EFFECTS OF GENISTEIN AND EXERCISE TRAINING ON ENDOTHELIAL DYSFUNCTION IN AGING MALE RATS

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ผลของเจนีสตีนและการออกกำลังกายต่อการสูญเสียหน้าที่ของเอนโดทีเลียม ในหนูแก่เพศผู้

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สุกัญญา เอกสกุลกล้า : ผลของเจนีสตีนและการออกกำลังกายต่อการสูญเสียหน้าที่ของเอนโดทีเลียมในหนูแก่เพศผู้. (EFFECTS OF GENISTEIN AND EXERCISE TRAINING ON ENDOTHELIAL DYSFUNCTION IN AGING MALE RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. น.พ. ประสงค์ ศิริวิริยะกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. สุทธิลักษณ์ ปทุมราช, ผศ. ดร. ดรุณวรรณ สุขสม, 120 หน้า.

การศึกษาวิจัยนี้ มีวัตถุประสงค์เพื่อศึกษาผลของเจนีสตีนและการฝึกออกกำลังกายต่อการสูญเสียหน้าที่ของเอนโดทีเลียม โดยใช้หนูแก่เพศผู้พันธุ์วิสตาร์ แบ่งออกเป็น 5 กลุ่ม ได้แก่ 1) หนูที่ได้รับตัวทำละลายเจนีสตีน, 2) หนูที่ได้รับเจนีสตีน (0.25 มก./กก./วัน, ฉีดเข้าทางใต้ผิวหนัง), 3) หนูที่ได้รับการแช่น้ำเป็นกลุ่มเปรียบเทียบกับกลุ่มออกกำลังกาย, 4) หนูที่ได้รับการออกกำลังกายโดยการว่ายน้ำ (40 นาที/วัน, 5 วัน/สัปดาห์) และ 5) หนูที่ได้รับทั้งเจนีสตีนและการออกกำลังกาย โดยทุกกลุ่มได้รับสารหรือการออกกำลังกายเป็นเวลาทั้งหมด 8 สัปดาห์ ทำการศึกษาหน้าที่ของเอนโดทีเลียมจากการตอบสนองของหลอดเลือดแดงรองที่กล้ามเนื้อครีแมสเตอร์ต่อสารอะเซทิลโคลีน (10^{-5} โมลาร์) และจากการวัดไนตริกออกไซด์โดยการใช้สาร 4,5-ไดอะมิโนฟลูออเรสซีน ไดอะซิเตท (3 ไมโครโมลาร์) ซึ่งวิเคราะห์ผลด้วยโปรแกรมอิมเมจโปรพลัส นอกจากนี้ ทำการตรวจวัดภาวะออกซิเดทีฟสเตรส ด้วยการวัดค่ามาลอนไดอัลดีไฮด์ของตับ และทำการตรวจวัดภาวะการอักเสบ ด้วยการวัดค่าทีเอ็นเอ็ฟ-แอลฟาในซีรั่ม

ผลการทดลองพบว่าในหนูแก่มีการสูญเสียหน้าที่ของเอนโดทีเลียม รวมทั้งมีการเพิ่มขึ้นของค่าความดันเลือดแดงเฉลี่ย ภาวะออกซิเดทีฟสเตรส และภาวะการอักเสบอย่างมีนัยสำคัญทางสถิติ โดยพบว่าการที่หนูแก่ได้รับเจนีสตีน การออกกำลังกาย หรือได้รับทั้งเจนีสตีนและการออกกำลังกายเป็นเวลา 8 สัปดาห์นั้นสามารถช่วยลดความดันเลือดแดงเฉลี่ย ภาวะออกซิเดทีฟสเตรส และภาวะการอักเสบ อีกทั้งยังช่วยเพิ่มการทำงานของเอนโดทีเลียมได้จากการที่เพิ่มการตอบสนองของหลอดเลือดแดงรองที่กล้ามเนื้อครีแมสเตอร์ต่อสารอะเซทิลโคลีนและมีปริมาณไนตริกออกไซด์เพิ่มมากขึ้นอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ ยังพบว่าการตอบสนองของหลอดเลือดแดงรองต่อสารอะเซทิลโคลีนมีความสัมพันธ์เชิงบวกกับค่าไนตริกออกไซด์อย่างมีนัยสำคัญทางสถิติ

ผลการศึกษานี้ชี้ให้เห็นว่าเจนีสตีนและการฝึกออกกำลังกายสามารถช่วยทำให้หน้าที่การทำงานของเอนโดทีเลียมในหนูแก่ดีขึ้นได้ โดยการลดการเกิดภาวะออกซิเดทีฟสเตรส ลดปริมาณทีอ็นเอ็ฟ-แอลฟาและเพิ่มปริมาณไนตริกออกไซด์

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต สุกัญญา เอกสกุลกล้า

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ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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SUKANYA EKSAKULKLA : EFFECTS OF GENISTEIN AND EXERCISE TRAINING ON ENDOTHELIAL DYSFUNCTION IN AGING MALE RATS. THESIS ADVISOR : ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D., THESIS CO-ADVISOR : ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., ASSIST. PROF. DAROONWAN SUKSOM, Ph.D., 120 pp.

The present study aims to investigate the effects of genistein, a potent phyto-antioxidant, and exercise training on age-induced endothelial dysfunction. Male Wistar rats (20-22-month old) were divided into five groups: 1) aged rats treated with corn oil (Aged+Veh), 2) aged rats treated with genistein (Aged+Gen, (0.25 mg/kg BW/day, s.c.)), 3) aged rats without exercise training (Aged+Without-Ex), 4) aged rats with exercise training (Aged+Ex, swimming 40 min/day, 5 days/week for 8 weeks), and 5) aged rats treated with both genistein and exercise training (Aged+Gen+Ex) group. The cremaster arterioles response to acetylcholine (Ach; 10^{-5}M, 5ml/5min) was accessed after 1-min norepinephrine preconstriction (10 μM). To determine NO bioavailability, the Krebs-Ringer buffer with 4, 5-diaminofluorescein-diacetate (3 μM DAF-2DA), and 10 μM Ach saturated with 95%N_2 and 5%CO_2 were used. Changes NO-associated fluorescent intensity along the cremaster arterioles were analyzed by the Image Pro-Plus Software. Liver malondialdehyde (MDA) level was measured by thiobarbituric acid reaction and used as an indicator for oxidative stress. TNF-α level was determined by ELISA kit.

The results showed that the mean arterial blood pressure (MAP), MDA level and TNF-α level of Aged groups (Aged+Without-Ex and Aged+Veh) were significantly increased when compared to controls. MAP, MDA level and TNF-α level of Aged-treated group (Aged+Gen, Aged+Ex and Aged+Gen+Ex groups) were significantly decreased as compared to their age-matched control groups ($P<0.05$). In these three groups of treatments, Ach-induced vasodilation after preconstriction with norepinephrine and NO-associated fluorescent intensity were significantly increased as compared to their age-matched control groups ($P<0.05$). The correlation between, Ach-induced vasodilatation and NO-associated fluorescent intensity was demonstrated and represented by a linear line: $y = 0.4276x - 2.0966$, $R^2=0.82$, ($P<0.01$).

These findings indicated that genistein and exercise training could improve age-induced endothelial dysfunction via reduced oxidative stress, reduced TNF-α and increased NO bioavailability.

Field of Study : Physiology ..... Student's Signature Sukanya Eksakulkla

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Co-Advisor's Signature .....

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CONTENTS

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

Ach	=	Acetylcholine
AMs	=	Adhesion molecules
β-AR	=	β-adrenergic receptor
BW	=	Body weight
CAD	=	Coronary artery disease
CHD	=	Coronary heart disease
COX	=	Cyclooxygenase
CRP	=	C-reactive protein
CVD	=	Cardiovascular disease
CYP 450	=	Cytochrome P450
DAF-2DA	=	4, 5-diaminofluorescein-diacetate
DAF-2T	=	Triazolofluorescein
E2	=	17β-estradiol
EDHF	=	Endothelium derived hyperpolarizing factor (s)
EETs	=	Epoxyeicosatrienoic acid
eNOS	=	Endothelial NO synthase
ER	=	Estrogen receptor
FDA	=	Food and Drug Administration
GPx	=	Glutathione peroxidase
H_2O_2	=	Hydrogen peroxide
IFN-γ	=	Interferon-gamma
IL-6	=	Interleukin-6
iNOS	=	Inducible NO synthase
MAP	=	Mean arterial blood pressure
MAPK	=	Mitogen-activated protein kinases
MDA	=	Malondialdehyde

LIST OF ABBREVIATION (Continue)

NE	=	Norepinephrine
NF-kB	=	Nuclear factor kappa B
NO	=	Nitric oxide
O_2^-	=	Superoxide
OD	=	Optical density
•OH	=	Hydroxyl radical
$ONOO^-$	=	Peroxynitrite
ORX	=	Orchidectomized
OVX	=	Ovariectomized
pg/ml	=	Picograms per milliliter
PGI_2	=	Prostacyclin
PKA	=	Protein kinase A
PTK	=	Protein tyrosine kinases
RS	=	Reactive species
ROS	=	Reactive oxygen species
RNS	=	Reactive nitrogen species
S.C.	=	Subcutaneous
SNP	=	Sodium nitroprusside
SOD	=	Superoxide dismutase
TGF-β	=	Transforming growth factor-β
TNF-α	=	Tumor necrosis factor-α
VCAM-1	=	Vascular cell adhesion molecule-1
VSMCs	=	Vascular smooth muscle cells
XO	=	Xanthine oxidase

CHAPTER I

INTRODUCTION

According to the report of United Nations (UN), it has been shown that aging population over 60 years old will increase up to about two folds from 2000 to 2050 as shown in Figure 1 (United Nations, 2002). Interestingly, it indicated that males have a considerably higher risk of cardiovascular disease (CVD) in comparison to premenopausal females. After menopause, the incidence of CVD in women increases, diminishing the gender difference in morbidity and mortality from CVD. Therefore, the significant role of sex hormones have been suggested to involve in the pathogenesis of atherosclerosis, either as independent risk factors or mediated via other risk factors, such as plasma lipids or insulin levels (Gyllenborg et al., 2001). Up to now, it is quite known that the presence of 17β-estradiol (E2) in premenopausal women attributes to be the protective agent that significantly retards the primary development of CVD through numerous cellular mechanisms (Mendelsohn and Karas, 1999).

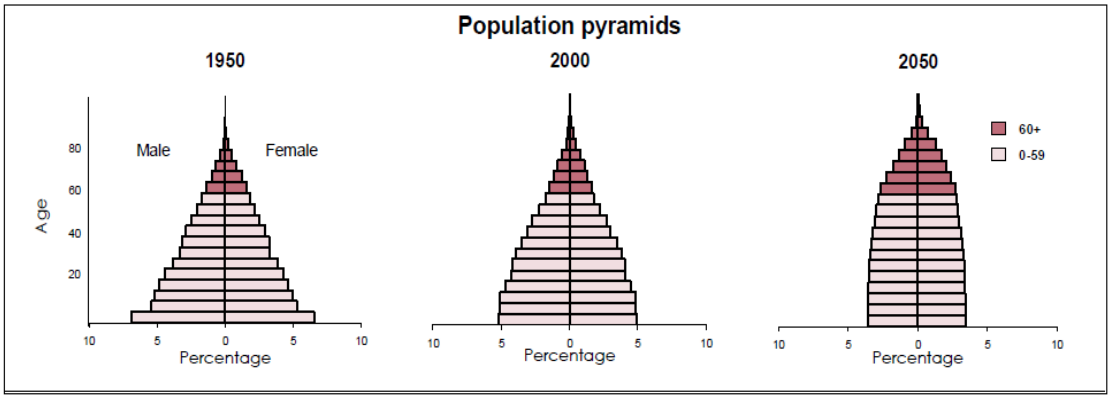


Figure 1.1 Population pyramids show world population aging 1950-2050 (United Nations, 2002)

E2 is strongly associated as a female hormone, the significance of this steroid in the male cardiovascular system is only recently emerging. Despite the lack of conclusive evidence elucidating E2's precise cardioprotective mechanisms in either men or women, endothelial dysfunction is a gender independent early contributory factor in the atherosclerotic process. Moreover, endothelial dysfunction is clinically defined as a loss of vasodilatation resulting from the partial or complete attenuation of bioavailable NO (Guetta et al., 1997). Besides, human males experiencing age-related decrement in testosterone and aromatase derived estradiol plasma levels may lose a vital cardioprotective mechanism that preserves proper endothelial function (Cho et al., 2003).

Several animal and human studies have highlighted the fact that aging is an important risk factor in cardiovascular disease (Amrani et al., 1996; Lyons et al., 1997; Singh et al., 2002; Wilson et al., 1998)**.** Aging is associated with a progressive decline in endothelium-dependent vasodilatation in resistance and conduit vessels (Egashira et al., 1993; Taddei et al., 2000). Endothelial dysfunction might have a pathogenic role in the development of atherosclerosis and its complications in the elderly. The experimental data suggested that nitric oxide and prostaglandin exhibit reduced activities with increasing age (Singh et al., 2002). Reduced basal endothelium-dependent dilatation might contribute to age-related increases in peripheral vascular resistance and systemic hypertension. Moreover, there is less nitric oxide produced by aged endothelium and that aging is as detrimental to endothelial function as clinically significant atherosclerosis (Al-Shaer et al., 2006).

Endothelium is a crucial cell that regulates vascular homeostasis through the release of a variety of autocrine and paracrine substances, such as nitric oxide (NO), prostacyclin (PGI_2), and the endothelium derived hyperpolarizing factor (s) (EDHF) (Moncada and Higgs, 1993).

The endothelium comprises a monolayer of endothelial cells and is in a strategic anatomical position within the vessel wall, located between the circulating blood and vascular smooth muscle cells of the media. Removal of the endothelium, most commonly using a balloon catheter, results in immediate deposition of platelets and white blood cells. After days to weeks intimal hyperplasia develops at the site of injury. This suggests that the endothelium also regulates vascular structure and that its presence is crucial in preventing migration and proliferation of vascular smooth muscle cells. Vascular structure is mainly determined by vascular smooth muscle cells and, in disease states, by white blood cells that invade the intima. Endothelial cells can have indirect and direct effects on vascular structure (Tanner et al., 2000).

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the ability of antioxidant systems to neutralize them. There are numerous reports of increased levels of O_2^- production with advancing age in epidemiological as well as experimental studies (Csiszar et al., 2002; Hamilton et al., 2001; Roberts and Reckelhoff, 2001; Taddei et al., 2001; van der Loo et al., 2000). Increased oxidative stress has been shown to contribute to the development of endothelial dysfunction in many forms of cardiovascular disease (Harrison, 1997). Superoxide (O_2^-) is a free radical that rapidly scavenges NO, thereby decreasing NO bioavailability (Gryglewski et al., 1986). Therefore, it is mostly documented that oxidative stress is an underlining cause of age-induced endothelial dysfunction. Thus, it is simply to say that oxidative stress should have the closed correlation with aging.

Interestingly, genistein, a phytoestrogen, may have estrogenic cardioprotective actions (Barnes, 1998) and enhances coronary vasoreactivity in macaque monkeys (Honore et al., 1997). The affinity of

genistein for the classic estrogen receptor (ER)-α present on reproductive organs is less than estrogen (Cassidy, 1999). However, genistein has a similar affinity as estrogen for the novel ER-β present in the vasculature. Genistein can be administered to both men and women without causing conventional estrogenic effects. Indeed, genistein is present in high concentrations in the East Asian diet (Adlercreutz et al., 1993), and it is possible that high plasma concentrations of genistein contribute to the strikingly low incidence of atherosclerosis and coronary heart disease (CHD) seen in East Asia (Barnes, 1998; Keys, 1970). Furthermore, phytoestrogens can act as antioxidants (Bingham et al., 1998) and may inhibit the oxidation of low-density lipoprotein (LDL) (Lissin and Cooke, 2000). A protective/antioxidant effect has been shown by phytoestrogen supplementation on the susceptibility of LDL to oxidation (Tikkanen et al., 1998). In addition, Walker et al (2001) reported that genistein produces acute NO-dependent vasodilation in the forearm vasculature of men and women with a potency similar to that 17β-estradiol and potentiates endothelium-dependent vasodilation (Walker et al., 2001).

Several studies have shown an aging-associated reduction in endothelial function in conduit and resistance arteries (Delp et al., 1995; Hongo et al., 1988; Tominaga et al., 1994). However, aerobic exercise training has been reported for its potential to ameliorate age-associated reduction in both central and peripheral cardiovascular functions (Ogawa et al., 1992). Moreover, it has been demonstrated that exercise training could reverse age-associated reductions in endothelium-dependent vasodilatation in humans (DeSouza et al., 2000; Taddei et al., 2000). There is an inverse relation between the level of physical activity and the incidence of cardiovascular diseases, and this relation persists after control for other risk factors for cardiovascular diseases (Oldridge et al., 1988; Vita and Keaney, 2000). Exercise training could improve

myocardial perfusion, coronary blood flow and increased shear stress on the surface of the endothelium (Schuler et al., 1992). Endothelial cells respond to short-term increase in shear stress by producing vasodilator compounds including prostacyclin and nitric oxide. Sustained increases in shear stress elicit an adaptive response in endothelial cells that is manifested, in part, by increased nitric oxide production (Sessa et al., 1994; Vita and Keaney, 2000). In addition, the adaptive responses of the endothelium in regular exercised subjects also benefit to coronary circulation (Hambrecht et al., 2000).

According to the above reviews, it become our interest to examine whether genistein and exercise training will be able to protect vascular function against age-induced endothelial dysfunction or not.

Research question

Can genistein and exercise training improve age-induced endothelial dysfunction in male rats?

Research objectives

1. To study the effects of genistein and exercise training on age-induced endothelial dysfunction.
2. To study the possible mechanism(s) that explains the effects of genistein and exercise on age-induced endothelial dysfunction.

Hypothesis

Genistein and exercise training can improve endothelial dysfunction in aging male rats by decreasing TNF-α and oxidative stress but increasing NO production.

