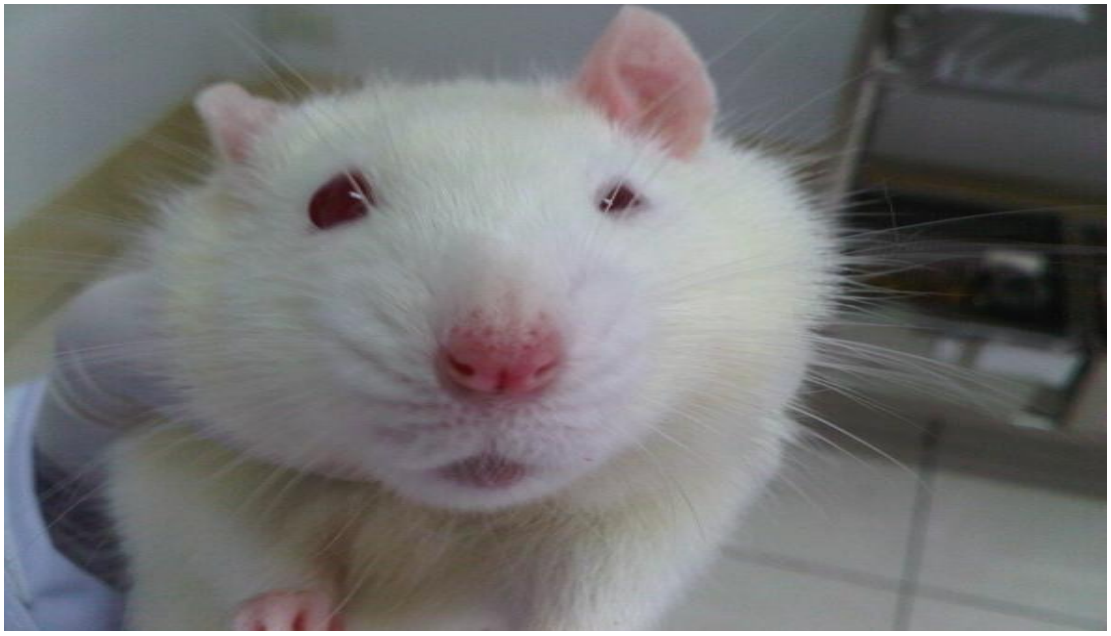


Figure 3.8 Photograph of post-operative the animal care. The animal was placed on a heating blanket to avoid hypothermia (I) and removed to her cage (J). The rat was ambulatory after 2 weeks surgery event, A symptom commonly demonstrated by balloon-injured rats is partial ptosis of the left eye due to nerve manipulation associated with the surgery and following malfunction of the eyelid elevator muscle (K) (18).

VI Histological and morphometric analyses for rat carotid artery balloon injury studies.

Histological and morphometric analyses were performed according to the method described previously (100),

In situ perfusion fixation

This process was performed to get rid of the blood from tissue of interest and to retain the tissues as close to their living state and preserve their integrity. Protocol - The animals were placed on their back on surgical trays with their distal sides towards operators. Arms and legs were fixed to the trays with a tapes. Midline incision was made from the substernal notch through sternum and ribcage, moving in a caudal direction to the diaphragm. The tip of the scissors was kept up to prevent heart puncture. When the animal carcass was in place, another incision was made at the right atrium to make an outflow. The stopcock was carefully opened and allowed the warm phosphate buffered saline (PBS) (150 ml.) flowing into the animal. The blood made an exit from the body through the incision at the right atrium. The color of the blood leaving the body would gradually clear as the blood was removed from the body and replaced by PBS. When, the fluid leaving the body was clear. The stopcock was switched to allow fixative solution (10% neutral formalin buffer 150 ml.) to flow through the system and ran in to the animal. Muscle twitching in tetany could be observed during fixation indicating adequate tissue fixation was occurring. After the tissues were completely fixed when rigor was observed. The catheter was then removed from the heart and placed the animal on an absorbent pad ready for tissue harvesting.

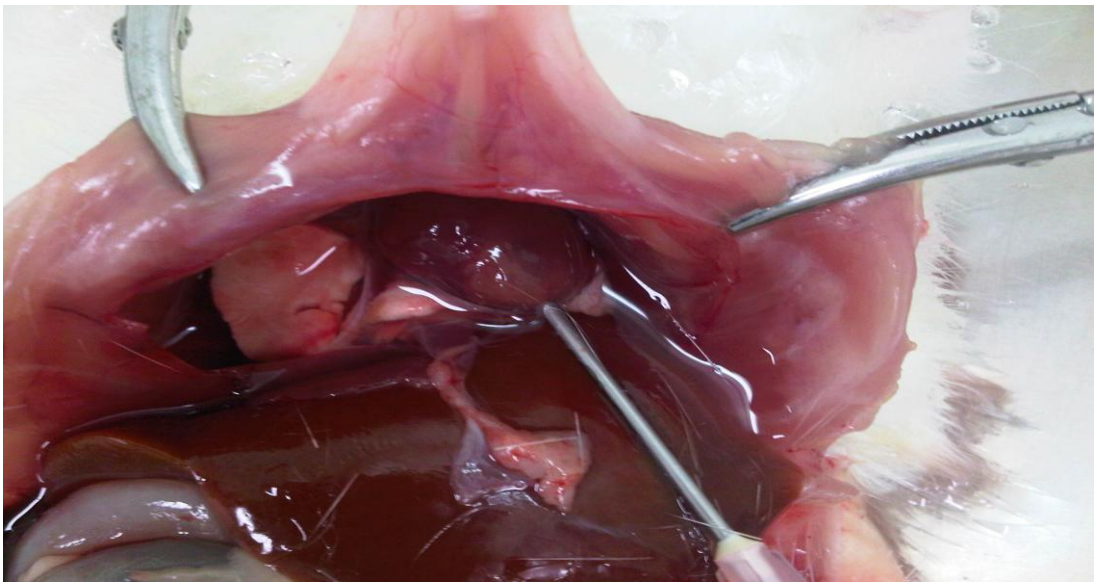


Figure 3.9 Photograph of *in situ* perfusion-fixation.

Tissue harvesting

Tissues and muscular fascia surrounding the left carotid artery were removed to reveal the entire vessel from the aorta to bifurcation. The artery was detached from adjacent tissues. Three centimetres long artery was cut from its aortic end. Only 1.5 cm long from its distal and was used for analysis. The artery was put in a petrie dish filled with fixative and cleaned throughly. Blood remained in the lumen was flushed with fixative by needle and syringe. Then the vessel was put into a vial obtaining the same fixative until it was processed by automated processing tissue. Uterus was also isolated and weighed to access the effects of ovariectomy and estrogen supplementation.

Sectioning

Sectioning of paraffin-embedded tissues is performed with a microtome that allows cut 3 micron in thickness. Five rounds cross-section per slide were stained by **Elastic Stain Kit** (Modified Verhoff's) method and preparation of immunostaining.