CHAPTER II

LITERATURE REVIEWS

Aging, testosterone, estrogen and CVD

Aging is a physiological process that proceeds via structural and functional alterations in the vessel wall, causing an increase in the incidence of pathological conditions such as hypertension, coronary heart disease, cardiac insufficiency, and postural hypotension (Dohi et al., 1995; Gerhard et al., 1996). In addition, aging has long been accepted as a non-modifiable risk factor leading to age-related cardiovascular disease. Moreover, male gender is one of the classic risk factors for coronary artery disease (CAD), and the average life expectancy for men with CAD is about 8 years less than that of women (Wu and von Eckardstein, 2003). The lifetime risk of developing CAD at age 40 years is 50% for men and 33% for women (Lloyd-Jones et al., 1999). Moreover, Figure 2.1 demonstrated that age-adjusted mortality rates for CAD by country and sex (age 35-74 yr) (Wu and von Eckardstein, 2003).

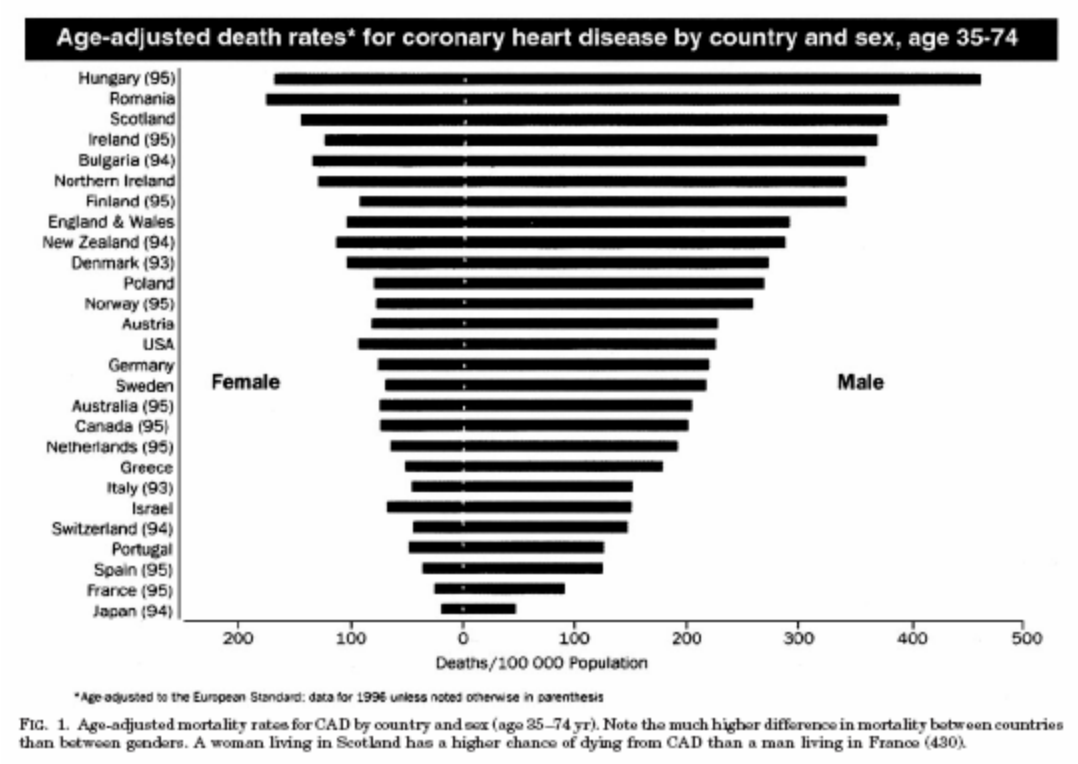


FIG. 1. Age-adjusted mortality rates for CAD by country and sex (age 35–74 yr). Note the much higher difference in mortality between countries than between genders. A woman living in Scotland has a higher chance of dying from CAD than a man living in France (430).

Figure 2.1 Age-adjusted mortality rates for CAD by country and sex (Wu and von Eckardstein, 2003)

In the 35 countries reporting mortality statistics, CVD is a persistent leading cause of death in both men and women. Independent of gender, the incidences of death and morbidity from CVD increase with advancing age but it is more prevalent in men than women before the age of 50 (Grady and Hulley, 2001). Although there is no analogous situation of complete gonadal hormone cessation in men starting at the age of 50, total serum and testosterone levels peak at age 30 and gradually decline thereafter (Lamberts et al., 1997). As shown in Figure 2.2, the decline of testosterone in men during the aging process (Cho et al., 2003). In 60% of elderly men over 65 years of age, free testosterone levels are below the normal values of men aged 30 to 35. Numerous studies of large populations of men have shown a marked rise in the incidence of CVD in

the same age group that exhibit declining free testosterone levels, with the highest incidences occurring in men aged 50 to 70 (Hak et al., 2002).

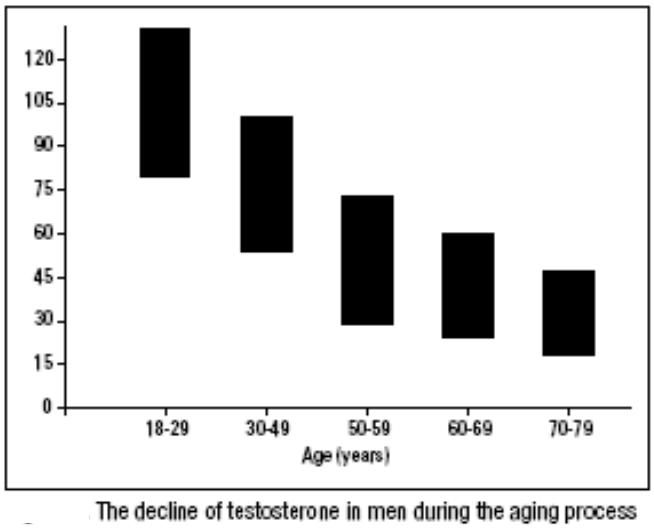


Figure 2.2 The decline of testosterone during aging process (Cho et al., 2003)

In trying to explain this causal relationship between testosterone and aortic atherosclerosis in aging men, it is proposed that age related decreases in testosterone does not directly promote CVD in elderly men but rather the loss of E2 signalling in the vasculature. Compared to menopausal women, healthy young men produce significantly higher levels of endogenous E2 (25–40 µg/24 hrs at a steady state concentration of 2–3 ng/dl) (Vermeulen et al., 2002). Because E2 in men arises primarily from the aromatase conversion of testosterone, the availability of E2 declines with increasing age as a result of low testosterone levels in most cases. In a study of 1,261 men of ages ranging from 20–75, free testosterone levels could be significantly correlated to age ($p<0.001$) while no statistically significant correlation of E2 levels to age could be determined. In 6% of these patients, low E2 levels (<10 pg/ml) were found in blood serum and 74% of this patient subset exhibited low testosterone levels whereas only isolated instances of low E2 levels were found in 1.6% of the total patient population (Derouet et al., 2000).

Therefore, circulating levels of E2 in men decline modestly as a result of age-related decline in testosterone and may significantly impact NO related events that maintain proper endothelial function.

Males generate estrogen by synthesizing it directly from testosterone in target tissues. The enzyme aromatase, a member of the P450 superfamily of enzymes, is responsible for the conversion of C_{19} androgenic steroids to the corresponding estrogens, a reaction known as aromatization, since it involves conversion of the A-ring of androgens to the corresponding phenolic A-ring characteristic of estrogens (Figure 2.3). Aromatase is widely expressed in ovary, placenta, hypothalamus, liver, muscle and endothelial cells (Bayard et al., 1995a; Bayard et al., 1995b; Harada et al., 1999; Simpson et al., 2002). Estradiol is therefore generated directly in the male vasculature, where it can activate ERs in both the cells in which it arises and in neighboring vascular smooth muscle and endothelial cells (Mendelsohn and Rosano, 2003).

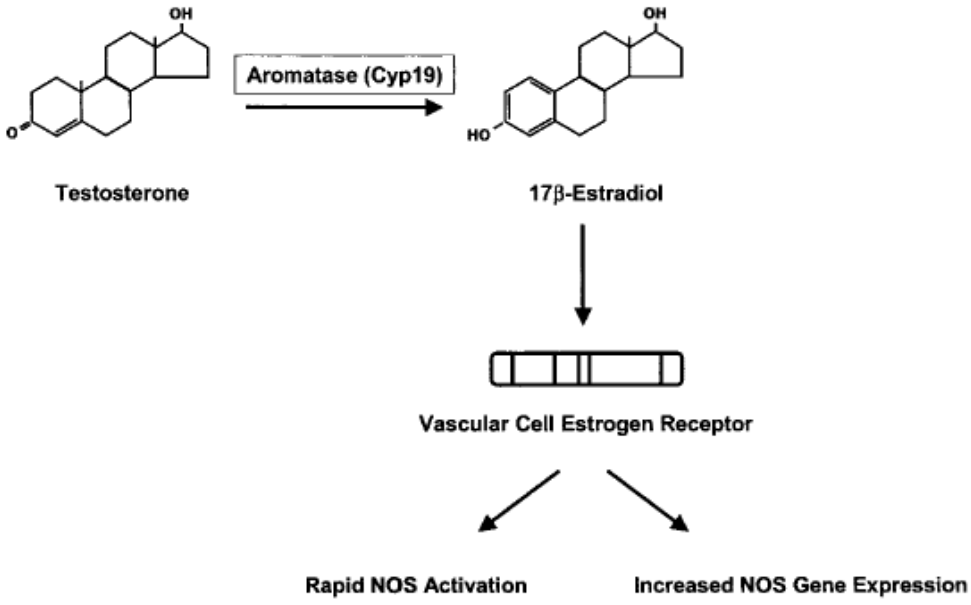


Figure 2.3 Estrogen synthesis directly from testosterone (Mendelsohn and Rosano, 2003)

Lew and colleagues report studies in healthy young men of the effect of aromatase inhibition on endothelial function. Study subjects were given anastrozole, a nonsteroidal inhibitor of aromatase used widely in the treatment of postmenopausal women with hormone receptor-positive breast cancer, to suppress local estrogen production. Anastrazole caused a significant decrease in flow-mediated brachial dilation compared with placebo in these healthy subjects (Lew et al., 2003). In addition, Kimura and coworkers present animal studies complementary to these human studies. They report that endothelium-dependent vasodilatation is significantly blunted in blood vessels from the male aromatase knockout (ArKO) mice (Kimura et al., 2003). These studies thus both support the hypothesis that aromatization of testosterone to estrogen is required for the maintenance of normal endothelial function and vascular tone in males.

In men estrogen is not solely an endocrine factor but instead is produced in a number of extragonadal sites and acts locally at these sites. These sites include the vascular endothelium, aortic smooth muscle cells, and numerous sites elsewhere in the human body. Within these sites, aromatase action can generate high levels of estradiol locally without significantly affecting circulating levels (Simpson and Davis, 2001). The following hypothesis has been postulated concerning the role of estrogen in men: Estrogen interacts with the vascular endothelium, causing an increase in NOS activity and the release of NO, which is now considered to be beneficial to the vascular system (Collins, 2001).

Aging and endothelial dysfunction

One of the major endothelial vasodilators, NO, is formed by endothelial NO synthase (eNOS) by conversion of the amino acid L-arginine the availability of which has been reported to be reduced in the

circulation of aged rats (Reckelhoff et al., 1994). NO-induced relaxation may involve different mechanisms. However, it acts primarily via activation of the soluble guanylyl cyclase in the underlying smooth muscle cells with a resulting increase in the content of cyclic GMP (Rapoport and Murad, 1983; Stoclet et al., 1998) (Figure 2.4). Thus, reduced endothelium-dependent relaxation in aged rats may be due to a decreased release of endothelium-derived NO.

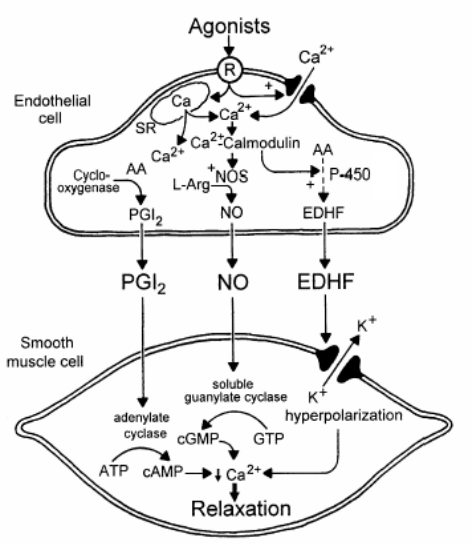


Figure 2.4 Nitric oxide pathway

Additionally, endothelium dysfunction is also a common feature of aging which leads to some alterations in these cells, resulting in reduced relaxation and increased constriction (Luscher et al., 1993). Moreover, hypercholesterolemia, diabetes mellitus, hypertension, cigarette smoking, sedentary lifestyle, and postmenopausal state, and established CAD are all associated with endothelial dysfunction (Cooke and Dzau, 1997; Taddei et al., 1996).

Relationship between endothelial cell senescence and nitric oxide

The activity of eNOS and the production of NO are diminished in senescent human endothelial cells (Sato et al., 1993). Induction of NO production by shear stress is also decreased in senescent endothelial cells (Matsushita et al., 2001). A decline in the eNOS activity of senescent endothelial cells appears to be attributable to a reduction in eNOS protein expression as well as in eNOS phosphorylation mediated by Akt (Hoffmann et al., 2001). Thus, aging down-regulates eNOS expression and activity and thus NO production in endothelial cells (Hayashi et al., 2008).

Aging and oxidative stress

Free radicals (particularly superoxide) inactivate NO, or cause direct endothelial damage. Other mechanisms for age-related endothelial dysfunction include reduced synthesis and impaired effector pathways for NO and other endothelium-derived relaxant factors (Barton et al., 1997). As shown in Figure 2.5, generation and metabolization of reactive oxygen species in the vasculature. Multiple enzymes may induce generation of superoxide (O_2^-) including NADH/NADPH oxidase, xanthine oxidase. lipoxygenase, cyclooxygenase, P-450 monooxygenase, and the enzymes of mitochondrial oxidative phosphorylation. Hydrogen peroxide (H_2O_2) is mainly formed by 3 forms of superoxide dismutase (SOD) and rapidly degraded by catalase and glutathione peroxidase. Hydroxyl radicals ($OH^{\bullet}$) are formed either by myeloperoxidase-catalyzed or by Fe^{2+} - catalyzed conversion of H_2O_2. NO produced by eNOS can react with O_2^- yielding peroxynitrite ($ONOO^-$) (Suvorava and Kojda, 2009). Therefore, oxidative stress is an important factor contributing to vascular

dysfunction with aging (Beckman and Ames, 1998; Gryglewski et al., 1986). To protect against the deleterious effects of oxiradicals and to prevent the lipid peroxidation process, both nonenzymatic and enzymatic antioxidant defense systems exits. The first one includes compounds such as α-tocopherol, ascorbate carotenoids, and the second concerns enzymes such as catalase, superoxide dismutase and the glutathione-dependent enzymatic system (Beckman and Ames, 1998). The organism possesses a delicate equilibrium between free radical production and antioxidant systems that can be altered in pathophysiological processes such as atherosclerosis and aging. Endothelial cells are vulnerable to oxidative stress due to their low antioxidant capacity and elevated metabolism of arachidonic acid (Marin and Rodriguez-Martinez, 1995).

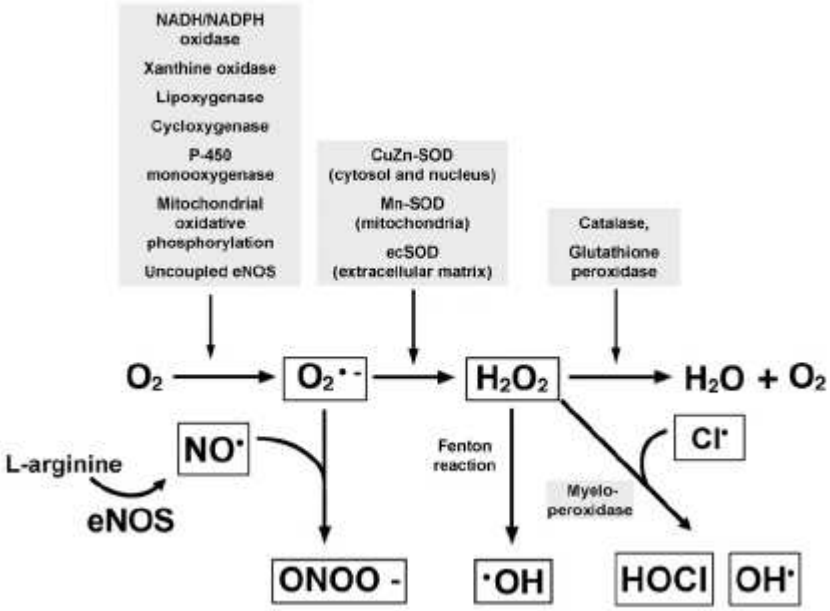


Figure 2.5 Free radicals inactivate NO (Suvorava and Kojda, 2009)

Age-related endothelial dysfunction may involve mechanisms such as alterations of antioxidant defense systems, increased oxidative injury or both. Rodriguez-Martinez et al., 1998 has recently shown that aging

enhanced the lipid peroxidation process, as indicated by an increase in the malondialdehyde (MDA) plasma levels in rats. MDA is a lipid peroxidation derivative resulting from oxidation of fatty acids such as arachidonic acid. The increase in plasma MDA levels is accompanied by an induction of lipid peroxide detoxification enzymes. The effects of MDA appeared to be mediated by superoxide anions. The changes in the blood prooxidant-antioxidant equilibrium with age contribute, at least partly, to the impairment of the relaxant response evoked by acetylcholine in the rat tail artery (Rodriguez-Martinez et al., 1998).

Thus, endothelial dysfunction is associated with both a decrease NO and, in some arteries, EDHF-related relaxation and an increase in vasoconstriction by cyclooxygenase products, mainly thromboxane A_2. The increase in oxidative stress plays a role in the deleterious effect of aging on the endothelium. This results not only in impairment of the pathways leading to the production of relaxant factors in the endothelium, but also to the destruction of the biologically active NO. Indeed, reactive oxygen species *per se* can produce vasoconstriction.

Aging and inflammatory cytokines

Aging is associated with increased inflammatory activity in the blood, including increased circulating levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, cytokine antagonists, acute-phase proteins and neopterin (Catania et al., 1997). Increased inflammatory activity in the elderly may reflect age-related pathological processes (Ross, 1999). For example, atherosclerosis is an age-related inflammatory disease reflected by secretion of cytokines such as TNF-α, IL-1, IL-6 and interferon-gamma (IFN-γ) and the presence of large numbers of

macrophages and activated $CD4^+$ T cells within inflammatory atherosclerotic plaques (Bruunsgaard et al., 2000).

TNF-α regulates NOS expression and/or activity, which exerts direct effects on NO production; for example, human aortic endothelial cells treated with TNF-α for 8 h had induced iNOS mRNA expression, but down-regulated eNOS expression (MacNaul and Hutchinson, 1993). Other studies have also shown that TNF-α significantly decreased eNOS expression in endothelial cells (Goodwin et al., 2007; Xia et al., 2006; Zhang et al., 1997). Unlike eNOS, iNOS is transcriptionally regulated and not normally produced in most cells. iNOS-derived reactive nitrogen species (RNS) initiate an $ONOO^-$ (peroxynitrite)-mediated mechanism and therefore contribute to nitrative stress and impair endothelial function.

TNF-α appears to decrease the bioavailability of NO by i) diminishing the production of NO (Ahmad et al., 2002; Goodwin et al., 2007; Greenberg et al., 1993; Picchi et al., 2006) and ii) enhancing the removal of NO (Gao et al., 2007). In addition to eNOS, other factors are also involved in regulating NO production, other factors are also involved in regulating NO production, and one of those factors is a functional citrulline/NO cycle (Goodwin et al., 2004; Hattori et al., 1994; Husson et al., 2003; Xie et al., 2000). The citrulline/NO cycle is regulated by ASS (argininosuccinate syntase). NO is synthesized from the conversion of L-arginine into L-citrulline mediated by eNOS, and ASS catalyses the rate-limiting step in the arginine regeneration through the citrulline/NO cycle and appears to be co-ordinately regulated with eNOS activity (Oyadomari et al., 2001).

Therefore, TNF-α reduces the production of NO through the inhibition of enzyme activities of ASS and eNOS, and enhances the

removal of NO through the increase in NADPH-dependent O_2^- production to react with NO to form $ONOO^-$. As a consequence, TNF-α decreases the bioavailability of NO induce relaxation of smooth muscle in the vasculature. TNF-α also diminishes epoxyeicosatrienoic acid (EETs), one of the candidate endothelium-dependent hyperpolarizing factors (EDHFs), via the inhibition of cytochrome P450 (CYP 450) enzyme activity (Figure 2.6) (Zhang et al., 2009).

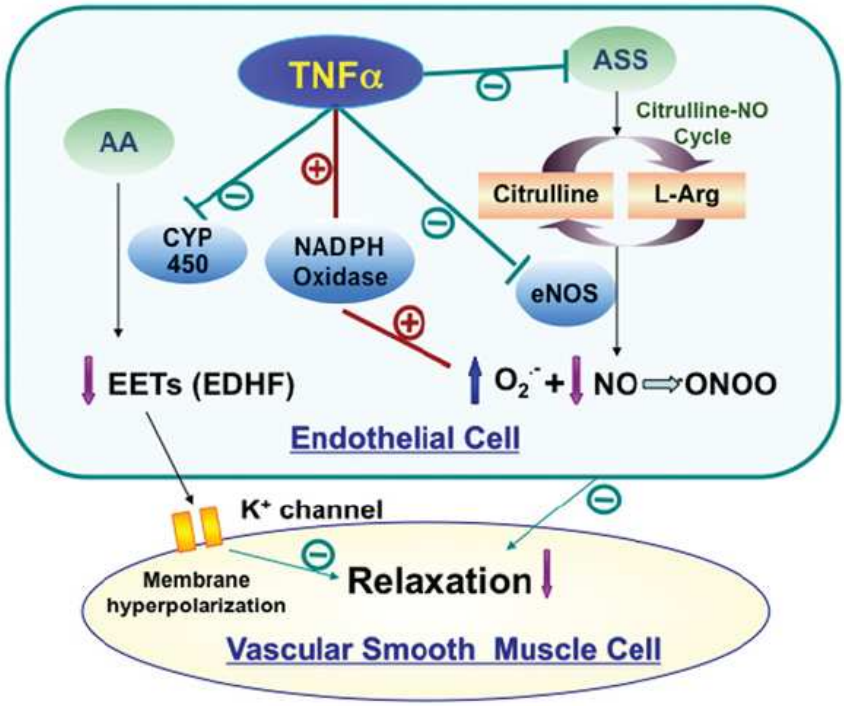


Figure 2.6 Role of TNF-α on endothelial cell (Zhang et al., 2009)

Beside, TNF-α is an early mediator of the acute-phase response and involved in the production of chemokines, IL-6 and C-reactive protein (CRP) as well as the recruitment of leukocytes during inflammatory reactions. TNF-α is also know to induce smooth muscle proliferation and to increase adherence of leukocytes to endothelial cells by inducing the expression of cell adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Furthermore, TNF-α

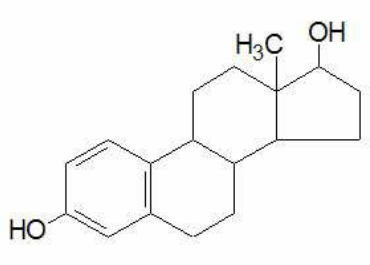
induces the expression of a wide range of cytokines, including chemokines an IL-6 by endothelial cells. TNF-α also has an important role in lipid metabolism by decreasing the activity of 7 α-hydroxylase and lipoprotein lipase and by stimulating the liver production of triglycerides (Bruunsgaard et al., 2000).

Genistein and endothelial function

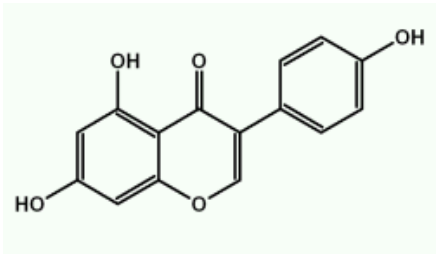
The Food and Drug Administration (FDA) has approved a health claim for soy based on clinical trials and epidemiological data indicating that high soy consumption is associated with a lower risk of CAD (1999). According to Adlercreutz (1990) (Adlercreutz, 1990) suggests that populations that consume a high phytoestrogen diet have a lower risk of cardiovascular disease and cancer. The lower incidence of cardiovascular disease in Asian countries and in vegetarians suggests that phytoestrogens may be cardioprotective. Thus, phytoestrogens have received widespread attention over the past few years because of their potential for preventing some highly prevalent chronic diseases, including cardiovascular disease (Cassidy and Griffin, 1999), osteoporosis (Setchell and Lydeking-Olsen, 2003), and hormone-related cancers (Sarkar and Li, 2002). Genistein, the primary soy-derived phytoestrogen, has various biological actions, including a weak estrogenic effect (Kim et al., 1998) and inhibition of tyrosine kinases (Nakashima et al., 1991). Studies demonstrate that genistein has antiatherogenic effects and inhibits proliferation of vascular endothelial (Fotsis et al., 1995) and smooth muscle cells (Dubey et al., 1999). Data from animal and *in vitro* studies suggest a protective role of genistein in the vasculature (Honore et al., 1997; Kapiotis et al., 1997; Karamsetty et al., 2001; Kondo et al., 2002; Nevala et al., 2002). Studies investigating its effect on plasma lipid profiles show either a moderate

positive effect (Anthony et al., 1996; Clarkson et al., 1998; Washburn et al., 1999) or a neutral effect (Hodgson et al., 1998; Nestel et al., 1997b). Some human intervention studies suggest a beneficial effect on atherosclerosis (Anthony et al., 1998), markers of cardiovascular risk (van der Schouw et al., 2000), vasomotor tone (Walker et al., 2001), vascular endothelial function (Squadrito et al., 2003), and systemic arterial compliance (Nestel et al., 1997a). Genistein also inhibits human platelet aggregation *in vitro* (Dobrydneva et al., 2002; Gottstein et al., 2003) and decreases TNFα-induced monocyte chemoattractant protein-1 secretion in human vascular endothelial cells (Gottstein et al., 2003).

Genistein is a principal isoflavone found in soy phytoestrogen, and it has structural similarity with 17β-estradiol (Figure 2.7). The key to understanding the health-protective potential of soy isoflavones may have been provided by Kuiper and colleagues, who first established the existence of a “novel” estrogen receptor, now known as estrogen receptor beta (ER-β) to distinguish it from the “classical” estrogen receptor for alpha (ER-α) (Kuiper et al., 1996; Kuiper and Gustafsson, 1997). These workers assessed the affinity of these receptors for a range of xenobiotic and phytochemical estrogenic compounds, including the soy isooflavones. The established that genistein has agonist activity for both ER-β and ER-α, but that genistein’s affinity for ER-β is considerably greater (Kuiper et al., 1997; Kuiper et al., 1998).



17β-estradiol



Genistein

Figure 2.7 Structure of 17β-estradiol and genistein

Phytoestrogen shows many anti-atherogenic activities. It decreases cholesterol concentration (Wong et al., 1998), lowers blood pressure (Lichtenstein, 1998) and increase HDL cholesterol (Lee et al., 2002). Genistein inhibits vascular smooth muscle cell proliferation (Dubey et al., 1999), improves arterial compliance (Nestel et al., 1997a; Walker et al., 2001) and it has an antioxidant action (Murkies, 1998; Yamakoshi et al., 2000). Therefore, dietary phytoestrogen may inhibit atherosclerosis through these actions. In addition, Liu et al (2004) reported that genistein had direct effects on eNOS activity in vascular endothelial cells, leading to eNOS activation and nitric oxide syntheis (Liu et al., 2004). According to, Rathel et al (2005) assessed the human endothelial cell line EA.hy926, which is a well-accepted human *in vitro* model, treated with genistein. They demonstrated that genistein could enhance NO production from endothelial cells after long-term administration *in vitro* (Rathel et al., 2005).

Moreover, there has been well documented that menopause and subsequent estrogen deprivation increase the risk of CVD in women. Before the menopause, women are at a decreased risk for CVD as compared to men, with men having between 3.5 and 4.5 times the risk over women. Within 10 years, following menopause, the risk of CVD in women increases to a level similar to that seen in men (Barrett-Connor,

1994; Kafonek, 1994). Khemapech et al (2003) reported that genistein supplementation 0.25 mg/kgBW/day was also able to restore Ach-induced endothelium-dependent vasodilatation in ovariectomized Wistar rat (Khemapech et al., 2003). In Table 2.1, lists of experiments indicate the effects of genistein supplementation on endothelial function.

Table 2.1 Representative animal studies examining the effects of genistein on endothelial function.

Author (s)	Animal model	Treatment genistein (mg/kgBW/day)	Treatment duration	Result
Squadrito et al. 2000 (Squadrito et al., 2000)	OVX Sprague-Dawleys rats	0.20, sc	4 wks	Prevent endothelial dysfunction
Khemapech et al. 2003 (Khemapech et al., 2003)	OVX Wistar rats	0.25, sc	4 wks	Prevent endothelial dysfunction
Molsiri et al. 2004 (Molsiri et al., 2004)	OVX Wistar rats	0.25, sc	10 wks	Prevent coronary endothelial dysfunction
Tang et al. 2005 (Tang et al., 2005)	OVX Sprague-Dawleys rats	0.50, sc	6 wks	Induced NO metabolic production

Genistein and antioxidant

Vina et al. (2006) suggested that genistein increased the expression of MnSOD and this effect was abolished when cells were incubated with genistein and an inhibitor of the MAP kinase pathway. They concluded that the pathway through which genistein acts to increase antioxidant capacity in cells is via interaction with estrogen receptor(s)-activation of MAP kinase activation and nuclear translocation of NFkB-over-expression of MnSOD and lowering of the intracellular levels of oxidants (Vina et al., 2006).

In addition, there is evidence that estrogen plays an important role in skeletal tissue in males as well as females. Wu et al. (2003) examined whether both interventions (exercise and genistein administration) exhibit cooperative effects on bone loss in androgen-deficient mice similar to those in estrogen-deficient mice. They demonstrated that the combined intervention of moderate exercise and genistein administration shows an additive effect in preventing bone loss in orchidectomized (ORX) mice similar to that in ovariectomized mice (Wu et al., 2003).

Exercise and endothelial function

Aging is linked with dysfunction of endothelium - the inner lining of the body's blood vessels, which plays a major role in maintaining a healthy circulation, and exercise is known to improve endothelial function. A lack of exercise (sedentary lifestyle) generally is considered a risk factor for atherosclerosis independent of its negative effects on body weight, blood pressure, and serum lipid values (Glasser et al., 1996). So important has physical activity and exercise come to be regarded in maintaining cardiovascular integrity that the American Heart Association has issued a position statement on its benefits (Fletcher et al., 1996). The statement affirms that physical inactivity is a recognized risk factor for

coronary artery disease and has been related to increased cardiovascular mortality. Hornig et al., 1996, has pointed out that chronic immobilization or lack of adequate physical activity, whether by choice or as a result of disease, may be associated with reduced expression of NO synthase and thereby decreased synthesis of NO (Hornig et al., 1996).

Several animal and human studies have demonstrated reduced NO activity with aging (Dohi et al., 1995; Dohi et al., 1990; Matsushita et al., 2001; Taddei et al., 1997). Basal release of endothelium-derived NO decreased with age in *in vitro* studies (Tschudi et al., 1996). Aging is also associated with reduced endothelium-dependent relaxation in response to vasoactive substances such as acetylcholine, histamine or adenosine (Dohi et al., 1995). Tanabe et al. (2003) showed that the expression of eNOS mRNA and eNOS protein in the aorta was markedly lower in the sedentary-aged group compared with the sedentary-young group, suggesting that eNOS production in the aorta is decreased by aging (Tanabe et al., 2003). This is in concordance with data from other groups indicating that NO activity decreases with age (Dohi et al., 1995; Dohi et al., 1990; Matsushita et al., 2001; Taddei et al., 1997). Therefore, it is considered that the aging-induced reduction of eNOS production may cause loss of endothelial function.

Because aging has been reported to produce impairment in endothelial function, and exercise training has been reported to produce an alteration in the function of vascular endothelial cells. Recent studies indicated that, in response to exercise training, an alternation in the function of vascular endothelial and smooth muscle cells occurs (Delp et al., 1993; Edwards et al., 1985; Rogers et al., 1991; Wang et al., 1993). In addition, exercise training has been reported to produce an alteration in the function of vascular endothelial cells (Delp et al., 1993). In previous study, it has been reported that the plasma nitrite/nitrate (NOx), the stable

end-product of NO, concentration significantly increased after exercise training for 8 weeks in healthy young humans (Maeda et al., 2001). In an animal study, Sessa et al. (1994) reported that exercise training increased the expression of NOS mRNA in young dog aorta (Sessa et al., 1994). Furthermore, Delp and Laughlin (1997) demonstrated that, in the aorta of young rats, the expression of NOS protein increased due to exercise training (Delp and Laughlin, 1997). Tanabe et al. (2003) demonstrated that, in the aorta of aged rats, the production of eNOS was increased by exercise training. Therefore, they suggested that swimming training (5 day/wk, 90 min/day, 8 wk) in aged rats may improve endothelial function in part through upregulation of the NO system (Tanabe et al., 2003). Moreover, Leosco et al. (2003) showed that age impairs β-adrenergic receptor (β-AR) vasorelaxation in rat carotid arteries through β-AR downregulation and desensitization. But, swimming exercise (5 day/wk, 40 min/day, 6 wk) restores this response and reverts age-related modification in β-ARs (Leosco et al., 2003). Furthermore, Hambrecht et al. (2003) indicated that exercise training improves endothelial function *in vivo* by up-regulating eNOS protein and by increased phosphorylation of this enzyme (Hambrecht et al., 2003). In addition, regular exercise simultaneously induces the up-regulation not only of eNOS but also of superoxide dismutase (SOD) expression (Fukai et al., 2000).

Effect of exercise on cytokines

Moreover, Keller et al (2004) found that exercise is likely to suppress TNF-α also via IL-6-independent pathways, as demonstrated by the finding of a modest decrease of TNF-α after exercise in IL-6 knockout mice (Keller et al., 2004). In addition, chronic exercise appears to suppress pro-inflammatory factors, such as TNF-α, CPR and IL-6 and

augment anti-inflammatory factors, including IL-4, IL-10, transforming growth factor-β (TGF-β) and adiponectin, even though these results showed discrepancies according to the modes, intensity and time duration of exercise (Bruunsgaard, 2005; Das, 2004).

Depend upon these beneficial effects of genistein and exercise training, therefore, it brings to our interest to examine whether genistein and exercise training can improve age-induced endothelial dysfunction.

The Figure 2.8 shown diagram that indicated the conceptual framework of this study.