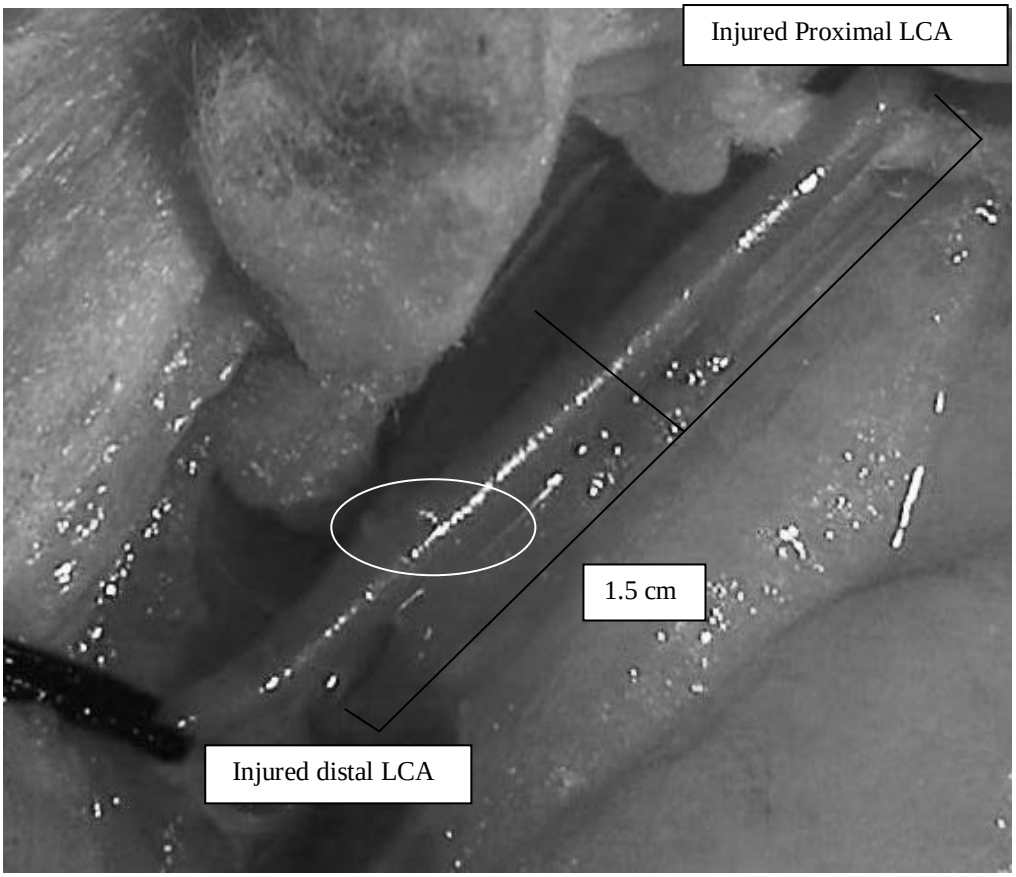


Figure 3.10 Photograph of the entire length of the left carotid artery (LCA) is balloon injured, yet only an approximately 1.5 cm longitudinal piece of the distal portion is subjected to a treatment of choice. ◯ Site where tissue samples can be taken (99).

Immunohistochemical Studies

To examine the localization of von Willebrand factor and endothelial NOS (eNOS) **for detecting the endothelial cells on the innermost surface** by Ventana automated immunostainer (Ventana, Tucson, AZ, USA). Antibody Von Willebrand factor [dilution 1:2500 in phosphate-buffered saline (PBS) and Primary Mouse monoclonal anti-eNOS dilution 1:100 PBS].

Reagents used in BenchMark® XT Instrument (Ventana, Medical System Inc, AZ, USA)

EZ prep™ for deparafinization

Liquid Coverslip (LCS™) for reagent evaporation prevention

Sodium Chloride sodium citrate pH 6.5-7.5 (SSC™)

Reaction buffer pH 7.6

Universal buffer pH 8.4

1% Hydrogen peroxide (H_2O_2, Ultra View ™ inhibitor) for endogenous peroxidase inhibition

Goat anti-Mouse IgG (Ultra View ™ HRP Multimer)

Ultra View ™ DAB chromogen

Ultra View ™ H_2O_2 (substrate)

Ultra View ™ Copper

Hematoxylin II

Lithium carbonate (Bluing reagent)

T**he localization immunostaining of inducible NOS (iNOS) and** asymmetric dimethylarginine (ADMA) as endogenous NOS inhibitor **to show their diminution if the blockage of subendothelial cells.** Their marker were performed and modified according to the method described previously (71), section 3 µm in thickness were deparaffinized and dehydrate. Antigen retrieval was performed by microwave hi-power 3 minutes and power 30% for 10 minute in the citrate buffer and incubate at room temperature 20 minutes then wash with phosphate buffer saline for 5 minutes . Endogenous peroxidase was block by 3% H_2O_2 /DW 5 minutes at room temperature and wash with distill water for 5 minutes and phosphate buffer saline for 5 minutes. Block non specific background incubate 3% normal horse serum for 20 minutes at room temperature, followed by incubation at 4˚ C primary mouse monoclonal antibody iNOS (dilution 1:50 in PBS) and primary rabbit monoclonal antibody ADMA (dilution 1:100 in PBS). Next, five sections were incubated in peroxidase labeled antibodies; Dako Envision kit (peroxidase/DAB) (DAKO, Carpinteria, CA) at room temperature for 30 minutes. Immunoreactivity was visualized by simple staining with DAB solution (3, 3 diaminobenzidine tetrahydrochloride anhydrous). Hematoxylin was used for counter-staining. The section were dehydrated and mounted with synthetic resin.

(A) (B)

Figure 3.11 Photograph of immunohistochemical staining of von Willebrand factor and eNOS by Ventana automated immunostainer (A). iNOS and ADMA immunostained with manual processing (B).

Assessment of Intimal Hyperplasia

The thickness of intimal area, medial area and the ratio of intimal area to medial area were calculated by pixel intensities values analysis and processing to regions of interest (ROI) using the image J (NIH) program version 1.44. By image J can convert the measurements from the number of pixel to the thickness of layer on carotid artery (μm^2).

(A) (B)

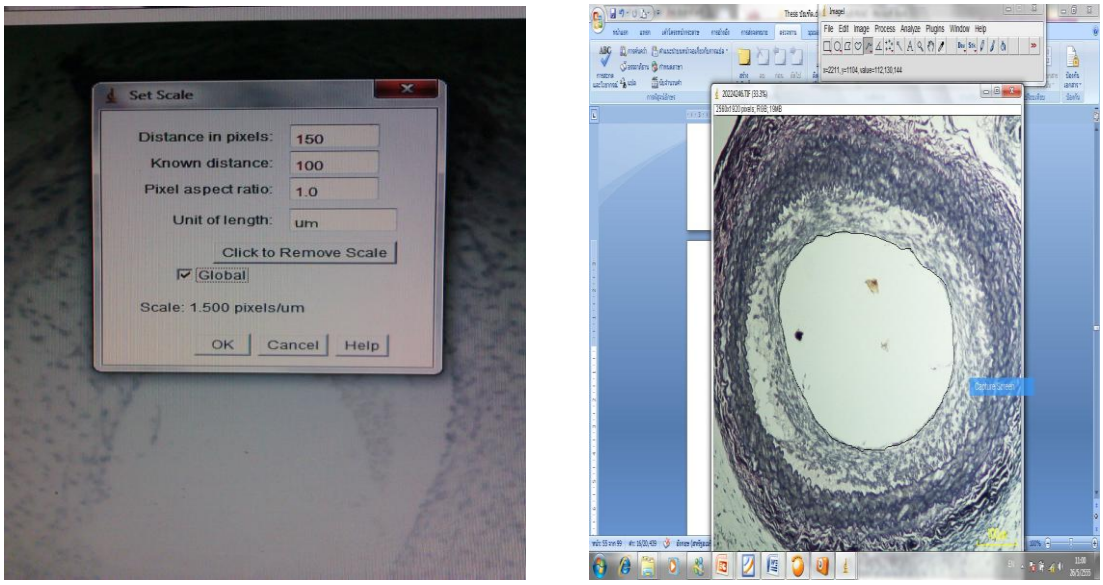


Figure 3.12 Setting the scale (A). Straight freehand line on the luminal surface, internal elastic lamina, and border between the medial layer and the adventitia were traced (B).

1. Setting the scale, select the measuring tool from the toolbar, right click and drag across a known distance in the image to be measured.
2. Click on analyze and select set the scale from the menu.
3. Select the unit " µm "
4. Enter the known length.
5. The program is ready to collect measurements that are read in pixels and convert them to µm.
6. Drag freehand line on the luminal surface, internal elastic lamina, and border between the medial layer and the adventitia were traced **Fig.3.12 B.**

7. Click on analyze and select tool and ROI manager.
8. The program can calculate area of intima and media.
9. **The intimal hyperplasia was measured the ratio of intimal area to medial area.**
10. The average of five sections was used for analysis as the value of each animal.

Assessment of the positively stained cells from immunohistochemical studies

A. Distribution and pattern staining

Immunoreactivity was visualized by simple staining with DAB solution showing brown color and counter-staining hematoxylin showing blue color. The number of von Willebrand factor- and endothelial NOS (eNOS) positive cells indicated on endothelial cells lining in the innermost surface of artery (**Fig. 3.13**).

Qualitative inducible NOS (iNOS) and asymmetric dimethylarginine (ADMA) as endogenous NOS inhibitor were expression in subendothelial cells (**Fig. 3.14**).