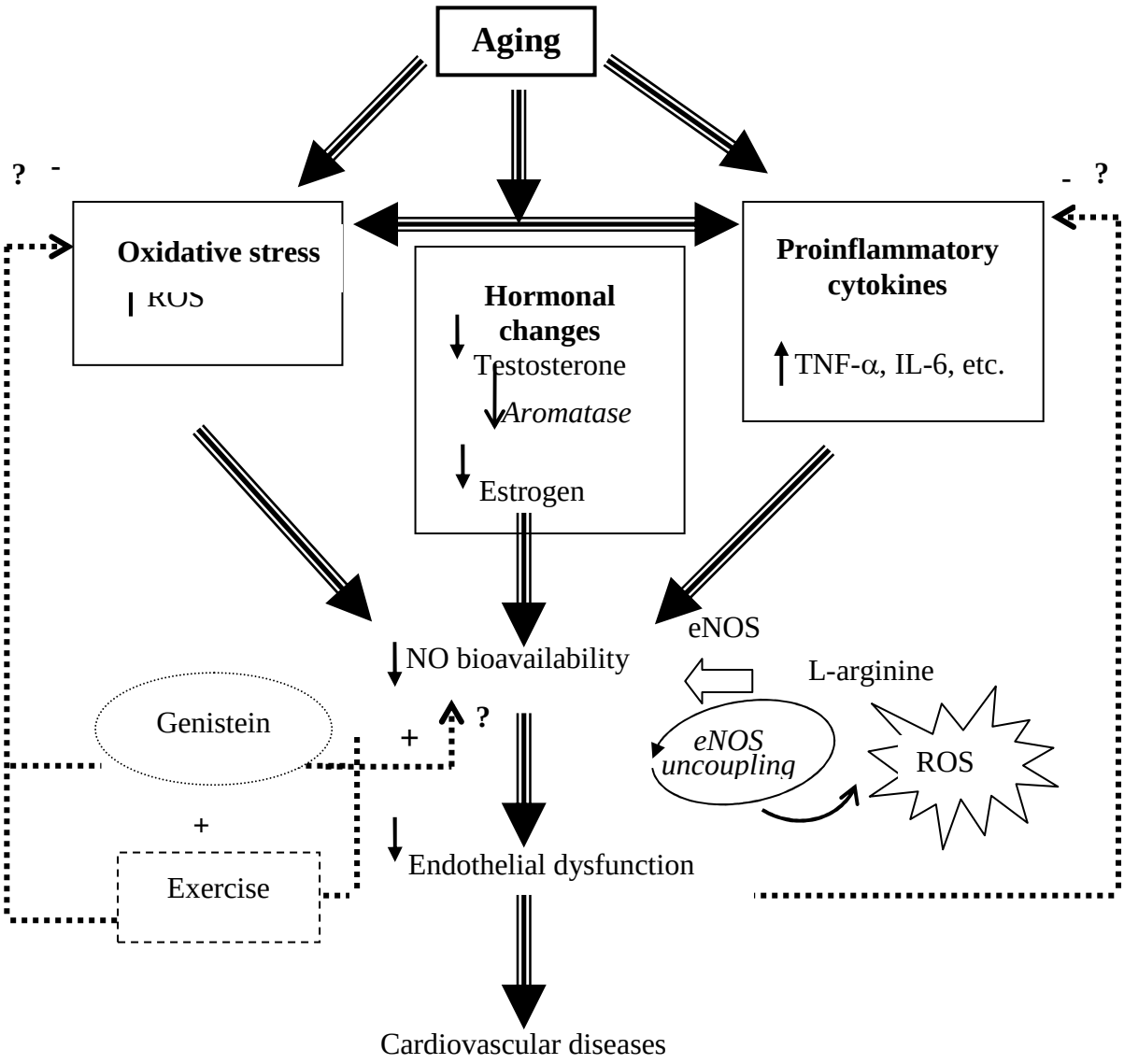


Figure 2.8 The conceptual framework showed that aging involved three main parts which were composed of hormonal, oxidative stress and immune system. All of them decline NO bioavailability and resulting in endothelial dysfunction and cardiovascular diseases.

CHAPTER III

MATERIALS AND METHODS

Animal preparation

The present study was conducted in accordance with the guidelines for animal experimentation of the National Research Council of Thailand and approved by Ethical Committee, Faculty of Medicine, Chulalongkorn University.

In this study, male Wistar rats were separated into young (4-6 months), adult (12-15 months) and aged (20-22 months). These animals which age 2 months were obtained from National Laboratory Animal Center, Salaya Campus, Nakhonprathom, Thailand, and housed in the animal laboratory center at Faculty of Medicine, Chulalongkorn University until used. The rats were fed standard chow and drank tap water *ad libitum* under the controlled temperature, 25°C, and a 12:12-light-dark cycle until them age 4-6 months, 12-15 months and 20-22 months.

The adult rats were randomly divided into five groups: adult rats treated with corn oil (Adult+Veh, (n=6)), adult rats treated with genistein (Adult+Gen, (n=6); 0.25 mg/kg BW/day, s.c.) (Khemapech et al., 2003), adult rats without exercise training (Adult+Without-Ex, (n=6)), adult rats with exercise training (Adult+Ex, (n=6)), and adult rat treated with combination of genistein and exercise training (Adult+Gen+Ex, (n=6)).

In the same manner as adult rats, the old rats were randomly divided into five groups: aged rats treated with corn oil (Aged+Veh, (n=6)), aged rats treated with genistein (Aged+Gen, (n=6); 0.25 mg/kg BW/day, s.c.), aged rats without exercise training (Aged+Without-Ex, (n=6)), aged rats with exercise training (Aged+Ex, (n=6)), and aged rat

treated with combination of genistein and exercise training (Aged+Gen+Ex, (n=6)).

Genistein supplementation

In control group, the rat were subcutaneously treated with vehicle (corn oil 100 μl/day; Sigma-Aldrich Co., USA). The genistein group was subcutaneously treated with 0.25 mg/kgBW/day of genistein (Sigma-Aldrich Co., USA) for 8 weeks. This study using the same dose of genistein that can prevent endothelial dysfunction in ovariectomized Wistar rats (Khemapech et al., 2003; Molsiri et al., 2004).

Exercise training protocol

In exercise groups (Adult+Ex and Aged+Ex), the swimming exercise training protocol was conducted in 2 phases, adaptation and training. The adaptation phase consisted of the first 5 days of training. On the first day, the animals exercised in the round plastic tank (diameter= 38.5 cm, depth= 35 cm, water temperature about 34-36ºC) for 10 minutes. The exercise period was extended by 10 minutes each day until the rats were swimming for 40 minutes. The training phase consisted of 40 min/day, 5 days/week for 8 weeks (Leosco et al., 2003). Swimming exercise has been used in this study because it has the advantage of not causing foot injuries, and causes less physically traumatic to the animal. In Adult+Without-Ex and Aged+Without-Ex groups, the rat were immersed in water as to make them wet for 30 min/day, 5 day/week for 8 weeks (Leosco et al., 2003). The rat were performed to swim or immerse at the same time through experimental study.

Experimental protocol

On week 8, all rats were anesthetized intraperitoneally with 50 mg/kg BW pentobarbital sodium. After a tracheotomy, a polyethylene tube was inserted into the carotid artery to measure arterial blood pressure. The jugular vein was cannulated for fluorescence tracer.

The present study was composed of two experimental protocols:

Experimental protocol 1: To study the effect of age on endothelial function, testosterone, oxidative stress and proinflammatory cytokine (Figure 3.1).

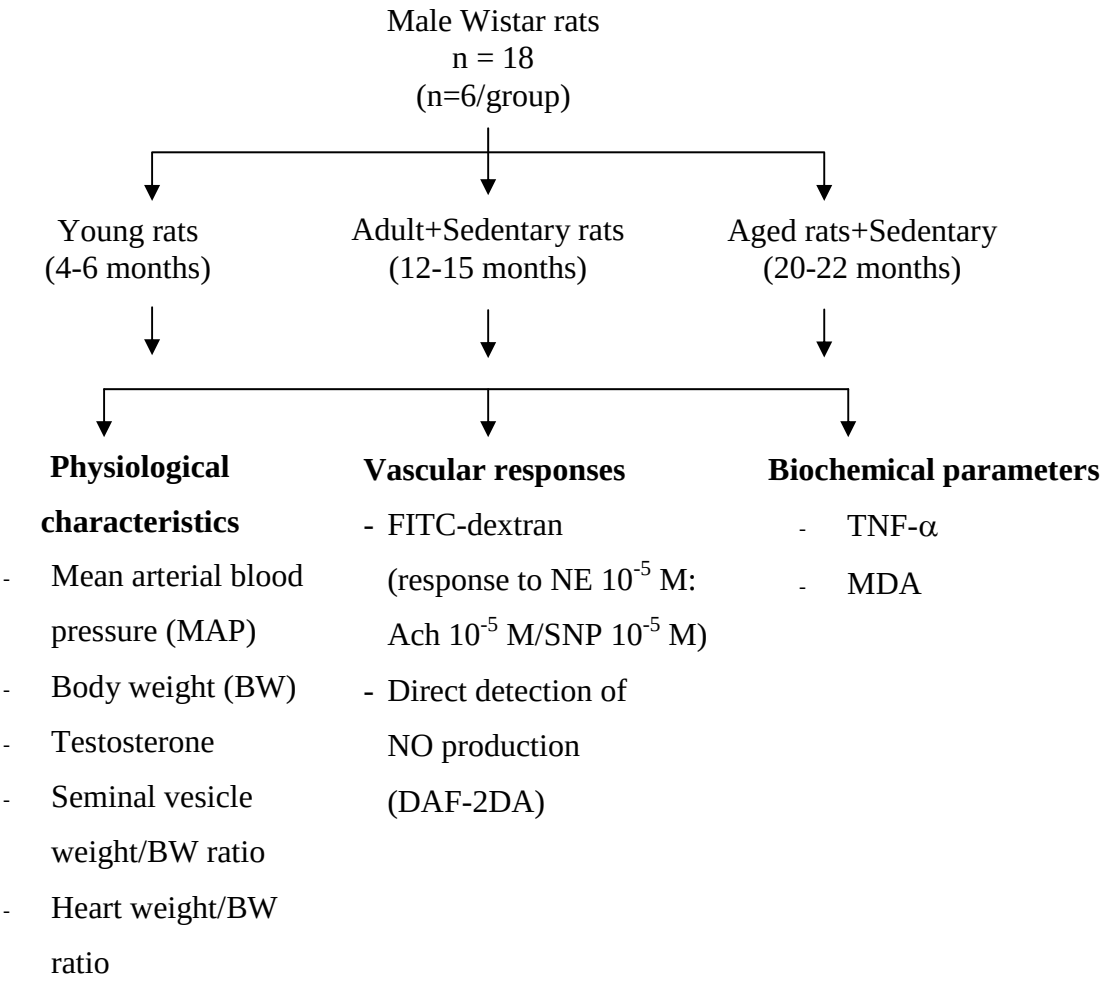


Figure 3.1 The experimental design was conducted in order to study effect of age on endothelial function, testosterone, oxidative stress and TNF-α

Experimental protocol 2: To study the effects of genistein and exercise training on age-induced endothelial dysfunction, testosterone, oxidative stress and proinflammatory cytokine (Figure 3.2).

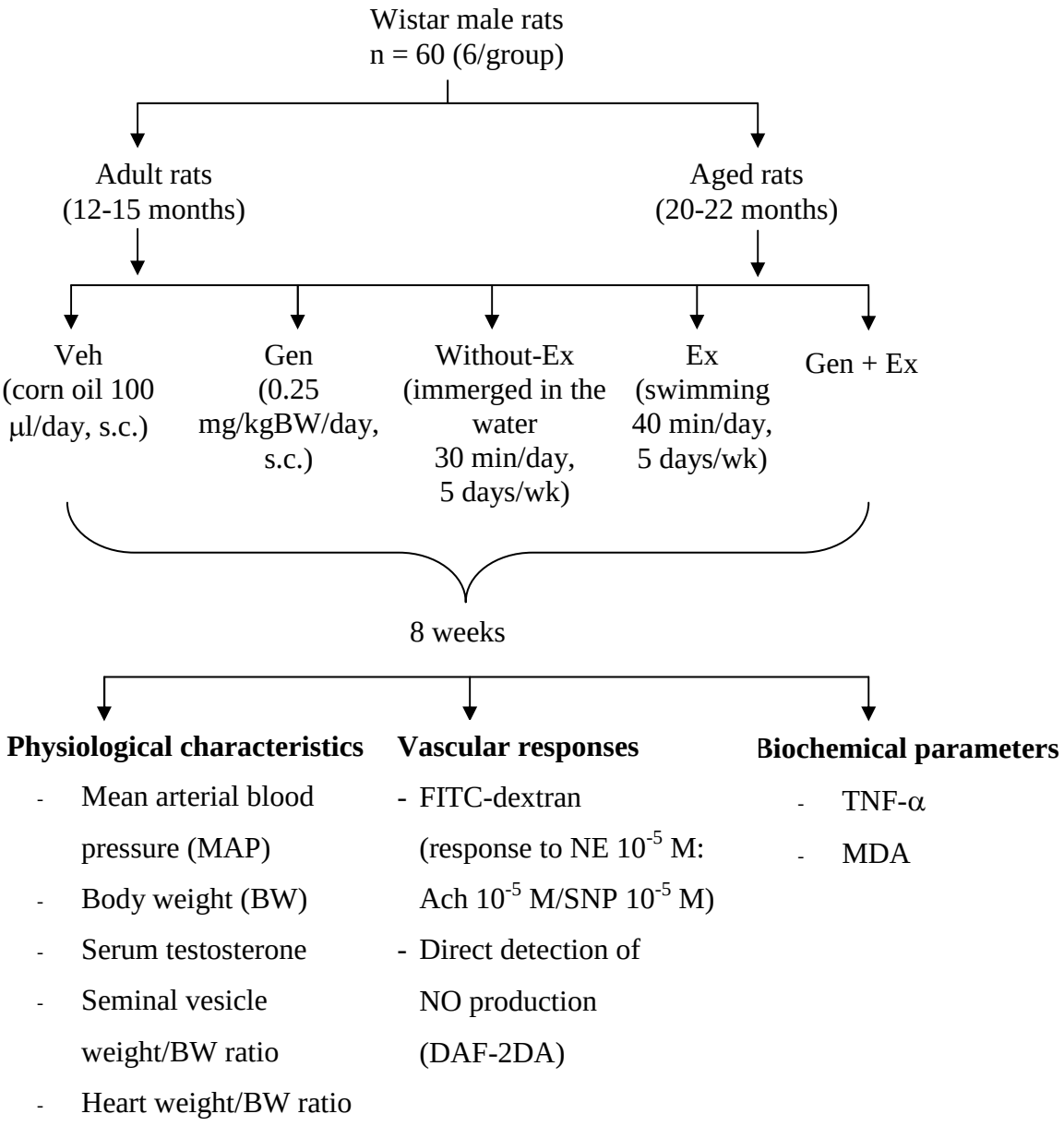


Figure 3.2 The experimental design was conducted in order to study effects of genistein and exercise training on age-induced endothelial dysfunction, testosterone, oxidative stress and TNF-α

These experimental protocols were separately performed as followed:

Physiological characteristics

In the present study, the physiological characteristics were determined including: mean arterial blood pressure (MAP), body weight (BW), heart weight/BW ratio and seminal vesicle weight/BW ratio. Arterial pressure was measured via a canular inserted into the carotid artery by using Polygraph system (Nikon Koden, Japan). Arterial pressure was reported in terms of mean arterial blood pressure (MAP) calculated by MAP = 1/3 (systolic blood pressure + 2diastolic blood pressure).

At the end of experiment, the arterial blood was withdrawn from abdominal aorta. Testosterone level was determined using ECL method by Bangkok RIA lab Co, Ltd.

Study of cremasteric microcirculation

In vivo microcirculatory observations were performed in the cremaster muscle according to the methods described by Gavins et al. (2004). Briefly, the cremaster muscle was carefully spread over a chamber that was continuously perfused with 37°C Krebs–Ringer buffer (composition in mmol/L: 135.7 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.18 KH_2PO_4, 1.64 $MgSO_4.7H_2O$, and 7.14 $NaHCO_3$) at pH 7.4 and equilibrated with 5%CO_2-95%N_2. The rate of perfusion was kept constant at 2 ml⁄min (Gavins and Chatterjee, 2004). The study of microcirculation comprised of arteriolar response to vasodilators (acetylcholine and sodium nitroprusside) and direct detection of NO production.

Arteriolar response to acetylcholine and sodium nitroprusside

The second or third-order cremasteric arterioles (15 to 35 μm in diameter) were labeled with 5% fluorescein isothiocyanate-labeled dextran (FITC-dextran 250, 5 μg/ml; Sigma-Aldrich Co., USA) which was injected into the jugular vein. After the arterioles were pre-constricted with norepinephrine (10^{-5}M NE; 0.1 ml/min), it was later dilated by applying acetylcholine (10^{-5}M Ach; 5 ml/5 min) topically (Figure 3.3). Under the same protocol, after the arteriole was selected washed by Krebs–Ringer solution (pH 7.4) until its diameter was return to normal. The sodium nitroprusside (SNP: 10^{-5}M; 5 ml/5 min), an endothelium-independent vasodilator, was applied topically on the arterioles after pre-constricted with NE (Figure 3.4).

The changes of vascular diameters were recorded real time throughout the experiment with a black and white video monitor (Sony, GM-1411 QM) and an epi-illumination fluorescence videomicroscopy system (Optiphot 2, Nikon, Japan) equipped with a 100 W mercury lamp, real time CCD camera (Hamamatsu C2400, Japan), a video recorder (VC-S5, Sharp, Japan) with a video timer (VTG-33, For-A, Japan) and a 20x objective lens (CF Plan Fluor, Nikon, Japan). Cremasteric arteriolar diameter was measured by using the software (Image-Pro Plus; Media Cybernatics, Inc, USA). The arteriolar diameter was calculated by averaging three measurements obtained from three different video frames using the same reference point as a marker for measuring each vessel in each frame (Figure 3.5; Equation 1). Arteriolar diameters were measured for five minutes after Ach or SNP administration. Vasodilatation responses were expressed as the percentage of maximal relaxation after norepinephrine (NE; 10^{-5}M) preconstriction (Equation 2).

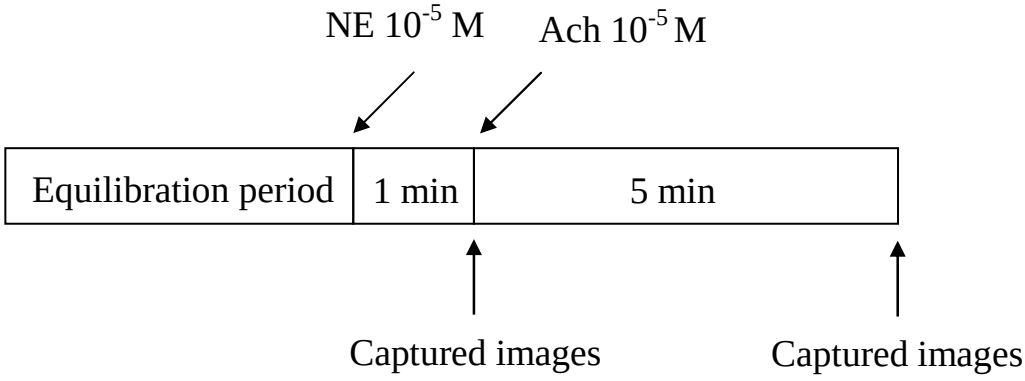


Figure 3.3 Schematic diagram for assessment arteriolar response to Ach

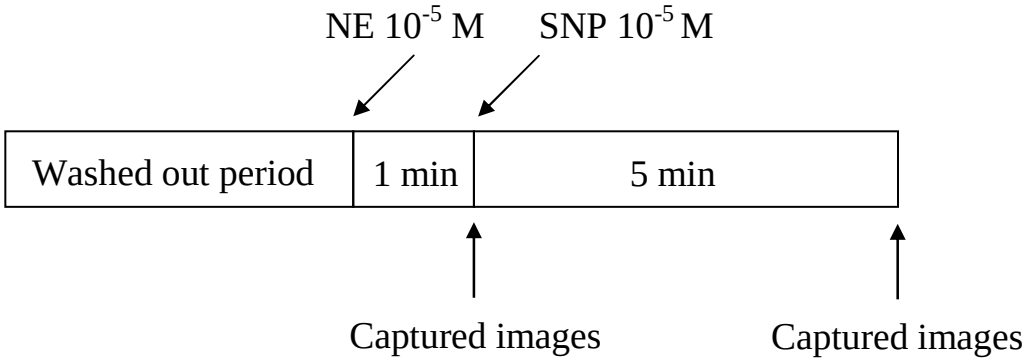


Figure 3.4 Schematic diagram for assessment arteriolar response to SNP

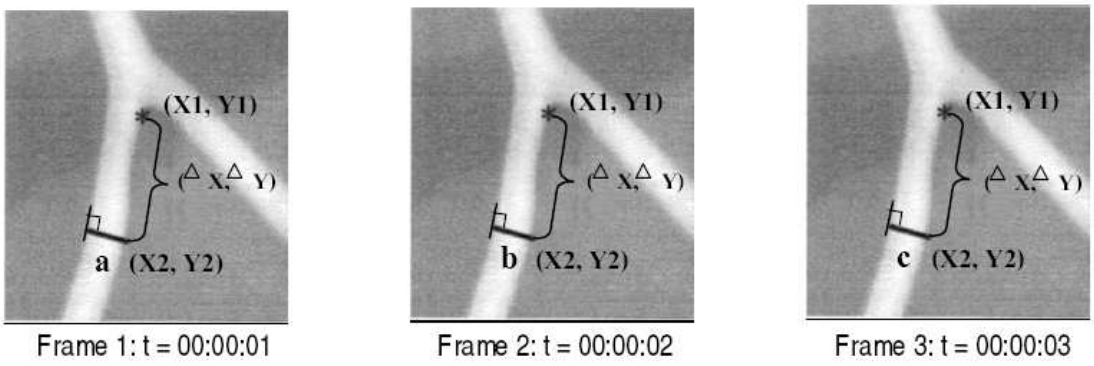


Figure 3.5 Method for measurement of arteriolar diameter (Jariyapongskul, 2000)

As shown in Figure 3.4, the arteriolar diameter was measured as followed by;

: x_1, y_1 or * where the reference position is locatedat the vessel branch

: x_2, y_2 where the diameter was measured

The diameter (d) was obtained by averaging diameters of three frames:

$$d = (a+b+c)/3 \quad \text{(Equation 1)}$$

The percentage of arteriolar diameter was calculated from the difference between diameter at 1 min after topically application with NE (d_1) and diameter at 5 min after the application of each vasodilator (d_2) (Jariyapongskul, 2000; Chakraphan, 2002; Sridulyakul, 2007). The changes of arteriolar diameters were calculated by equation 2.

$$\% \text{ changes of arteriolar diameter} = [(d_2-d_1)/d_1]\times 100 \quad \text{(Equation 2)}$$

Direct detection of NO production

On the experiment day, the cremaster microcirculation was observed under intravital fluorescent video microscope with a 20x objective lens and a 10x eyepieces. To visualize microvascular distribution of NO, diaminofluorescein-2 (DAF-2DA, Cayman Chemical Company, Michigan, USA), a NO-sensitive fluoroprobe, was used. After the cremaster was superfused with Krebs-Ringer buffered solution containing 3 μM DAF-2DA and 10^{-5}M Ach, NO levels from Ach-activated endothelial cells were analyzed at two different time points, 0 min ($I_{0\ min}$) and 20 min ($I_{20\ min}$) (Kashiwagi et al., 2002; Sridulyakul et al., 2008) (Figure 3.6; Equation 3).

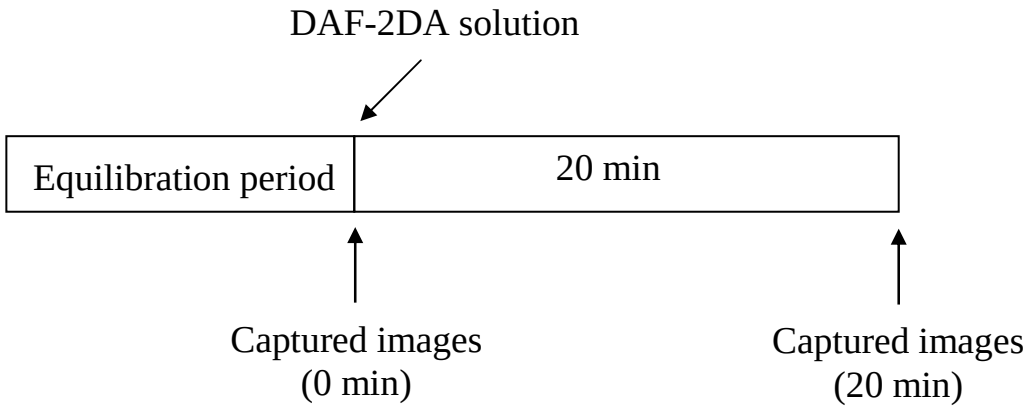


Figure 3.6 Schematic diagram for assessment NO production

DAF-2DA can readily enter the cells and hydrolyzed by cytosolic esterase to DAF-2, which is trapped inside the cells. In the presence of NO, the relatively non-fluorescent DAF-2 is converted into a highly green fluorescent triazole form, DAF-2T (shown in diagram below). Thus the increases in DAF-2T fluorescent intensity represent of the cremasteric microcirculation would indicate an elevation of nitric oxide having by an excitation and emission wavelengths of 488 and 538 nm, respectively (Kashiwagi et al., 2002; Kojima et al., 1998; Matsubara et al., 2006).

$$\text{DAF-2DA} \xrightarrow{\text{Esterase}} \text{DAF-2} + \text{NO} \longrightarrow \text{DAF-2T}$$

Non-fluorescent Triazolofluorescein

The cremasteric microcirculation was epi-illuminated having an excitation wavelength of 488 nm and emission wavelength of 538 nm. The microscopic field containing arterioles (15 to 35 μm in diameter) sharing the same focusing plane were selected and recorded for further analysis using Image Pro-Plus V. 5 software (Media Cybernatics, Inc, USA). From fourteen small working window frames (7x3 μm^2 each window), the fluorescent intensity of each arteriolar vascular wall was

averaged (Figure 3.7). Assuming that DAF-2T intensity is linearly related to the intracellular NO content, the difference in fluorescent intensity between $I_{0\ min}$ and $I_{20\ min}$ was calculated according to the following equation and represented as the percent increase in NO released during the first 20 minutes:

% changes of NO-associated fluorescent intensity

$$= [(I_{20\ min} - I_{0\ min}) / I_{0\ min}] \times 100 \text{……..(Equation 3)}$$

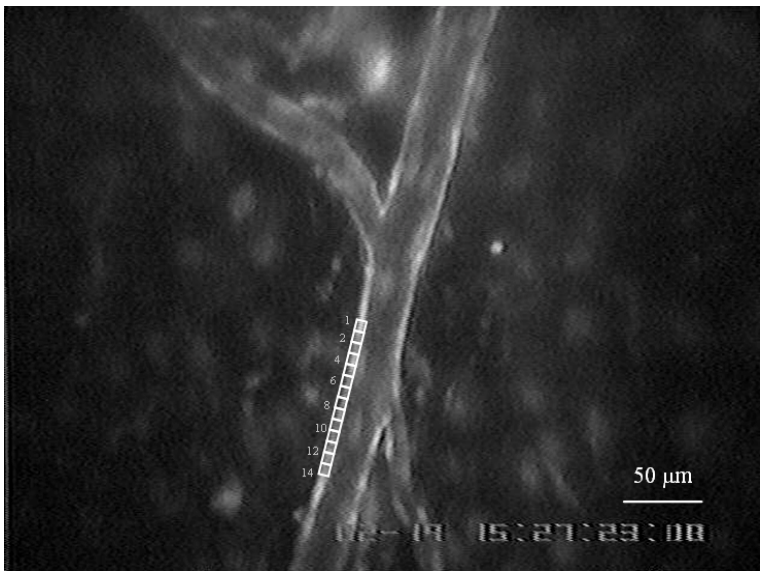


Figure 3.7 A videoimage of NO-associated fluorescent intensity taken from cremasteric arteriole of a young rat by using 20 x-objective lenses. From 14 frames of working windows, the Histogram Menu in Image Program Software was used to define the mean value of fluorescent intensity of each window, and then the averaged intensity was calculated for all 14 small working window frames (7x3 μm^2 each window).

Biochemical parameters

At the end of microcirculation experiment, the blood sample of each rat was collected for testosterone, TNF-α level determination and then collected the liver for malondialdehyde (MDA) evaluation as followed:

1. The testosterone level was analyzed from serum using ECL method by Bangkok RIA Lab Co., Ltd.

2. In order to evaluate serum TNF-α concentrations, blood samples were collected in polypropylene tubes, store at room temperature for 2 hour and then centrifugation at 3,000 rpm at 4°C for 20 minutes, each sample was stored at -20°C until analysis. To measurement of TNF-α in serum a commercially available ELISA-Kit (R&D Systems, USA) was used. The results were calculated from a standard curve and expressed in optical density (OD) and picograms per milliliter (pg/ml).

3. Immediately after collected the blood, the liver was perfused with ice-cold phosphate buffer saline (PBS); pH 7.4 and then the liver was immediately excised. Subsequently, fat and fibrous tissues on the isolated liver were removed before weighing. The liver was kept at -80°C for later malondialdehyde (MDA) analyses using the thiobarbituric acid reaction as described by Ohkawa et al., 1979.

Statistical analyses

Data are expressed as means ± SD. For comparison among groups of animals, one-way analysis of variance (one-way ANOVA) and a two-sided alpha level of 0.05 adjusted by Tukey's procedure for multiple comparisons were used. $P<0.05$ were considered statistically significant.

CHAPTER IV

RESULTS

This chapter was composed of two major parts of results which were: 1) the effect of age on endothelial function, testosterone, oxidative stress and proinflammatory cytokine and 2) the effects of genistein and exercise training on age-induced endothelial dysfunction, testosterone, oxidative stress and proinflammatory cytokine.

4.1 The effect of age on endothelial function, testosterone, oxidative stress and proinflammatory cytokine.

4.1.1 Physiological characteristics

In Table 4.1 mean arterial pressure (MAP), the results showed that there were no significant difference between young and Adult+Sedentary group (122.77±4.28 mmHg and 127.82±8.35 mmHg, respectively). However, it seems to incline the MAP value in Adult+Sedentary group. On the other hand, MAP value for Aged+Sedentary was significantly higher than the young group (142.84±16.30 mmHg and 122.77±4.28 mmHg, respectively) (P<0.01).

Body weight of Adult+Sedentary and Aged+Sedentary were significantly higher than young group (696.67±61.54 g, 662.00±49.70 g and 481.78±18.22 g, respectively) (P<0.05).

The testosterone level, the results showed that there was no significant difference between young and Adult+Sedentary group (2.21±1.24 ng/mL and 1.51±0.56 ng/mL, respectively). However, it appears to decline the testosterone level in Adult+Sedentary group. In contrast, the testosterone level of Aged+Sedentary was significantly

lower than the young group (0.60±0.59 ng/mL and 2.21±1.24 ng/mL, respectively) (P<0.01) (Table 4.1 and Figure 4.1).

Heart weight/body weight ratio of Adult+Sedentary and Aged+Sedentary were significantly lower than young group (Table 4.1) (P<0.05). It might be resulting from age effect. The seminal vesicle weight/BW ratio of Adult+Sedentary and Aged+Sedentary were significantly lower than the young group (Table 4.1).

4.1.2 Arteriolar response to acetylcholine (Ach) and sodium nitroprusside (SNP)

As shown in Table 4.2 and Figure 4.2, arteriolar dilatation to Ach was significantly impaired in Aged+Sedentary (12.28±2.78%) when it was compared to the young (27.11±5.14%) and Adult+Sedentary (24.86±9.22%) groups (P<0.01 and P<0.05, respectively).

To ensure that impairment of arteriolar dilatation to Ach did not interfere with the function of the smooth muscle cells, the vasodilatory response to endothelium-independent vasodilatation was examined by using SNP (10^{-5}M). The results showed that there was no significant difference among the young (24.41±4.89%), Adult+Sedentary (26.13±7.10%) and Aged+Sedentary (25.05±6.37%) groups (Table 4.2 and Figure 4.2).

4.1.3 Direct detection of NO production

The NO production of Adult+Sedentary (61.68±8.68%) and Aged+Sedentary (47.83±16.53%) group were significantly attenuated as compared with young (85.27±13.49%) group (P<0.05 and P<0.01, respectively) (Table 4.3 and Figure 4.3).

4.1.4 Proinflammatory cytokine

As showed in Table 4.4 and Figure 4.4, the TNF-α level of Adult+Sedentary (12.54±9.44 pg/mL) was as same as young (13.42±5.91 pg/mL) group. However, the TNF-α level of Aged+Sedentary (39.50±14.02 pg/mL) was significantly increased as compared to young group (P<0.01). Likewise, the TNF-α level of Aged+Sedentary was significantly increased as compared to Adult+Sedentary (12.54±9.44 pg/mL) group (P<0.01).

4.1.5 Levels of liver malondialdehyde

The MDA level, the indicator of oxidative stress, for Aged+Sedentary was significantly increased when compared to the young group (3.69±0.54 µmol/g wet weight and 2.42±0.48 µmol/g wet weight, respectively) (P<0.05) (Table 4.5 and Figure 4.5).

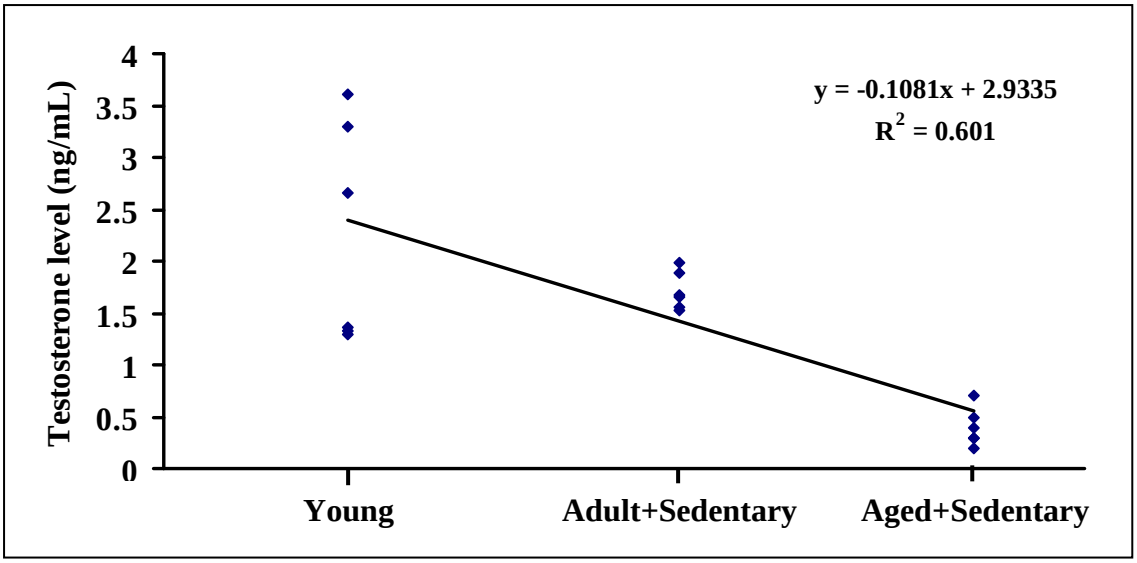


Figure 4.1 Effect of age-induced decline the testosterone level. The correlations between age and testosterone level were examined by using Pearson's Correlation and the best-fitting linear regression. All data were taken from values of each group: young, Adult+Sedentary and Aged+Sedentary (Pearson's correlation=0.60, P<0.01).

Table 4.1 Mean arterial blood pressure (MAP) value, body weight (BW), testosterone level, seminal vesicle weight/BW ratio and heart weight/BW ratio were demonstrated for young, Adult+Sedentary and Aged+Sedentary groups.

Group	MAP (mmHg)	Body weight (g)	Testosterone (ng/mL)	Seminal vesicle weight/BW ratio	Heart weight/BW ratio
Young	122.77±4.28	481.78±18.22	2.21±1.24	0.0044±0.0007	0.0032±0.0003
Adult+Sedentary	127.82±8.35 ^{NS}	696.67±61.54**	1.51±0.56 ^{NS}	0.0033±0.0003*	0.0028±0.0002*
Aged+Sedentary	142.84±16.30*	662.00±49.70**	0.60±0.59**	0.0029±0.0009**	0.0029±0.0003*

Values are mean±SD (n=6)

$^{*}P<0.05$ compared to young group, ** $P<0.01$ compared to young group, ^{NS} not significant difference as compared to young group.

Table 4.2 The percentage of increase in arteriolar diameters induced by Ach and SNP for young, Adult+Sedentary and Aged+Sedentary groups.