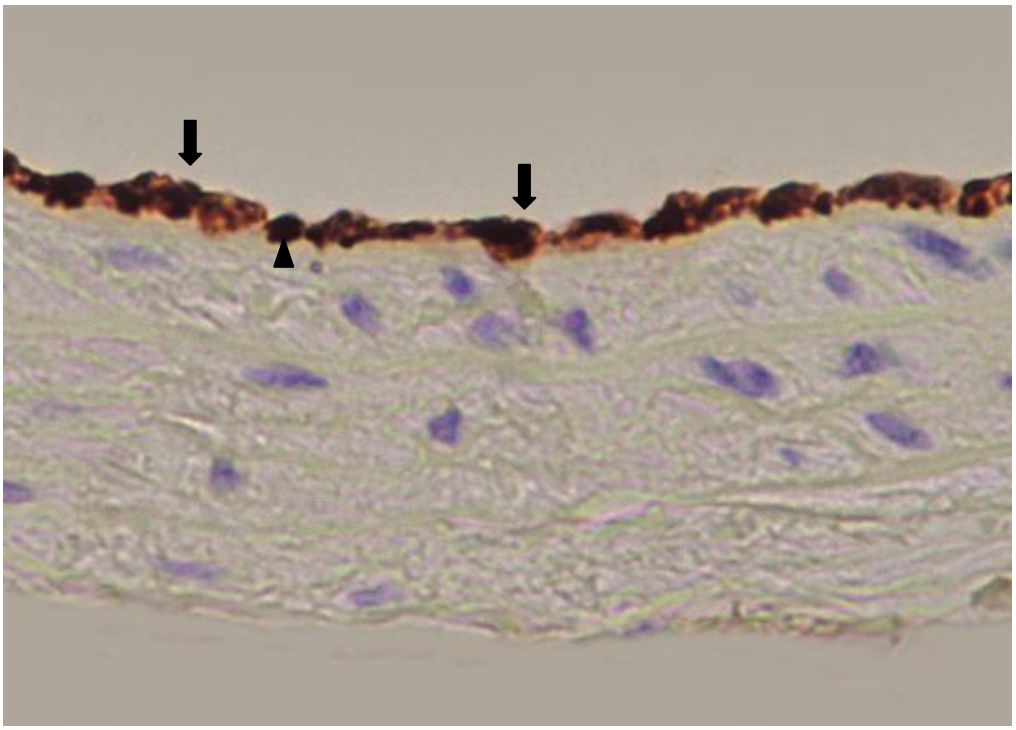


Figure 3.13 Immunohistochemical localization of **von Willebrand factor**-positive cell on the endothelial cells.

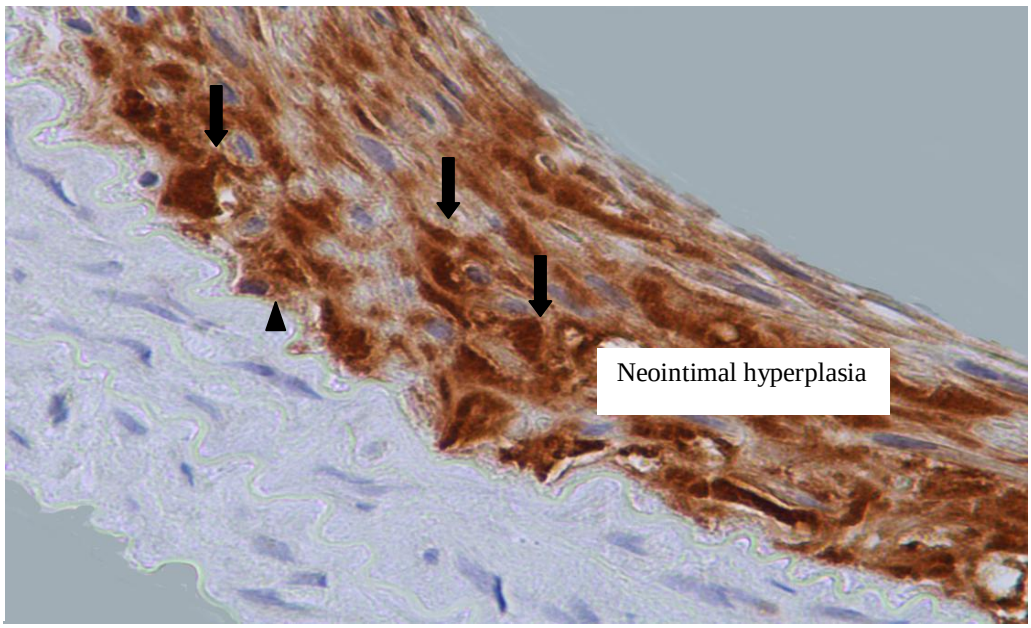


Figure 3.14 Immunohistochemical localization of ADMA-positive cell within **subendothelial cells**.

Determination of the number of positive cells was performed by image J program as measures count in threshold and analyzes particle.

1. File open
2. Click image type 8 bit
3. Click process and select sharpen.
4. Click image select adjust and threshold, analysis is performed on the existing area selection or on the entire image if no selection is present.
5. Click analyze and select analyze particle. Result display count as the number of positive cells.
6. The average of five sections was used for analysis as the value of each animal.

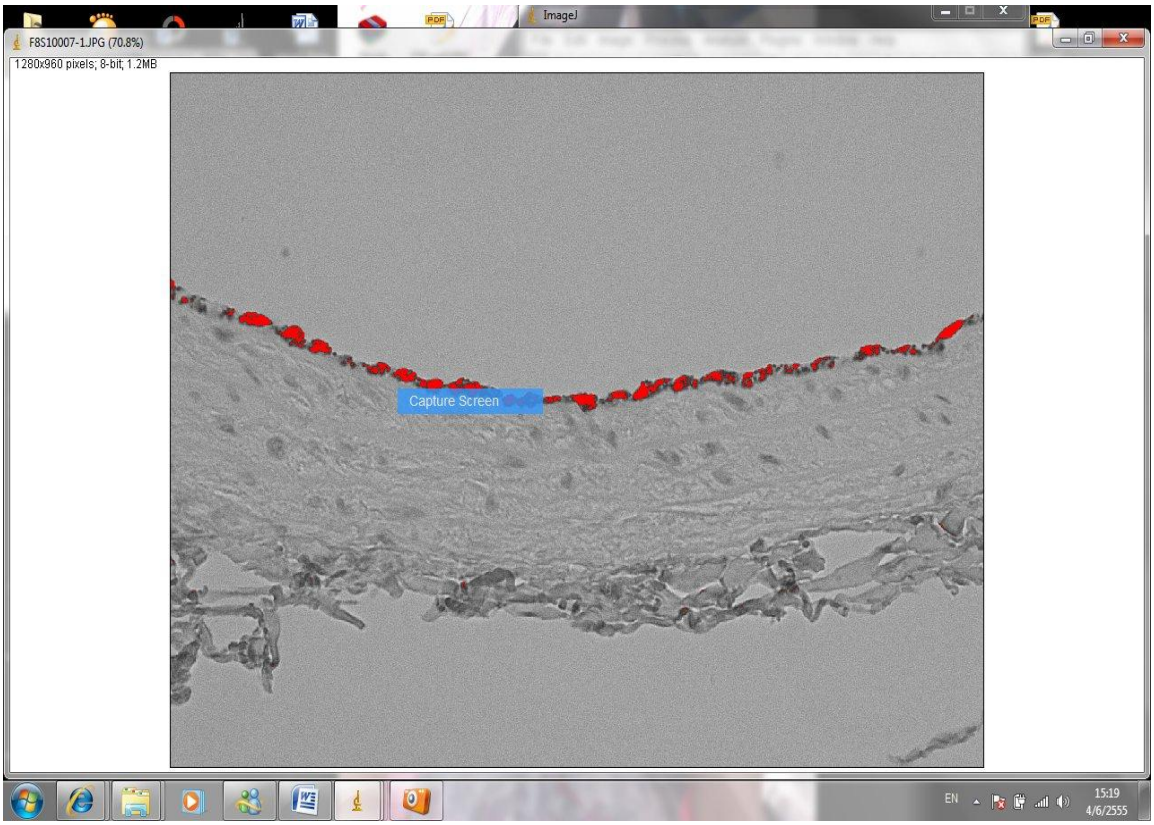


Figure 3.13 Immunohistochemical localization of **von Willebrand factor**-positive cell on the innermost surface. Determination of the number of positive cells was performed by image J program.

VII Serum concentration of Cholesterol (Chol), Triglyceride (TG), High-Density Lipoprotein Cholesterol (HDL), Low-Density Lipoprotein cholesterol (LDL) and estradiol

Blood sample were collected 2 weeks post surgery event. Plasma was separated from whole blood by centrifugation at 3500 rpm at 4˚C for 15 minutes, then it was stored at a -20˚C before being analyzed by contract laboratories (Department of laboratory, ISO 15189 Accreditation number 4006/47, Chulalongkorn hospital) according to an automatic analysis technique on a chemical analyzer enzymatic, colorimetric method (CHOD/PAP). 17β-estradiol was analyzed by contract laboratories (BRiA laboratories, ISO 15189 Accreditation number 4006/47) using chemiluminescent microparticle immunoassay (CMIA). Reference range 11- 44 pg/ml.