Group	% changes of arteriolar diameters	
	Ach	**SNP**
Young	27.11±5.14	24.41±4.89
Adult+Sedentary	24.86±9.22 ^{NS}	26.13±7.10^{NS}
Aged+Sedentary	12.28±2.78$^{**, \#}$	25.05±6.37^{NS}

Values are mean±SD (n=6)

$^{**}P$<0.01 compared to young group, $^{\#}P$<0.05 compared to Adult+Sedentary group, ^{NS} not significant difference as compared to young group.

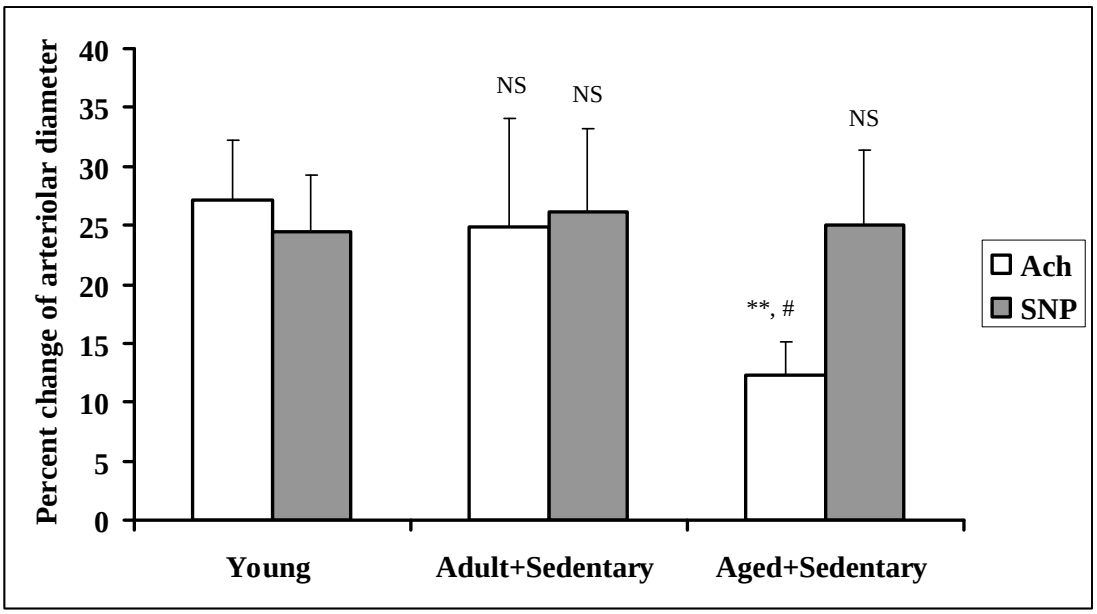


Figure 4.2 The percentage of increase in arteriolar diameters induced by Ach and SNP for young, Adult+Sedentary and Aged+Sedentary groups.

Values are mean±SD (n=6)

$^{**}P$<0.01 compared to young group, $^{\#}P$<0.05 compared to Adult+Sedentary group, ^{NS} not significant difference as compared to young group.

Table 4.3 The percentage change of NO-associated fluorescent intensity for young, Adult+Sedentary and Aged+Sedentary groups.

Group	% changes of NO-associated fluorescent intensity
Young	85.27±13.49
Adult+Sedentary	61.68±8.68*
Aged+Sedentary	47.83±16.53**

Values are mean±SD (n=6)

*P<0.05 compared to young group, **P<0.01 compared to young group.

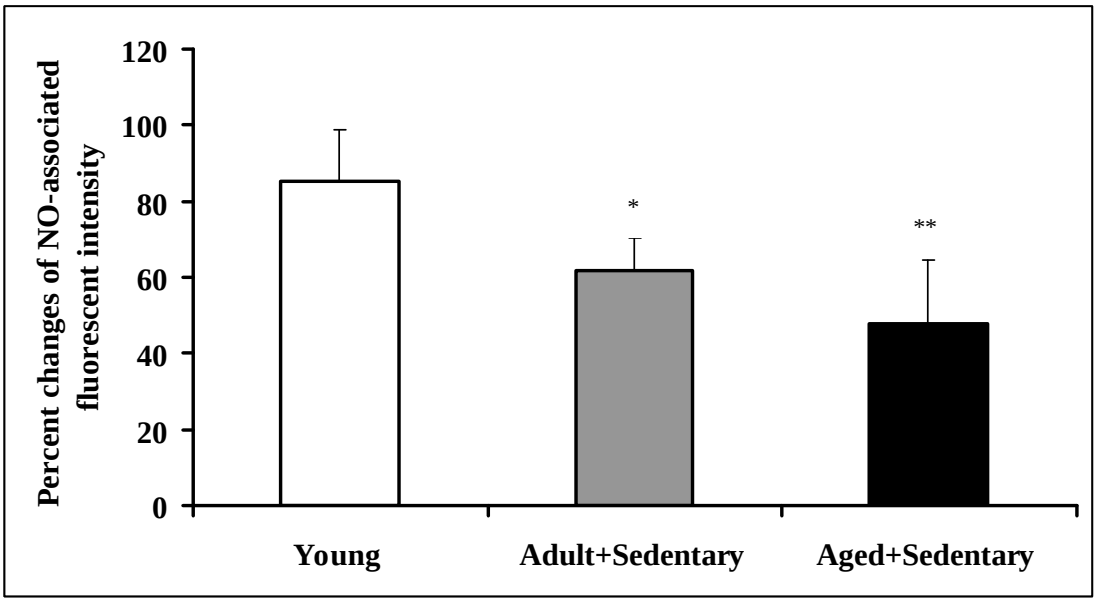


Figure 4.3 The percentage change of NO-associated fluorescent intensity for young, Adult+Sedentary and Aged+Sedentary groups

Values are mean±SD (n=6)

*P<0.05 compared to young group, **P<0.01 compared to young group.

Table 4.4 TNFα level was shown for young, Adult+Sedentary and Aged+Sedentary groups.

Group	TNF-α level (pg/mL)
Young	13.42±5.91
Adult+Sedentary	12.54±9.44^{NS}
Aged+Sedentary	39.50±14.02$^{**, \#\#}$

Values are mean±SD (n=6)

$^{**}P$<0.01 compared to young group, $^{\#\#}P$<0.01 compared to Adult+Sedentary group, ^{NS} not significant difference as compared to young.

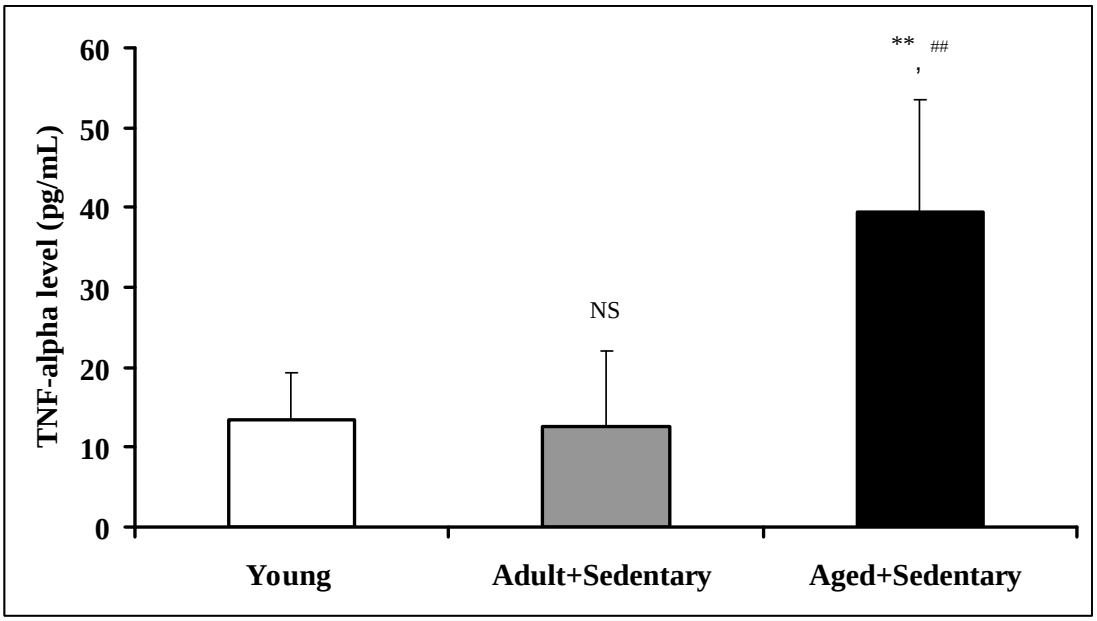


Figure 4.4 TNF-α level for young, Adult+Sedentary and Aged+Sedentary groups

Values are mean±SD (n=6)

$^{**}P$<0.01 compared to young group, $^{\#\#}P$<0.01 compared to Adult+Sedentary group, ^{NS} not significant difference as compared to young.

Table 4.5 MDA level was shown for young and Aged+Sedentary groups.

Group	MDA level of liver (µmol/g wet wt.)
Young	2.42±0.48
Aged+Sedentary	3.69±0.54*

Values are mean±SD (n=5)

*P<0.05 compared to young group.

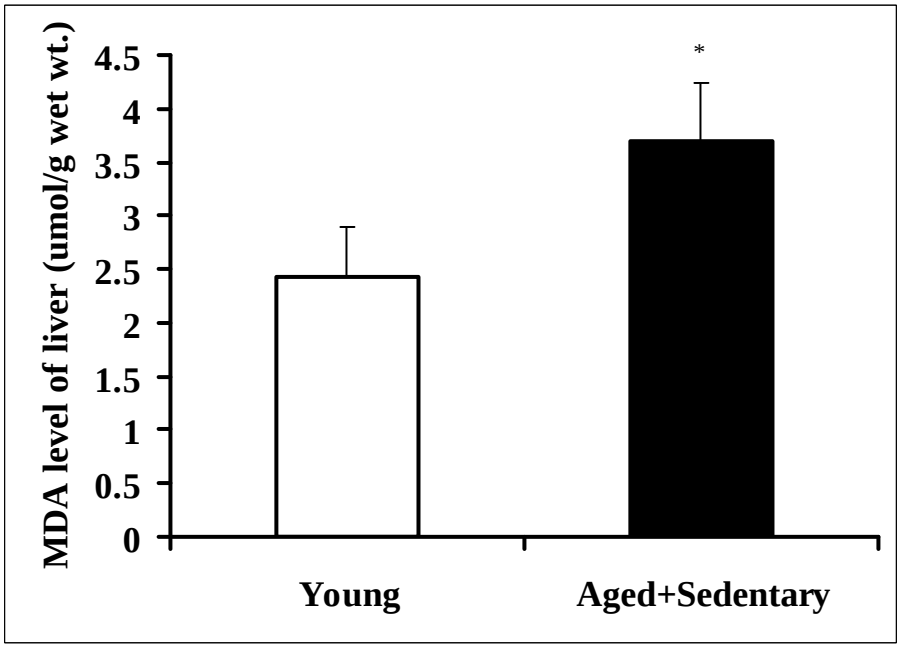


Figure 4.5 MDA level for young and Aged+Sedentary groups

Values are mean±SD (n=5)

*P<0.05 compared to young group.

4.2 The effects of genistein and exercise training on age-induced endothelial dysfunction, testosterone, oxidative stress and proinflammatory cytokine.

4.2.1 Physiological characteristics

The MAP of Adult treated with genistein, exercise and combination with genistein and exercise, there were no significant different among all groups of rats. Nevertheless, there was a tendency to attenuate the MAP in adult-treated group when compared to aged-match control group (Table 4.6). There was no significant different among all group of rats for body weight and heart weight/BW ratio. In adult treated with genistein, exercise and combination with genistein and exercise training had no effect on hormonal change, seminal vesicle weight/BW ratio and heart weight/BW ratio (Table 4.6).

Interestingly, MAP from aged treated with genistein, exercise and combination with genistein and exercise (Aged+Gen (124.26±2.29 mmHg), Aged+Ex (122.74±5.95 mmHg) and Aged+Gen+Ex (125.13±11.67 mmHg)) groups reduced significantly when they were compared to their age-matched control (Aged+Veh (145.57±5.73 mmHg) and Aged+Without-Ex groups (143.81±16.68 mmHg) (P<0.01, P<0.01 and P<0.05, respectively). Body weight of Aged+Ex (610.68±88.61 g) and Aged+Gen+Ex (618.20±73.56 g) groups were significantly lower than Aged+Without-Ex (728.33±50.37 g) group (P<0.05).

Genistein supplementation showed no effects on both testosterone level and the ratio of seminal vesicle /body weight. In addition, exercise training had no effect on hormonal change. However, in Aged+Ex and Aged+Gen+Ex groups, the ratio of seminal vesicle/body weight were significantly higher than the Aged+Without-Ex group (P<0.05).

Moreover, heart weight/BW ratio of Aged+Gen+Ex was significantly higher than Aged+Veh group (P<0.05) (Table 4.7).

4.2.2 Arteriolar response to acetylcholine (Ach) and sodium nitroprusside (SNP)

Both the dilatory responses of the arterioles to Ach and SNP of adult treated with genistein, exercise and combination with genistein and exercise, there were no significant different among all groups of rats, (Table 4.8, Figure 4.6 and 4.7).

The results showed that, the dilatory responses of the arterioles to Ach significantly increased in Aged+Gen (26.87±7.94%), Aged+Ex (31.02±4.11%) and Aged+Gen+Ex (30.02±12.14%) groups when compared to their age-matched controls (P<0.05, P<0.01 and P<0.01 respectively). However, there was no significant difference in the percentage changes of arteriolar diameters to SNP among all group of rats (Table 4.9, Figure 4.8 and 4.9).

4.2.3 Direct detection of NO production

The results showed that there was no significant different between Adult+Gen and Adult+Veh group (78.63±22.52% and 59.51±17.11%, respectively). Interestingly, the NO production of Adult+Ex was significantly increased as compared to Adult+Without-Ex group (98.62±4.70% and 63.63±13.15%, respectively). However, there was no different between Adult+Gen+Ex and their aged-matched control group (76.18±8.95%, 59.51±17.11% and 63.63±13.15%, respectively) (Table 4.10 and Figure 4.10).

Interestingly, the NO production, in Aged+Gen (74.98±17.05%), Aged+Ex (76.34±17.82%) and Aged+Gen+Ex (70.22±16.05%) groups

had a significant enhancement of the NO level when compared to their age-matched controls (Aged+Veh and Aged+Without-Ex) (P<0.01, P<0.01 and P<0.05, respectively). It is noted that genistein and exercise training could enhance NO bioavailability in aging rats (Table 4.11 and Figure 4.11).

4.2.4 Proinflammatory cytokine

There was no significant difference in the TNF-α level among all group of adult rats (Table 4.12 and Figure 4.12).

Table 4.13 and Figure 4.13 showed that, the TNF-α significantly decreased in Aged+Gen (11.16±5.68 pg/mL), Aged+Ex (14.40±11.03 pg/mL) and Aged+Gen+Ex (10.25±5.91 pg/mL) groups when compared to their age-matched controls (P<0.01, P<0.05 and P<0.01 respectively).

4.2.5 Levels of liver malondialdehyde

Interestingly, the MDA levels of three treated groups, Aged+Gen (2.53±0.59 µmol/g liver wet weight), Aged+Ex (2.51±0.49 µmol/g liver wet weight) and Aged+Gen+Ex (2.48±0.29 µmol/g liver wet weight), were significantly decreased when compared to their age-matched controls (P<0.05) (Table 4.14, Figure 4.14).

In Figure 4.15, linear regression was performed to establish the relationship between the Ach-induced increase in arteriolar diameters and the percent increased in NO-associated fluorescent intensity for the Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups. The linear equation obtained was: $y = 0.4276x - 2.0966$, $R^2 = 0.82$, (P<0.01), where x is the mean of NO-associated fluorescent intensity and y is the mean percentage of diameter change.

Table 4.6 Mean arterial blood pressure (MAP) value, body weight (BW), testosterone level, seminal vesicle/BW ratio and heart weight/BW ratio were demonstrated for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Group	MAP (mmHg)	Body weight (g)	Testosterone (ng/mL)	Seminal vesicle weight/BW ratio	Heart weight/BW ratio
Adult+Veh	129.23±6.27	745.00±58.22	1.60±0.94	0.0028±0.0003	0.0024±0.0001
Adult+Gen	126.01±10.31^{NS}	710.00±55.86^{NS}	1.51±0.15^{NS}	0.0032±0.0007^{NS}	0.0029±0.0002^{NS}
Adult+Without-Ex	129.22±5.06	686.00±44.19	1.47±0.53	0.0034±0.0004	0.0026±0.0006
Adult+Ex	123.64±5.74^{NS}	678.00±24.90^{NS}	1.59±0.70^{NS}	0.0041±0.0010^{NS}	0.0027±0.0003^{NS}
Adult+Gen+Ex	122.50±6.56^{NS}	678.33±54.19^{NS}	2.01±0.19^{NS}	0.0036±0.0001^{NS}	0.0027±0.0002^{NS}

Values are mean±SD (n=6)

^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

Table 4.7 Mean arterial blood pressure (MAP) value, body weight (BW), testosterone level, seminal vesicle/BW ratio and heart weight/BW ratio were demonstrated for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups.

Group	MAP (mmHg)	Body weight (g)	Testosterone (ng/mL)	Seminal vesicle weight/BW ratio	Heart weight/BW ratio
Aged+Veh	145.57±5.73	715.00±37.28	0.66±0.67	0.0021±0.0007	0.0029±0.0004
Aged+Gen	$124.26\pm2.29^{\#\#}$	670.00 ± 10.95^{NS}	1.02 ± 0.71^{NS}	0.0036 ± 0.0013^{NS}	0.0033 ± 0.0003^{NS}
Aged+Without-Ex	143.81±16.68	728.33±50.37	0.77±0.79	0.0022±0.0013	0.0032±0.0004
Aged+Ex	$122.74\pm5.95^{\dagger\dagger}$	$610.68\pm88.61^{\dagger}$	0.36 ± 0.79^{NS}	$0.0040\pm0.0004^{\dagger}$	0.0035 ± 0.0007^{NS}
Aged+Gen+Ex	$125.13\pm11.67^{\#,\dagger}$	$618.20\pm73.56^{\dagger}$	1.35 ± 0.67^{NS}	$0.0039\pm0.0004^{\#,\dagger}$	$0.0038\pm0.0006^{\#}$

Values are mean±SD (n=6)

$^{\#}P<0.05$ compared to Aged+Veh group, $^{\#\#}P<0.01$ compared to Aged+Veh group, $^{\dagger}P<0.05$ compared to Aged+Without-Ex group, $^{\dagger\dagger}P<0.01$ compared to Aged+Without-Ex group, ^{NS} not significant difference as compared to Aged+Veh or Aged+Without-Ex groups.

Table 4.8 The percentage of increase in arteriolar diameters induced by Ach and SNP for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Group	% changes of arteriolar diameters	
	Ach	**SNP**
Adult+Veh	26.72±10.30	29.35±5.07
Adult+Gen	24.95±5.22^{NS}	21.82±5.84^{NS}
Adult+Without-Ex	23.82±8.07	22.40±5.91
Adult+Ex	30.93±3.40^{NS}	23.23±6.68^{NS}
Adult+Gen+Ex	32.00±6.81^{NS}	29.77±5.75^{NS}

Values are mean±SD (n=6)

^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

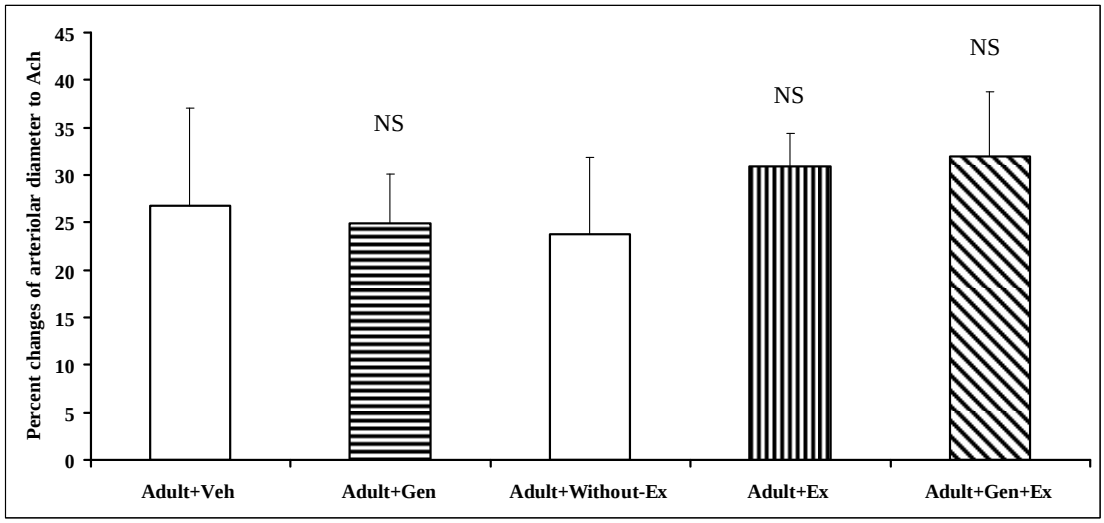


Figure 4.6 The percentage of increase in arteriolar diameters induced by Ach for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Values are mean±SD (n=6)

NS not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

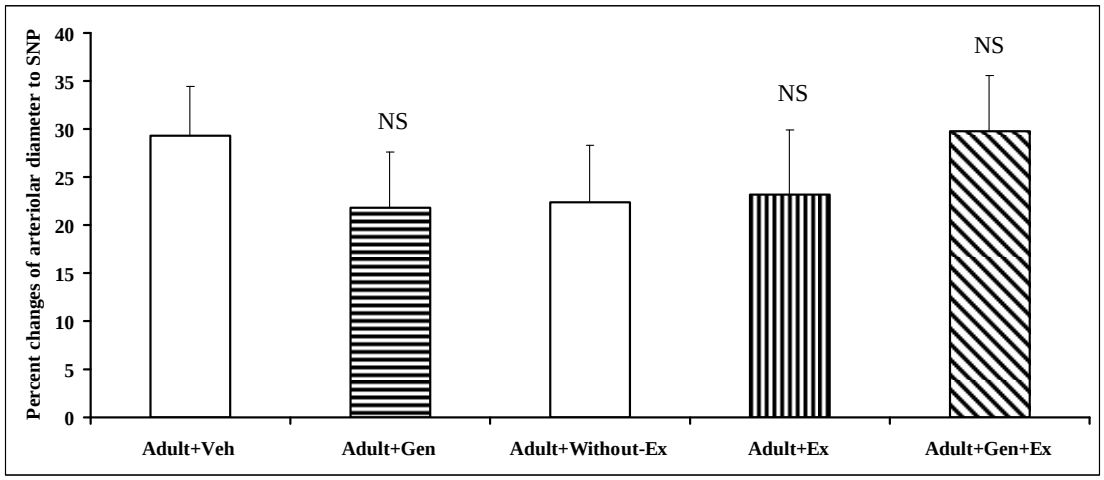


Figure 4.7 The percentage of increase in arteriolar diameters induced by SNP for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Values are mean±SD (n=6)

NS not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

Table 4.9 The percentage of increase in arteriolar diameters induced by Ach and SNP for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups.

Group	% changes of arteriolar diameters	
	Ach	**SNP**
Aged+Veh	12.65±4.63	27.77±11.58
Aged+Gen	26.87±7.94$^{\#}$	31.81±12.34^{NS}
Aged+Without-Ex	14.41±4.13	30.14±15.62
Aged+Ex	31.02±4.11††	29.53±6.93^{NS}
Aged+Gen+Ex	30.02±12.14$^{\#\#, \dagger\dagger}$	26.30±6.45^{NS}

Values are mean±SD (n=6)

$^{\#}P<0.05$ compared to Aged+Veh group, $^{\#\#}P<0.01$ compared to Aged+Veh group, $^{\dagger\dagger}P<0.01$ compared to Aged+Without-Ex group, ^{NS} not significant difference as compared to Aged+Veh or Aged+Without-Ex groups.

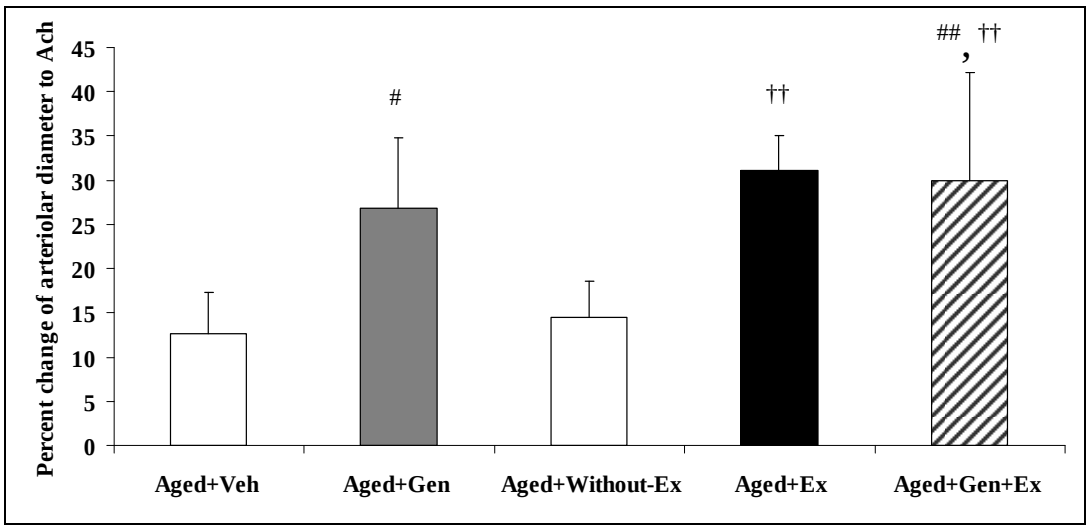


Figure 4.8 The percentage of increase in arteriolar diameters induced by Ach for aged rats with vehicle (Aged+Veh), genistein (Aged+Gen), without exercise training (Aged+Without-Ex), exercise training (Aged+Ex) and combination with genistein and exercise training (Aged+Gen+Ex). $^{\#}P<0.05$ compared to Aged+Veh group, $^{\#\#}P<0.01$ compared to Aged+Veh group, $^{\dagger\dagger}P<0.01$ compared to Aged+Without-Ex group.

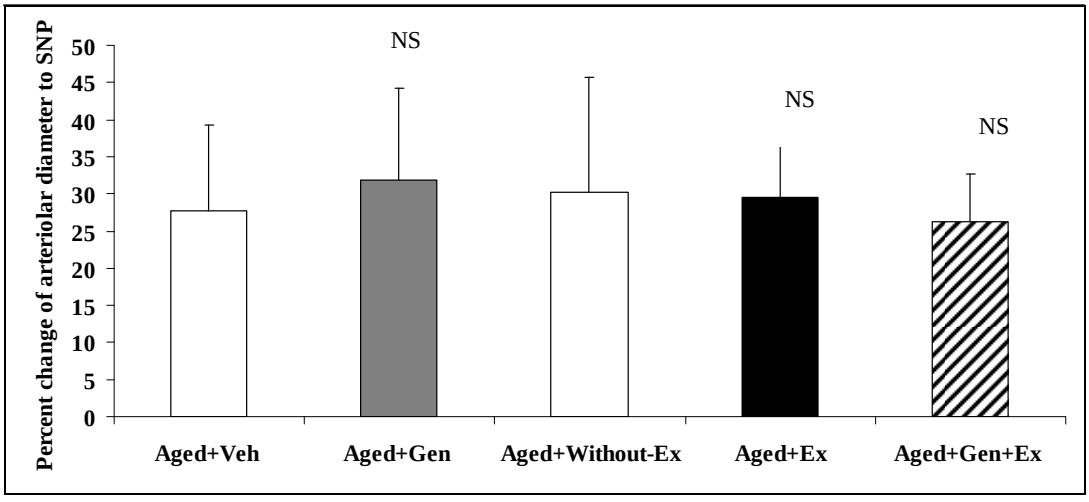


Figure 4.9 The percentage change of arteriolar diameters induced by SNP for aged rats with vehicle (Aged+Veh), genistein (Aged+Gen), without exercise (Aged+Without-Ex), exercise (Aged+Ex) and combination with genistein and exercise training (Aged+Gen+Ex).

^{NS} not significant difference as compared to Aged+Veh or Aged+Without-Ex groups.

Table 4.10 The percentage change of NO-associated fluorescent intensity for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Group	% changes of NO-associated fluorescent intensity
Adult+Veh	59.51±17.11
Adult+Gen	78.63±22.52^{NS}
Adult+Without-Ex	63.63±13.15
Adult+Ex	98.62±4.70††
Adult+Gen+Ex	76.18±8.95^{NS}

Values are mean±SD (n=6)

$^{††}P<0.01$ compared to Aged+Without-Ex group**,** ^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

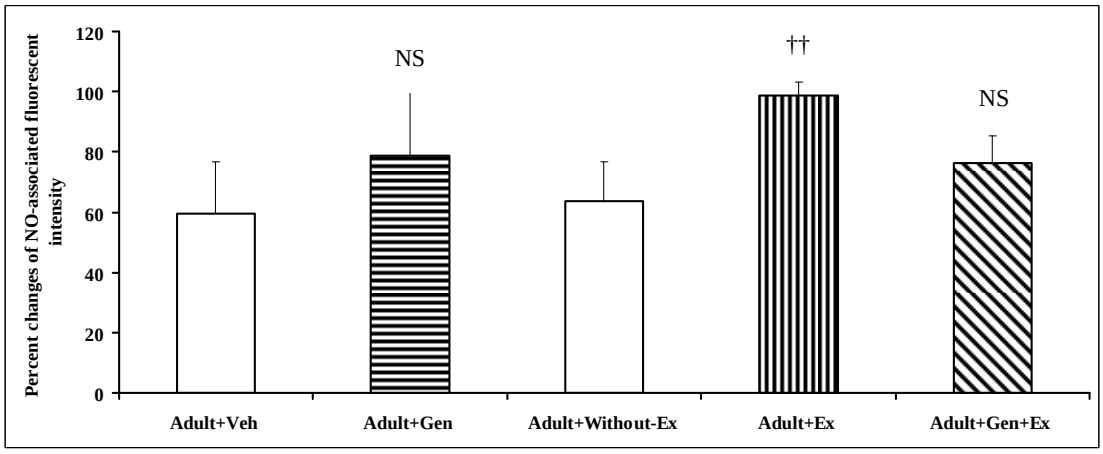


Figure 4.10 The percentage change of NO-associated fluorescent intensity for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups**.**

Values are mean±SD (n=6)

$^{††}P<0.01$ compared to Aged+Without-Ex group**,** ^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

Table 4.11 The percentage change of NO-associated fluorescent intensity for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups.

Group	% changes of NO-associated fluorescent intensity
Aged+Veh	35.15±8.69
Aged+Gen	74.98±17.05$^{\#\#}$
Aged+Without-Ex	39.68±7.15
Aged+Ex	76.34±17.82††
Aged+Gen+Ex	70.22±16.05$^{\#\#,\dagger}$

$^{\#\#}P$<0.01 compared to Aged+Veh group, $^{\dagger}P$<0.05 compared to Aged+Without-Ex group, $^{\dagger\dagger}P$<0.01 compared to Aged+Without-Ex group.

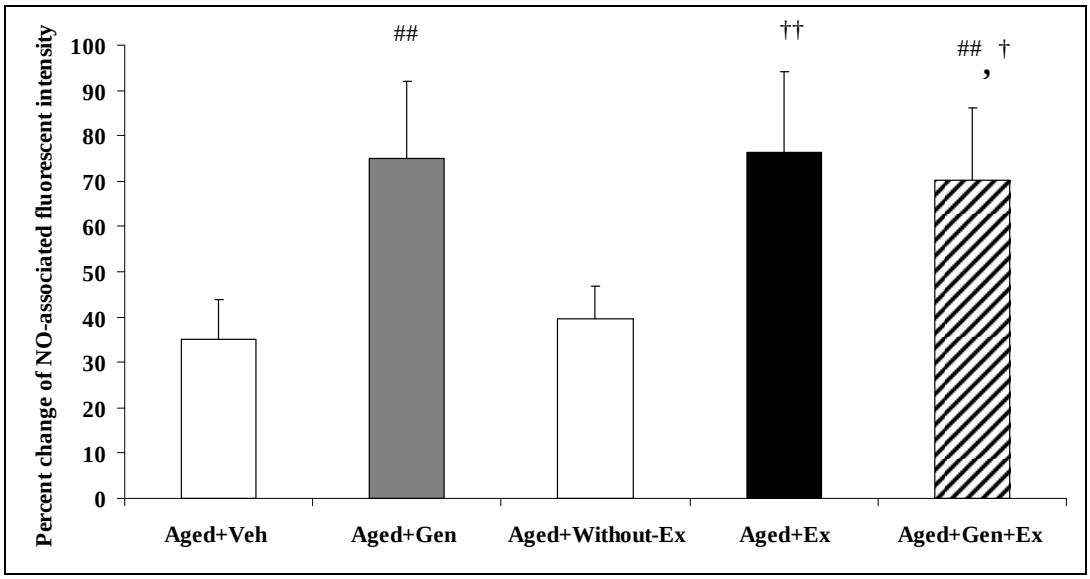


Figure 4.11 The percentage change of NO-associated fluorescent intensity for aged rats with vehicle (Aged+Veh), genistein (Aged+Gen), without exercise (Aged+Without-Ex), exercise (Aged+Ex) and combination with genistein and exercise training (Aged+Gen+Ex).

Values are mean±SD (n=6)

$^{\#\#}P$<0.01 compared to Aged+Veh group, $^{\dagger}P$<0.05 compared to Aged+Without-Ex group, $^{\dagger\dagger}P$<0.01 compared to Aged+Without-Ex group.

Table 4.12 TNF-α level was shown for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Group	TNF-α (pg/mL)
Adult+Veh	16.25±7.64
Adult+Gen	10.22±7.81^{NS}
Adult+Without-Ex	15.64±4.17
Adult+Ex	14.03±13.01^{NS}
Adult+Gen+Ex	13.94±7.01^{NS}

Values are mean±SD (n=6)

^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

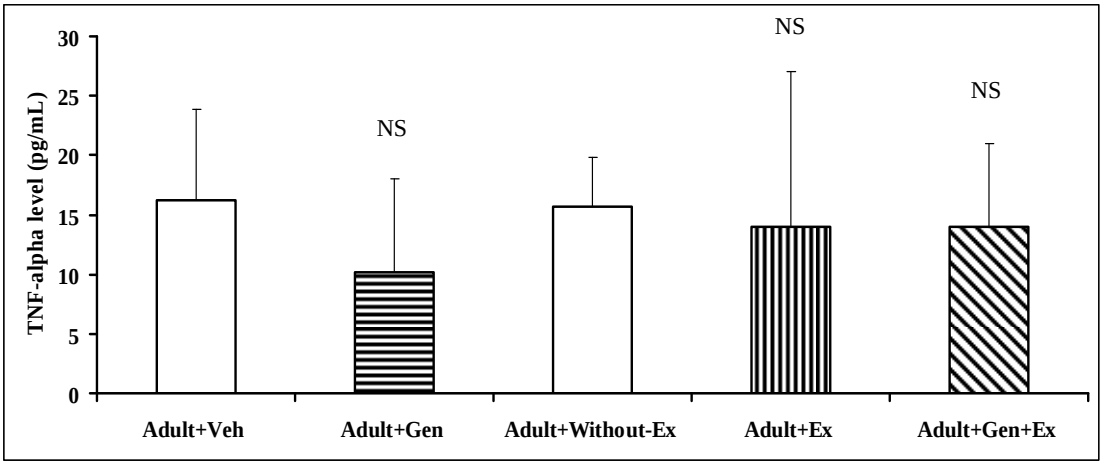


Figure 4.12 TNF-α level was shown for Adult+Veh, Adult+Gen, Adult+Without-Ex, Adult+Ex and Adult+Gen+Ex groups.