VIII Chemicals

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

Chemical	Company
Dimethyl sulfoxide ≥ 99.9%:C_2H_6OS	D8418, Lot No.11596, Sigma, USA
17β-estradiol purified ≥ 98% : $C_{18}H_{24}O_2$	E8875, Lot No.060M0146V, Sigma, USA
Genistein purified ≥ 98% : $C_{15}H_{10}O_5$	G6649, Lot No.10181,Sigma, USA
Normal saline	A.N.B.Laboratories, Thailand
Pentobarbiturate sodium (Nembutal ^{R})	Sanofi,Thailand
Lidocaine hydrochloride (Xylocaine ^{R})	Recipharm Monts, France
Elastica Van Gieson kit	KT 012-IFU, Diagnostic Biosystems, USA
Antibody Von Willebrand Factor , Anti-Human/Human	Code No.A0082, Lot No.A00820210510-1001 DAKO A/S, Denmark
Purified Mouse Anti-eNOS/NOS type III: Endothelial NOS (eNOS)	Material No. 610296, Lot No.02491,BD Transduction Laboratories, USA
Purified Mouse Anti-iNOS/NOS type II: Inducible NOS (iNOS)	Material No.610328, Lot No.20082,BD Transduction Laboratories, USA
Rabbit anti-ADMA(Asymmetric dimethylarginine)	Catalog No.A3886, Lot No.L11092371, US biology , USA

Statistical Analysis

All results are expressed as mean ± SEM. Data were analyzed the difference of means by one-way analysis of variance (ANOVA) followed post Hoc tests by Bonferroni multiple comparison test and independent sample T-test to compare the means of two independent samples using SPSS statistic program version 17. The statistical differences were considered at the probability level (*p*-value) of lower than 0.05.

CHAPTER IV

RESULTS

4.1 Study of vaginal cytology in the female Wistar rats to indicate estrogen deficiency

The vaginal cytology of the sham group and the ovariectomized group at baseline demonstrated predominant superficial and intermediate cells (**Fig. 4.1**) in contrast with the parabasal cell dominant profile in ovariectomized rats at 4 weeks after the operation (**Fig. 4.2**). The average count of parabasal cells among the ovariectomized group is 79.88 ± 1.70 % indicating estrogen deficiency state (**Table 4.1**). The cut-off for lack of estrogen state is parabasal cells >75% (102).

A)

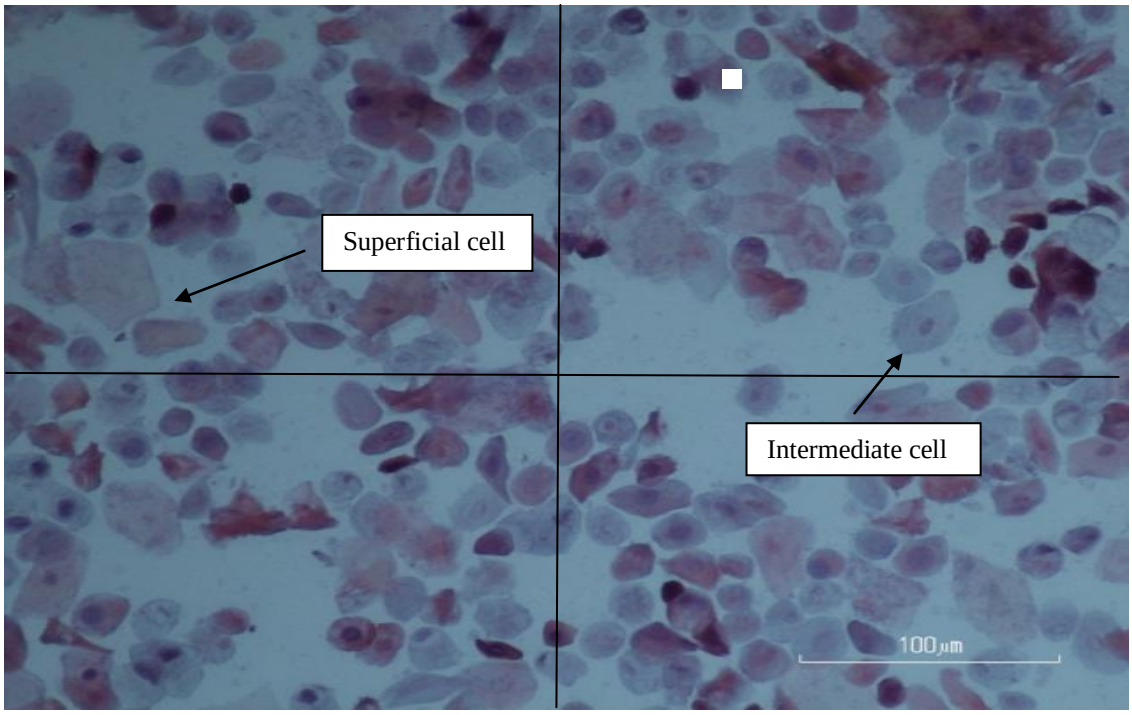


Figure 4.1. The baseline cytology showing predominant superficial cells and intermediate cells of squmaous epithelia. (Papanicolaou stain, original magnification 400 x)

B)

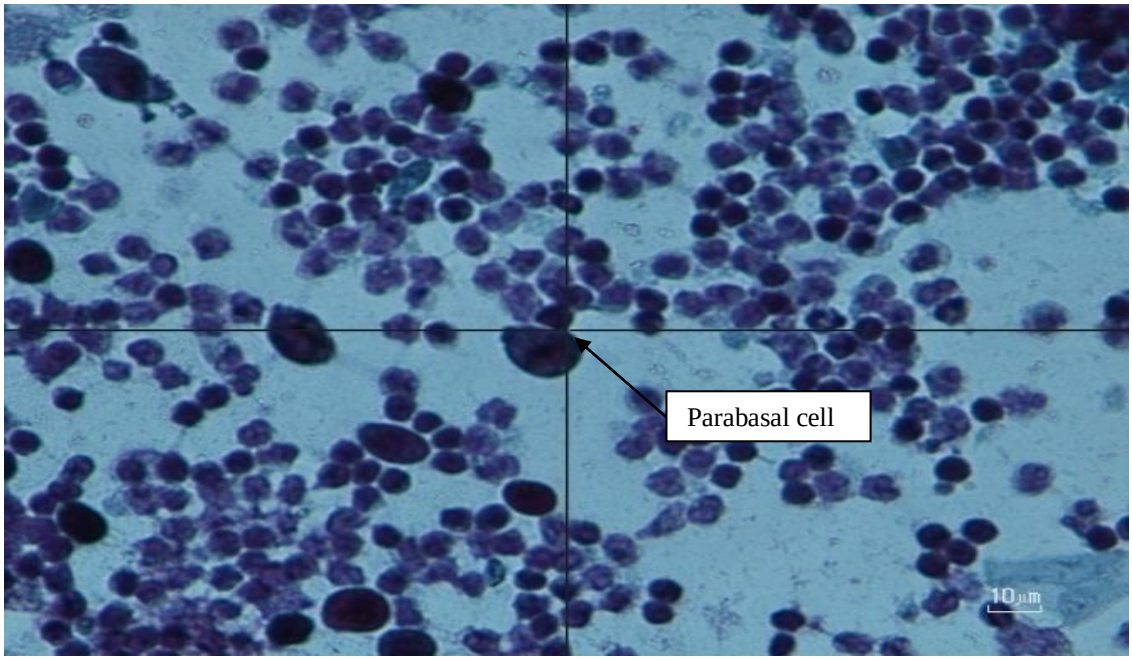


Figure 4.2. The estrogen deficit cytology in an ovariectomized rat showing predominant parabasal cells of squmaous epithelia. (Papanicolaou stain, original magnification 400 x)

Table 4.1. Percentage of parabasal cells in sham group and ovariectomized group at baseline and four weeks after operations.

Groups	Percentage of parabasal cells (%)	
	Baseline	4-week after operation
Sham (n=8)	25.00 ± 7.08	23.38 ± 3.79
OVX (n=24)	25.92 ± 3.69	79.88 ± 1.70*

4.2 Effect of ovariectomy on body weight of the female Wistar rats.

The initial and weekly follow-up body weights are demonstrated in the **Table 4.2**. The body weights were significantly increased for the ovariectomized rats starting at week 3. The effect of the ovariectomy is evident on body weight change gain, 24.84% for the ovariectomized group comparing to 6.38% for the sham group at the fourth week.

Table 4.2. Body weights of the female Wistar rats in first four weeks comparing between sham and ovariectomized (OVX) groups**.** The results are expressed as mean ± S.E.M. * (p<0.05).

Groups	**Body weight (g)**					**%body weight change**
	Baseline	**Week 1**	**Week 2**	**Week 3**	**Week 4**	
Sham (n=8)	251.00.±2.33	247.25±2.39	255.75±3.71	262.00±3.23	267.00±3.40	6.38 ±1.10
OVX (n=24)	250.20±2.81	252.91±3.46	270.63±4.23	298.67±3.99*	311.75±4.54*	24.84±1.05*

4.3 Effect of genistein on body weight after balloon injury of left carotid artery of female Wistar rats

At week 5 the ovariectomized rats had been allocated into three arms that were DMSO, genistein and estrogen subgroups. All of them were got balloon injury of the left carotid artery at week 8. To demonstrate the effect of genistein on body weight, the body weights at week 10 were recorded and compared among the four groups **(Table 4.3)**. The data show no significant difference indicating genistein did not affect body weight.

Table 4.3. Body weights of the Wistar rats at week 10 (two weeks after balloon injury of left carotid artery for the ovariectomized (OVX). The results are expressed as mean ± S.E.M.

Groups	Body weight (g)
Sham+DMSO	270.25 ± 3.69
OVX+DMSO	304.25 ± 7.79
OVX+Gen	303.25 ± 8.87
OVX+E2	286.25 ± 11.97

4.4 Effect of genistein on serum lipid profile after balloon injury of left carotid artery of female Wistar rats

The result showed that genestein did not affect changes in serum lipid profile. There was an increase in high-density lipoprotein cholesterol (HDL) for the OVX+E2 group. **(Table 4.4)**

Table 4.4. Comparison on Cholesterol (Chol), Triglyceride (TG), High-Density Lipoprotein cholesterol (HDL), Low-Density Lipoprotein cholesterol (LDL) among groups of the Wistar rats. The results are expressed as mean ± S.E.M. # (p<0.05)

Groups	Chol (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Sham+DMSO	79.4 ± 8.31	50 ± 5.06	25.8 ± 2.38	3.3 ± 0.45
OVX+DMSO	90.1 ± 4.67	31.75 ± 2.79	22.4 ± 2.74	4.3 ± 0.59
OVX+Gen	86.5 ± 3.77	36 ± 3.12	27.5 ± 1.51	4.4 ± 0.42
OVX+E2	92.1 ± 3.09	59 ± 4.97	30.1 ± 0.69#	3.5 ± 0.26

4.5 Effect of genistein on serum estradiol level after balloon injury of left carotid arteries of female Wistar rats

The results showed that genistein did not change serum estradiol level. There was statistical difference between control positive (OVX + DMSO) and control negative (Sham+DMSO) (41.00 ± 4.84 and 61.50 ± 6.98 pg/ml, respectively, p= 0.062) (**Table 4.5)**.

Table 4.5. Serum estradiol level of the Wistar rats in each group. The results are expressed as mean ± S.E.M.

Groups	Estradiol level (pg/ml)
Sham+DMSO	61.50 ± 6.98
OVX+DMSO	41.00 ± 4.84
OVX+Gen	47.25 ± 2.53
OVX+E2	44.63 ± 5.72

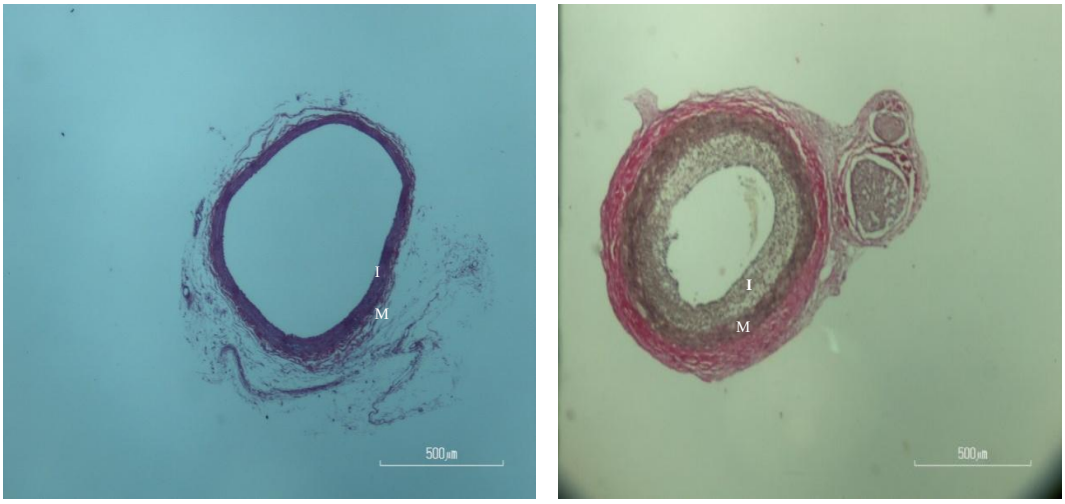
4.6 Effect of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats

The results showed that the medial area was not different among groups. The intimal area and the intima/media ratio were significantly increased in the OVX+DMSO group compared with the Sham+DMSO group (2,805.27 ± 168.15 μm^2, 1.27 ± 0.07 and 1,102.05 ± 29.76 μm^2, 0.47 ± 0 respectively; $p<0.005$). The intimal area and its ratio to media were significantly decreased in the OVX+Gen group and the OVX+E2 group as compared with the OVX+DMSO group (1,762.37 ± 282.40 μm^2, 0.76 ± 0.11 and 1,298.00 ± 152.02 μm^2, 0.58 ± 0.07 respectively; $p<0.005$). See **Table 4.6. , Fig. 4.3** (A, B, C and D) and **Fig. 4.4**.

Table 4.6. Morphometric analyses of medial area, intimal area and intima/media ratio of rats' carotid arteries in the four groups. The results are expressed as mean ± S.E.M. $^{*}p<0.005$ versus Sham+DMSO, $^{\#}p<0.005$ versus OVX+DMSO.

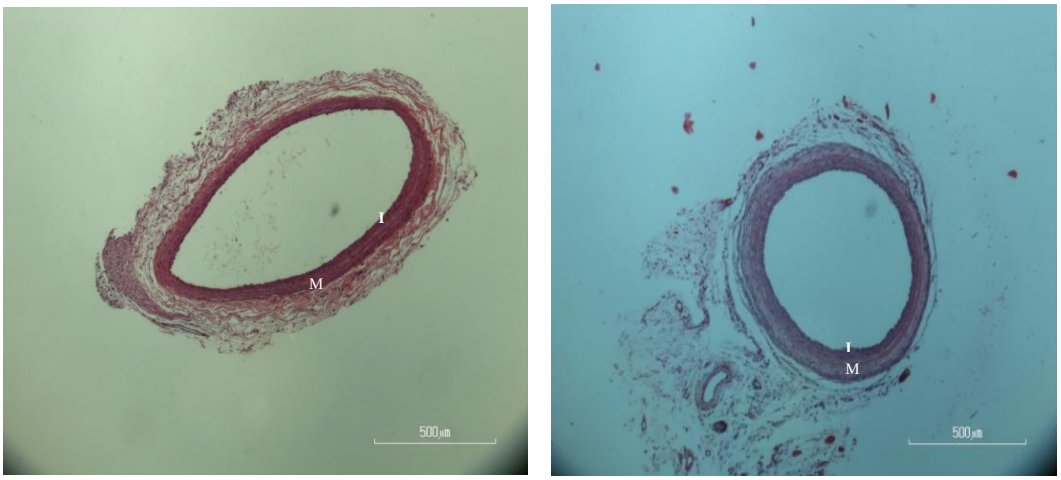
Groups	Medial area (μm^2)	Intimal area (μm^2)	Intima/media ratio
Sham+DMSO	2,340.16 ± 67.75	1,102.05 ± 29.76	0.47 ± 0.00
OVX+DMSO	2,281.27 ± 71.73	2,805.27 ± 168.15 *	1.27 ± 0.07 *
OVX+Gen	2,305.19 ± 61.87	1,762.37 ± 282.40 #	0.76 ± 0.11 #
OVX+E2	2,244.72 ± 62.98	1,298.00 ± 152.02 #	0.58 ± 0.07 #

A) B)



Sham+DMSO OVX+DMSO

C) D)



OVX+Gen OVX+E2

Figure 4.3 Representative cross-sections of the rat carotid artery at two weeks after balloon injury and sham operation. Sham +DMSO (A), OVX+DMSO (B), OVX+Gen and OVX+E2 (D). Scale Bar: 500 µm. Elastic stain, original magnification, 100 x. M indicates the layer of media, I indicates the layer of intima.

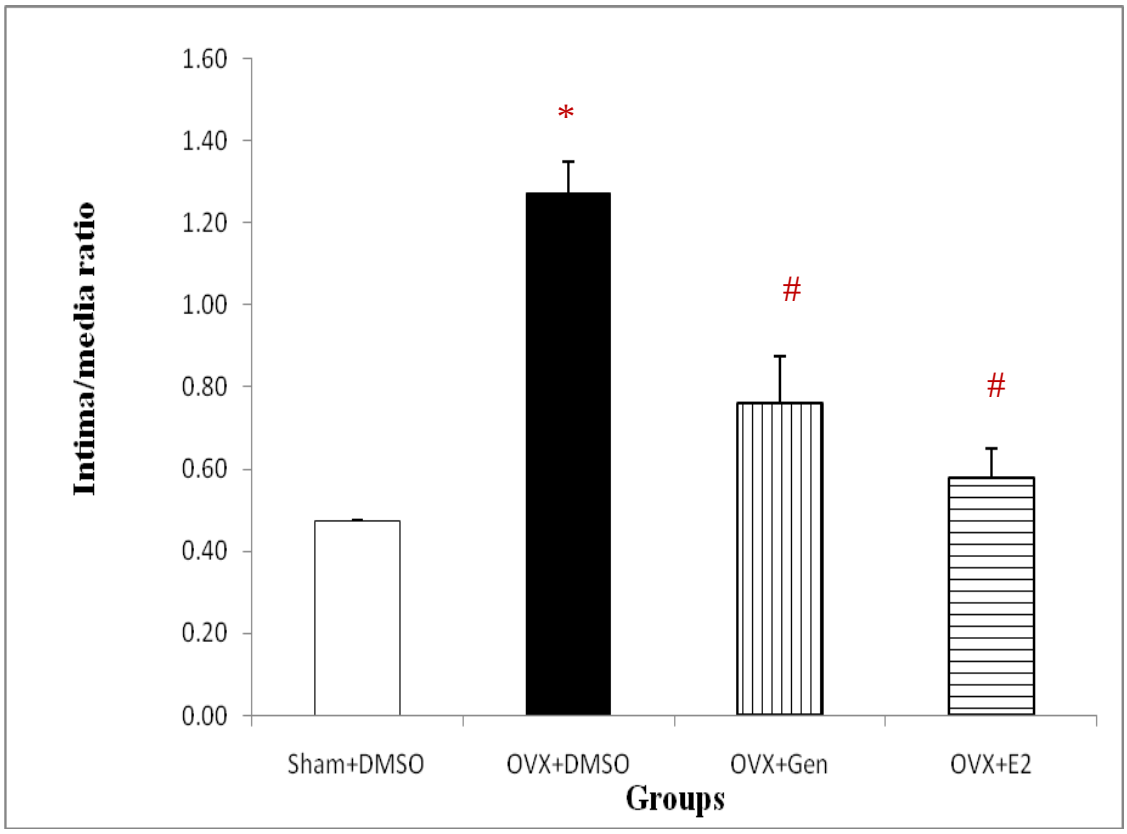


Figure 4.4. Bar graph demonstrating intima/media ratio of the carotid arteries of the four groups, $^{*}p<0.005$ versus Sham+DMSO, $^{\#}p<0.005$ versus OVX+DMSO.

4.7 Immunohistochemetry localization

The result disclosed that the number of von Willebrand factor- and endothelial nitric oxide synthase (eNOS) positive cells were not significantly different among groups (**Table 4.7**). The immunostaining for inducible nitric oxide synthase (iNOS) and asymmetric dimethylarginine (ADMA) were significantly increased in the OVX+DMSO group compared with the Sham+DMSO group (77.5 ± 7.9%, 82.5 ± 7.9% respectively; p <0.05). Meanwhile, the number of iNOS and ADMA positive cells were significantly decreased in the OVX+Gen group and the OVX+E2 group compared with the OVX+DMSO group (12.5 ± 5.2%, 10 ± 3.7% and 25 ± 5%, 18.7 ± 6.3% respectively; p <0.05). See **Table 4.8 and Fig. 4.5.**

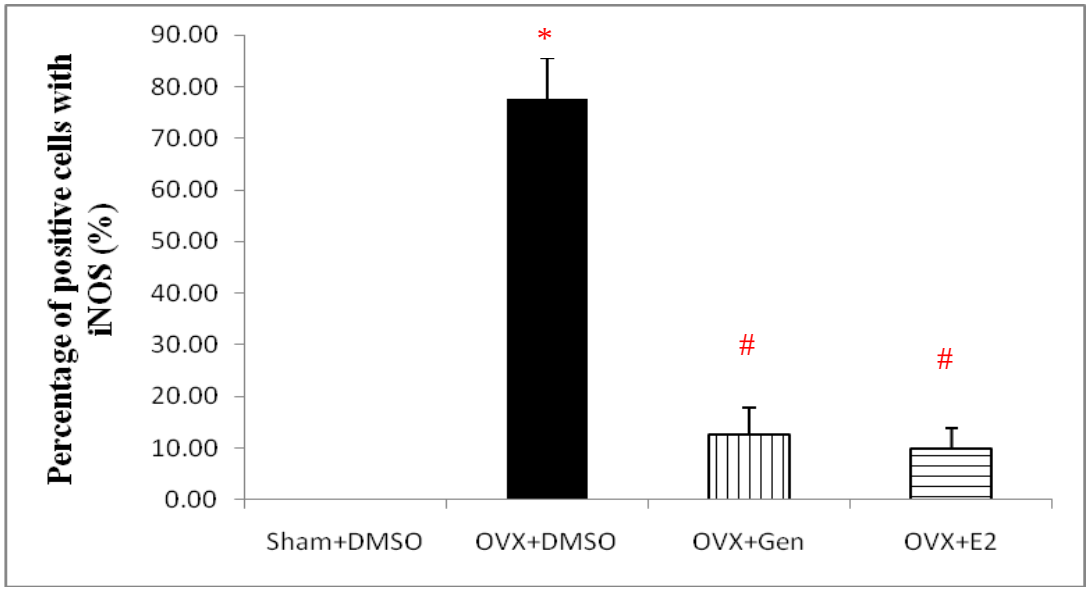
Table 4.7. Average percentages of positive cells for von Willebrand and eNOS in the innermost layer of intima of carotid arteries among the four groups.

Groups	Percentage of positive cells for von Willebrand factor (%)	Percentage of positive cells for eNOS (%)
Sham+DMSO	100 ± 0.00	100 ± 0.00
OVX+DMSO	92.5 ± 3.66	85 ± 5.98
OVX+Gen	96.25 ± 3.75	95 ± 3.27
OVX+E2	89.38 ± 4.06	85 ± 6.48

Table 4.8. Average percentages of positive cells for iNOS and ADMA within **subendothelial intimal layer of the carotid arteries among the four groups**.

Groups	Percentage of positive cells for iNOS (%)	Percentage of positive cells for ADMA (%)
Sham+DMSO	0.00 ± 0.00	8.75 ± 5.15
OVX+DMSO	77.5 ± 7.96.*	82.5 ± 7.96.*
OVX+Gen	12.5 ± 5.26#	25.00 ± 5.00#
OVX+E2	10.00 ± 3.78#	18.75 ± 6.39#

A)



(B)

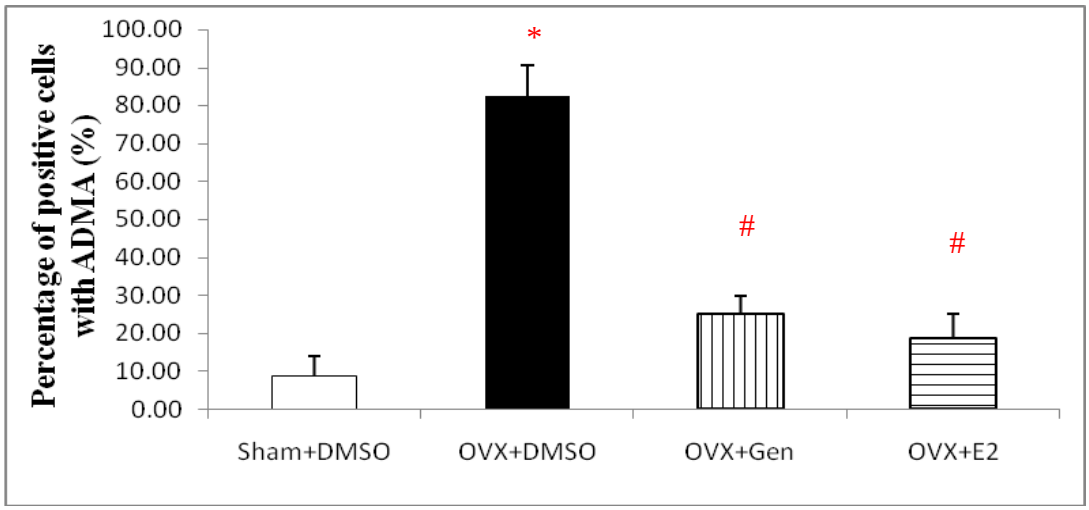


Figure 4.5. Bar graph demonstrating percentages of positive cells for **iNOS**-and **ADMA** within **subendothelial intimal layer** of the carotid arteries. The results are expressed as mean percentages ± S.E.M. (* p<0.005 versus Sham+DMSO, $^{\#}$ p<0.005 versus OVX+DMSO).

4.8 Effect of genistein on uterine weight per body weight

The results showed that genistein did not affect uterine weight and uterine weight per body weight. The rats in this group had weight and uterine weight per body weight not different from the control ovariectomized rats. Unlike genistein, the ovariectomized rats after received estradiol exhibited weight gain to the level of control negative **(Table 4.9).**

Table 4.9. Uterine weight and uterine weight per body weight of the Wistar rats in each group. The results are expressed as mean ± S.E.M. * $p<0.05$

Groups	Uterine (g)	Percentage of uterine weight per body weight (%)
Sham+DMSO	0.58 ± 0.08	0.20 ± 0.03
OVX+DMSO	0.14 ± 0.08*	0.04 ± 0.00*
OVX+Gen	0.19 ± 0.02*	0.06 ± 0.05*
OVX+E2	0.52 ± 0.02#	0.16 ± 0.09#

CHAPTER V

DISCUSSION AND CONCLUSION

This animal experiment research was conducted to investigate the effect of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats and its related mechanism. From the results, it could be discussed as follows.

5.1 Evaluation of ovariectomy

The result showed that at the start, percentage of parabasal cells in sham group and ovariectomized group were about 25%. At the week four after ovariectomy, the percentage of parabasal cells in ovariectomized group increased to an average of 79%, significantly different from the control group. On the other hand, the baseline body weights were about 250 g. At the third and fourth weeks of ovariectomy, body weight of ovariectomized rats gained significantly with an average body weight change of 25% compared with 6% of the control group.

Ovariectomy leads to estrogen deficiency which was confirmed by vaginal cytology assay. The proportion of parabasal cell to superficial and intermediate cells exceeds 75% due to lack of squamous maturation that is influenced by the estrogen hormone on vagina and ectocervix. On the other hand**,** ovariectomy caused the weight gain in rats. It is explained that ovariectomized rats had hyperphagia and increased adiposity (103). Estrogen acts to reduce food intake and increase energy expenditure. It's deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y; the mechanism may contribute to excess fat accumulation (104). Moreover, in female rats, discrete silencing of ER-α in the ventromedial hypothalamus (VMH) causes hyperphagia and reduces physical activity and thermogenesis leading to increased adiposity (105). Furthermore, the reduction in uterine weight in ovariectomized rats could be explained that loss of estrogen causes endometrium undergrowth and decreases water retention.

5.2 Effect of genistein on neointimal changes after balloon injury of carotid artery in ovariectomized rats

The results showed that the media area was not different among groups. The intimal area and the intimal/medial area ratio were significantly increased in the control ovariectomized rats group, so-called neointima existence. The intimal area and its ratio to media were significantly less in the ovariectomized rats treated by either genistein or estradiol, so-called neointima inhibition. The innermost layer cells were endothelium, verified by positive immunostaining for von Willebrand factor and endothelial nitric oxide synthase (eNOS). The subendothelial cells in the intimal layer showed different expressions of inducible nitric oxide synthase (iNOS) and

asymmetric dimethylarginine (ADMA) between the ovariectomized rats with neointima and without neointima. According to immunhistochemistry, the number of iNOS and ADMA positive cells were significantly less in the so-called neointima inhibition groups compared with the so-called neointima existence group.

Upon basic knowledge, the vascular endothelial cells function on estrogen and estrogen receptors. Estrogen induces vasodilation via increasing NOS expression (106-107). Activation of genomic ERs may cause up-regulation of eNOS (108). The acute effect of estrogen on eNOS activity and NO release is dependent on Ca^{2+}. Experiments on pressurized rat coronary arteries have shown that increase in Ca^{2+}i cause activation of eNOS with a similar slope and half – activation constant in arteries of both female and male rats. However, at $Ca^{2+}{}_i > 100$ nM, eNOS activity is higher in females than in males (107). Interestingly, E2 may promote the association of heat shock protein 90 with eNOS and thereby reduce the Ca^{2+} required for eNOS activation (109).

It is known that NO mediates endothelium-dependent vasodilation and inhibits platelet adhesion and aggregation, monocyte and leukocyte adhesion, smooth muscle cell growth, and endothelin production as a potent mitogen (110). Thus, the impaired NO production may contribute to the pathogenesis of atherosclerosis.

Recent study, Ishibahshi and colleague (71) found that von Willebrand factor- and eNOS positive cells could covered all the innermost surface of an injured vessel within 2 week after the balloon injury in ovariectomized rats. The result from their study has suggested that regeneration of endothelial cells is complete within 2 weeks after surgery. The endothelial progenitor cells were thought to have positive influence on endothelialization and vascular repair. Meanwhile, the NO-mediated relaxation and cyclic GMP production in injured carotid artery specimens were significantly decreased because the ability of regenerated endothelial cells to produce/release NO was lower.

The neointimal hyperplasia was significantly increased in OVX+DMSO group suggested that the ovariectomy may rapidly accelerate neointimal formation by the enhanced impairment of the endothelium-dependent relaxation, cyclic GMP production and accumulation of asymmetric dimethylarginine (ADMA). The endothelium- independent relaxation caused by an NO donor remained unaltered even in the OVX group (71).

Balloon injury caused neointimal hyperplasia which was associated with increased iNOS and ADMA based on immunohistochemical analysis in OVX+DMSO group.

The balloon injury induces migration/proliferation of adventitial fibroblasts as target cell for activation and migration into media and neointimal formation after

endothelial injury (50). An early inflammatory reaction which leukocytes penetrate into the arterial wall from the periadventitial tissues may play an important role in initiation of vascular injury response and ultimately, in determining the extent of neointimal formation (55). The rapid up-regulation of iNOS in vascular injury was also a characteristic feature of balloon angioplasty. Up-regulation of iNOS at the mRNA level was observed in the rat carotid artery by 24 hours post injury and it was sustained throughout 14 days (114). The appearance of inflammatory cells is associated with expression of cytokines such as IL-1β activate NF-κB is required for iNOS expression (64-65). Increasing evidence indicates that the mitogen activated protein (MAP) kinase family of protein kinases (ERK1/2, c-Jun Kinase, p38), were important modulators of pro-inflammatory cytokine-dependent expression of inducible nitric oxide synthase (iNOS) in multiple cell types (115-117). On one hand, iNOS expression in vascular smooth muscle cells may be beneficial as a compensatory mechanism for the lack of endothelial NO synthesis (118).

The authors speculate that iNOS may be protective up to the point when enzyme activity yields biologically active NO, but not in situations of excessive activity in which oxidizing or nitrating species are released. Thus, the results found that increased NOS activity does not mean an increase in bioactive NO either because NO is being inactivated via increased superoxide production or because the enzyme itself is generating superoxide instead of NO (NOS uncoupling) (118).

Increase of ADMA may be related to decreasing level of L-arginine (119-120) and also reducing of dimethylarginine dimethlaminohydrolase (DDAH) activity (121), which is a hydrolyzing enzyme of ADMA. The phenomenon is associated with decreased NO production/release from regenerated endothelial cells and neointimal formation.

Administration of genistein could inhibit neointimal changes after balloon injury of carotid artery in ovariectomized rats via reducing iNOS and ADMA. These results suggest that genistein may be accelerated the recovery of endothelial function and inhibits the intima thickening, and that similar to the antiatherogenic action of estrogen is possibly mediated in part through direct effect on endothelial cells (71). Genistein may partly act via an estrogen receptor dependent pathway. Genistein shares structural features with 17β-estradiol, particularly the phenolic ring, which confers ability to bind to estrogen receptor (61). In rat carotid artery and aorta (46-47), the mRNA of both estrogen receptor types are constitutively expressed at low levels in the smooth muscle cells. But after denudation, the expression of ER-β mRNA increases while that of ER-α remains low. Therefore, some studies used endothelial denudation as a technique to promote atherogenesis. Seven days after injury, expression of both the ER-α and ER-β were increased, but ER-β overexpressed to a much greater extent (approximately 30 times). Treatment of various subcutaneous

doses (0 to 2.5mg/kg) of either 17β-estradiol or genistein in rats resulted in protection against neointimal formation (46).

On the other hand, genistein may exhibit anti-inflammatory properties, genistein inhibits endothelial and monocyte activation (59), pro-inflammatory cytokine and chemokine expression at the local inflammation site of lesion formation (96,122). Furthermore, genistein possibly suppressed activation and subsequent adhesion of inflammatory cells (123-124).

Watanabe and others (99) have reported that the low-dose estrogen effectively prevented the neointimal hyperplasia after balloon injury of carotid artery in the ovariectomized rats. Of note, the effect of estrogen was dose-dependent and more importantly, high dose of estrogen (≥ 20 μg/kg/day of estrogen subcutaneously) have adverse effects such as uterine hyperplasia and dyslipidemia. Ishibahshi and colleague found that the enhanced impairment of NO production, which possibly results from the accumulated ADMA and lack of reendothelialization, may contribute to the acceleration of intimal hyperplasia by ovariectomy and that estrogen replacement effectively improved the intimal hyperplasia by restoring the impaired NO production through reducing endogenous NOS inhibitor and facilitating reendothelialization (71).

5.3 Effect of genistein on serum lipid profile

Genistein did not elicit changes on serum lipid profile in this study. There is evident that genistein binds with higher affinity to ER-β than to ER-α receptor (48) while only the latter are present in rat hepatocytes (125-127). This may explain inactive role of genistein on lipid profile. On contrary, administration of 0.2 μg/kg/day of 17β-estradiol was associated with increased high density lipoprotein cholesterol (HDL), may partly be explained from the binding to estrogen α receptor in hepatocytes, stimulating uptake of serum lipoproteins as well as production of coagulation factors (128-129). Another study found that the administration of ≥20 μg/kg/day of 17β-estradiol subcutaneously exhibited many adverse effects including dyslipidemia and uterine hyperplasia (99).

5.4 Effect of genistien on uterotrophic activity

Uterotrophic activity is manifested by increased uterus weigh and percentage of uterine weight to body weight. The parameters were significantly decreased in OVX+DMSO and OVX+ Gen on comparing to control negative. The activity was maintained in OVX+E2 rats. It is supported that physiological concentrations of genistein may be safe for uterine endometrium. Like the lipid profile above, the uterotrophic effect appeared to be mediated solely by ER-α which is the dominant type of receptor in uterine endometrium (119).

5.5 Evaluation of serum estradiol level

Estradiol level in OVX + DMSO was lower than Sham+DMSO (41.00 ± 4.84 and 61.50 ± 6.98 pg/ml *p*= 0.062, respectively). Administration 0.25 mg/kg /day of genistein did not elevate estradiol in circulation Like effect of estrogen, genistein could inhibit neointimal formation but did not increase uterine wieght. In this study, we did not determine FSH and LH level because limitations of measurement methods.

On the other hand, administration 0.2 μg/kg/day of 17β-estradiol in this study also did not increase the serum estradiol level (44.63 ± 5.72) and could inhibition of neointimal formation. But it is related to increased uterine weight. However, in other study, serum estradiol was correlated to the doses of estrogen administration (103).

Further study

We have demonstrated that genistein can prevent neointimal changes following balloon injury of carotid artery in ovariectomized rats. The probable mechanism is via reducing iNOS and endogenous NOS inhibitor as same as the estrogenic action. On the advantage side, genistein elicits no effects on uterine hyperplasia and lipid profile. Clinical research on the benefit of genistein is encouraging.

CONCLUSION

Genistien at a dose of 0.25 mg/kg/day via subcutaneously route for 4 weeks can prevent ovariectomized rats from neointima formation that induced by balloon injury of carotid artery. Its action imitates estrogen and is associated with reduced iNOS and ADMA expressions of the subendothelial intimal cells. Unlike estrogen, genistein does not affect serum lipid profile and uterotrophic parameters.

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APPENDIX

Genistein, synthetic

Product Numer G6649

Storage Temperature -0˚C

Product Description

Molecular Formula: C15H10O5

Molecular Weight: 270.2

CAS Number:446-72-0

Synonyms: 5, 7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one;4',5,7-Trihydroxyisoflavone

Preparation Instruction

Genistein is soluble in chloroform:methanol (1:1[v:v],10 mg/ml), yielding a clear, faint to light yellow solution. Stock solution of Genistein in DMSO (up to a concentration of 100 mM) have been prepared. Genistein is practically insoluble in cold water; slightly soluble in hot water, hot ethanol, and hot methnol and soluble in hot 80% ethanol, hot 80% methanol, hot acetone, and pyridine.

Storage/Stability

Genistein has been dissolved in DMSO and frozen in aliquots until ready for use.

Asymmetric Dimethylarginine

Catalog No.A3886

Lot No.L11092371

Control No.C11092371

Storage and stability:

Lyophyilized powder may be stored at -20˚C. Stable for 12 months at -20˚ C. Reconstitute with sterile dd$H_2$0. Aliquot to avoid repeated freezing and thawing. Store at -20˚C. Reconstituted product is stable for 12 months at -20˚C. For maximum recovery of product,centrifuge the original vial after thawing and prior to removing the cap. Further dilution can be made in assay buffer. Purified by Protein G affinity chromatography.

Form :

Supplied as a lyophilized powder from 50mM Tris, pH 7.4. Reconstitute with 10 ul sterile dH_2O

β-Estradio

Product Number : E8875

Brand : Sigma

CAS-No. : 50-28-2

Synonyms : 3,17β-Dihydroxy-1,3,5(10)-estratriene 1,3,5-Estratriene-3,17β-diol Dihydrofolliculin 17β-Estradiol

Formula : C18H24O2

Molecular Weight : 272,38 g/mol

Purified Mouse Anti-eNOS/NOS type III

Material Number	610296
Clone:	3/eNOS/NOS type III
Immunogen	Human eNOS aa 1025-1203
Isotype	Mouse IgG1
Target MW	140 kDa
Storage Buffer	Aqueous buffered solution containing BSA, glycerol, and ≤0.09% sodium azide

Preparation and storage

The monoclonal antibody was purified from tissue culture supernatant or ascites by affinity chromatography. Store undiluted at -20˚C.

Purified Mouse Anti-iNOS/NOS type II

Material Number	610328
Clone	6/iNOS/NOS type II
Immunogen	Mouse iNOS aa.961-1144
Isotype	Mouse IgG2a

Elastic Stain Kit (Modified Verhoff's) (Diagnostic Biosystems,KT 012-IFU)

Preparation of Reagents Prior to Beginning

1. Prepare working Elastic stain Solution by mixing 30 ml Hematoxylin Solution (5%) with 12 ml Ferric Chloride Solution (10%) and 12 ml Lugol's lodine Solution. Mixed solution may be used for 24 hours.

Procedure (Standard):

1. Deparaffinize section if necessary and hydrate to distilled water.
2. Place slides in working Elastic Stain Solution for 15 minutes.
3. Rinse in running tap water unit no excess stain remains on slide.
4. Dip slides in Ferric Chloride (2%) Differentiating Solution 15-20 time and rinse and rise in tap water.
5. Check slides microscopically for proper differentiation. Repeat step 4 if required.
6. Rinse in running tap water.
7. Place slides in Sodium Thiosulfate Solution (5%) for 1 minute.
8. Rinse in tap water.
9. Stain slide using Van Gieson's Solution for 2-5 minutes.
10. Rinse in two changes of 95% alcohol.
11. Dehydrate in absolute alcohol.
12. Clear, and mount in synthetic resin.

Biography

Name Miss Jirawan Mala

Birth 19th January 1982

Status Single

Qualifications Bachelor of Nursing Science with the second class honors from Mahidol University in 2005.

Honor The 90 th Anniversary of Chulalongkorn University Fund for support research

The Third prize presentation award winner at the 34th Pharmacological and therapeutic society of Thailand

Recent Abstract

Mala, J., Udayachalerm,W., Sangaunrungsirikul, S., and Sampatanukul, P. (2012). Effects of Genistein on Neointimal Changes after Balloon Injury of Carotid Artery in Ovariectomized Rats. Thai Journal of Pharmacology. 34: 54. (proceeding)

Mala, J., Udayachalerm,W., Sangaunrungsirikul, S., and Sampatanukul, P. (2012). Genistein acts like estrogen on inhibiting intimal hyperplasia from vascular injury in ovarietomized rats. Journal of Physiological and Biomedical Sciences. 25: 58. (proceeding)