Values are mean±SD (n=6)

^{NS} not significant difference as compared to Adult+Veh or Adult+Without-Ex groups.

Table 4.13 TNF-α level was shown for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups.

Group	TNF-α (pg/mL)
Aged+Veh	33.97±7.66
Aged+Gen	11.16±5.68$^{\#\#}$
Aged+Without-Ex	36.29±14.41
Aged+Ex	14.40±11.03†
Aged+Gen+Ex	10.25±5.91$^{\#\#,\dagger\dagger}$

Values are mean±SD (n=6)

$^{\#\#}P<0.01$ compared to Aged+Veh group, $^{\dagger}P<0.05$ compared to Aged+Without-Ex group, $^{\dagger\dagger}P<0.01$ compared to Aged+Without-Ex group.

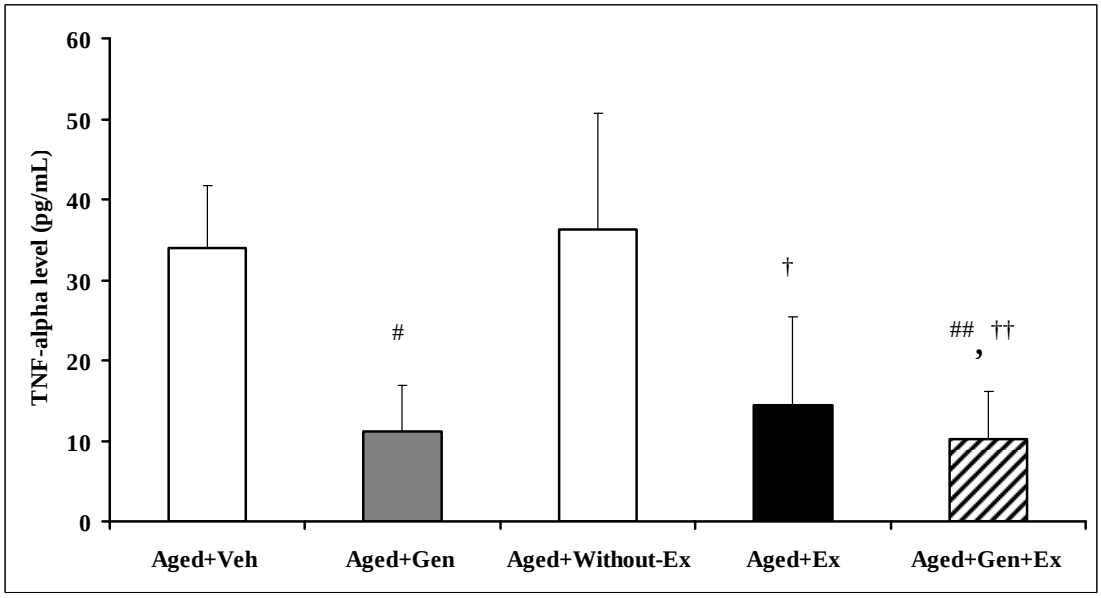


Figure 4.13 The TNF-α level for aged rats with vehicle (Aged+Veh), genistein (Aged+Gen), without exercise (Aged+Without-Ex), exercise (Aged+Ex) and combination with genistein and exercise training (Aged+Gen+Ex). Values are mean±SD (n=6)

$^{\#\#}P<0.01$ compared to Aged+Veh group, $^{\dagger}P<0.05$ compared to Aged+Without-Ex group, $^{\dagger\dagger}P<0.01$ compared to Aged+Without-Ex group.

Table 4.14 MDA level was shown for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex groups.

Group	MDA level of liver (µmol/g wet wt.)
Aged+Veh	4.81±1.17
Aged+Gen	2.53±0.59$^{\#}$
Aged+Without-Ex	4.33±2.00
Aged+Ex	2.51±0.49†
Aged+Gen+Ex	2.48±0.29$^{\#,\dagger}$

Values are mean±SD (n=5)

$^{\#}P$<0.05 compared to Aged+Veh group, $^{\dagger}P$<0.05 compared to Aged+Without-Ex group.

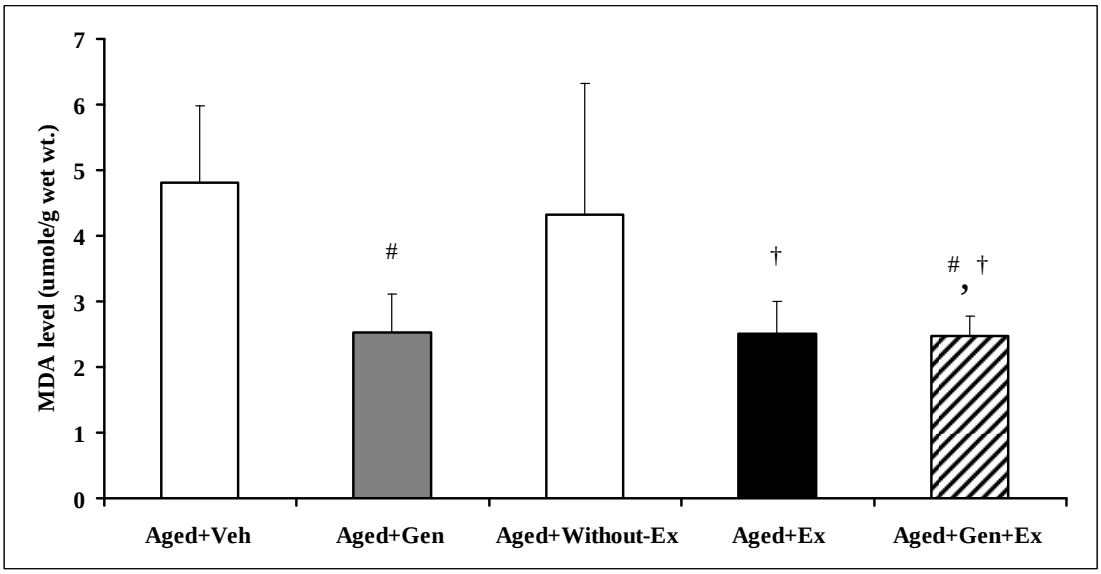


Figure 4.14 The MDA level for Aged+Veh, Aged+Gen, Aged+Without-Ex, Aged+Ex and Aged+Gen+Ex.

Values are mean±SD (n=5)

$^{\#}P$<0.05 compared to Aged+Veh group, $^{\dagger}P$<0.05 compared to Aged+Without-Ex group.

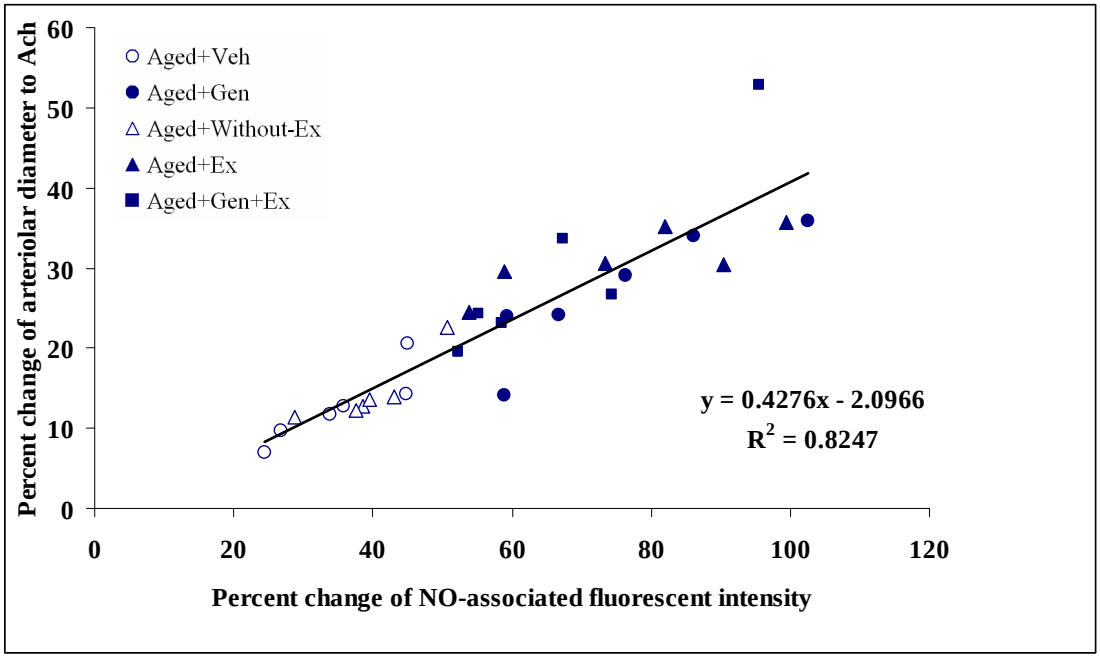


Figure 4.15 The correlations between percent changes of NO-associated fluorescent intensity and the percent arteriolar diameter changes were examined by using Pearson's Correlation and the best-fitting linear regression. All data were taken from values of each groups: aged+vehicle (Aged+Veh), aged+genistein (Aged+Gen), aged+without exercise (Aged+Without-Ex), aged+exercise (Aged+Ex) and aged+genistein+exercise (Aged+Gen+Ex) (Pearson's correlation=0.82, $P<0.01$).

CHAPTER V

DISCUSSION

In the present study, the intravital experiments in cremaster microcirculation were conducted to investigate the effects of genistein and exercise training on endothelial dysfunction in aged male rats. The results showed that aged rats developed hypertension, decreased testosterone, impaired endothelium dependent vasodilation, decreased NO bioavailability, increased oxidative stress and increased proinflammatory cytokine. Our finding showed that genistein and exercise training have a potential role in improvement these deleterious physiological and vascular functions by means of similar pathways. Discussion is expressed in relation to the heading topics as followed.

Effects of aging on physiological and biochemical parameters

In male, androgen is an important steroid hormone that regulates metabolism in many organs, and its effect is obvious in male sex organs. During aging the diminution of the blood testosterone level parallels a decline of several physiological parameters including cardiovascular functions. The results showed that the blood testosterone level progressively declined (young=2.21±1.24 ng/mL, Adult+Sedentary=1.51±0.56 ng/mL and Aged+Sedentary=0.60±0.59 ng/mL), in accordance with the ratios of seminal vesicle/BW in aged group as compared to young group (young=0.0044±0.0007, Adult+Sedentary=0.0033±0.0003 and Aged+Sedentary=0.0029±0.0009).

Similar to human aging process in association with hypertension, the result showed that, the mean arterial blood pressure (MAP) was significantly elevated in Aged+Sedentary rat as compared to young group

(142.84±16.30 mmHg and 122.77±4.28 mmHg, respectively). Although, in Adult+Sedentary group that it seems to incline the MAP value (127.82±8.35 mmHg). Changes in vascular structure and function, and thereby in total peripheral resistance (TPR) were believed to be the underlining cause of this increase MAP. In our study, the result of vascular response to Ach supported this proposed idea. We found the abnormality of arteriolar response to Ach significantly in aged cremaster microcirculation. Therefore, it implied that the phenomenon of age-induced endothelial dysfunction may be widespread and applies to the whole vascular bed (Taddei et al., 1995). Moreover, the declined Ach response was confirmed by the decrease in NO, endothelium-dependent vasodilator with age (Taddei et al., 2001). Therefore, it is possible to increase the TPR and MAP in the aged group.

Age-induced endothelial dysfunction

The results of Ach-activated vascular dilatation demonstrated that there was significant reduction in the endothelium-dependent vasodilation in cremaster arterioles in Aged+Sedentary group as compared to young group (12.28±2.78% and 27.11±5.14%, respectively). In addition, by using 4, 5-diaminofluorescein-diacetate (3 μM DAF-2DA) to detect *in situ* Ach (10^{-5} M)-induced NO-associated fluorescent intensity, the results showed that there was significant reduction in the NO-associated fluorescent intensity in Aged+Sedentary rat as compared with young group (47.83±16.53% and 85.27±13.49%, respectively). Furthermore, our result also showed that, there was significant reduction in the NO-associated fluorescent intensity level in Adult+Sedentary as compared to young group (61.68±8.68% and 85.27±13.49%, respectively). However, there was no significant different between Adult+Sedentary and young group when activated by Ach (10^{-5} M) (27.11±5.14% and 24.86±9.22%,

respectively). It might be interpret that in adult rat the endothelial dysfunction was just initiated by aging process.

Besides, the present study also showed that, the response of arteriolar to SNP, a NO donor, did not indicate any changes in aged group. There is evidence indicating that aging per se leads to an attenuated generation and release or enhanced breakdown of endothelial autocoids (Brandes et al., 2005). According to report of Singh et al., 2002, they found that generalized abnormality of basal endothelial function in older people, with similar impairment of NO and prostanoid dilator pathways. It has been reported that the activity of eNOS and the production of NO are diminished in senescent human endothelial cells (Sato et al., 1993). A decline in the eNOS activity of senescent endothelial cells appears to be attributable to a reduction in eNOS protein expression as well as in eNOS phosphorylation (Hoffmann et al., 2001). Hence, aging down-regulates eNOS expression and activity and thus NO production in endothelial cells (Hayashi et al., 2008). Thus, our finding has already confirmed the effect of aging process on endothelial function that may contribute to age-induced endothelial dysfunction.

Age-induced TNF-α

The results of TNF-α detection showed that: 1) There was no significant difference between young and Adult+Sedentary groups (13.42±5.91 pg/mL and 12.54±9.44 pg/mL, respectively). 2) There was significantly elevated the TNF-α level in Aged+Sedentary as compared to young and Adult+Sedentary group (39.50±14.02 pg/mL, 13.42±5.91 pg/mL and 12.54±9.44 pg/mL and respectively).

Recent scientific studies have advanced the notion of chronic inflammation as a major risk factor underlying aging and age-related

diseases. Plasma levels of TNF-α in endotoxin-challenged animals have been found to be higher in aged than in young animals. Also, TNF-α production by endotoxin-stimulated macrophages from aged animals was increased when compared with that from young mice. Accumulated data strongly suggested that continuous (chronic) upregulation of pro-inflammatory mediators (e.g., TNF-α, IL-1b, IL-6, COX-2, iNOS) are induced during the aging process due to an age-related redox imbalance that could further activate many proinflammatory mediators, including the NF-kB signalling pathways (Chung et al., 2009).

The age-related inflammatory hypothesis is edited with two established findings: (1) a dysregulation of the immune system with age, and (2) altered redox status during aging. Both processes lead to increase in systemic inflammatory status probably due to the activation the several inflammatory mediators and mainly under oxidative stress-induced redox imbalance. The age-related redox imbalance is likely caused by the net effect of weakened anti-oxidative defense systems, and incessantly increasing production of reactive species (RS), such as superoxide (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2), reactive nitric oxide (NO), peroxynitrite ($ONOO^-$) and reactive lipid aldehydes. Overproduced and unregulated RS during aging are a major causative factor in the activation of immune systems (Brod, 2000), as exemplified in over-reactive macrophages in the inflammatory process. The salient point of the molecular inflammation hypothesis is that unresolved chronic inflammation during aging may act as the patho-physiologic link that drives normal functional changes to become many of the age-related degenerative diseases (Chung et al., 2002).

Age-induced oxidative stress

The present study showed that liver malondialdehyde (MDA), an oxidative stress indicator, was significantly increased in Aged+Sedentary group when compared to young group (3.69±0.54 µmol/g wet weight and 2.42±0.48 µmol/g wet weight, respectively). It is well know that aging is a biological process characterized by time-dependent, progressive, physiological declines accompanied by the increased incidence of age-related diseases. Over the past several decades, a number of theories have been proposed to define the causality and the underlying mechanisms of aging (Dice, 1993). Currently and widely accepted idea is the ‘‘oxidative stress hypothesis’’ (Yu and Yang, 1996), that modified and advanced from the free radical theory of aging (Harman, 1956). According to the oxidative stress hypothesis, oxidative damage is not only elicited by the uncontrolled production of reactive oxygen species (ROS) as proposed in the original free radical theory, but also by other oxidants, including reactive nitrogen species (RNS) and reactive lipid species. More importantly, the oxidative stress hypothesis emphasizes the essential role of anti-oxidant defenses as the crucial component of the overall redox balance of the organism, which was not considered in the original free radical theory. Oxidative stress is reinforced by a number of reactive species (RS), such as H_2O_2, $\bullet O_2^-$ and singlet oxygen, and other radicals as well as non-radicals, which are formed continuously in the body as a consequence of aerobic metabolism, thereby potentially modifying cellular activity and basic structural components including nucleic acids, proteins, and lipids (Barry, 1993; Davies and Goldberg, 1987; Yu and Yang, 1996). Biological sources of RS production vary widely depending on various cellular activities related to lipoxygenase, COX, plasma membrane-associated NADPH oxidase; mitochondrial electron transport

system, ubiquinone, NADH dehydrogenase; cytochrome P450, cytochrome b5, microsomal electron transport; flavoproteins and oxidases in peroxisome; and xanthine oxidase (XO) in cytosol (Chung et al., 2009).

Oxidative stress that plays a key role in endothelial dysfunction associated with superoxide (O_2^-) and nitric oxide (NO), and thereby produces ROS in form of $ONOO^-$. Besides, under endothelial dysfunction condition, it is more believed that superoxide could be produced much more by uncoupling eNOS (Heistad, 2006).

The NF-kB transcription factor can be viewed as the master regulator of the inflammatory process and can be activated by oxidative stimuli. Indeed, the activation of NF-kB-dependent genes is a major culprit responsible for the wide-spread systemic inflammatory process (Makarov, 2000). Under activated conditions, pro-inflammatory genes encode pro-inflammatory proteins, such as cytokines, growth factors, adhesion molecules (AMs) or chemokines. As shown Figure 5.1, NF-kB is known to regulate the transcription of proinflammatory molecules, such as TNF-α, interleukins (IL-1b, IL-2, and IL-6), chemokines (IL-8 and RANTES), adhesion molecules (ICAM-1, VCAM, E-selectin) and enzymes, including inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Bohrer et al., 1997; Brand et al., 1996). NF-kB activity is modulated by upstream signalling pathways such as IkB kinase (IKK) and MAPKs. The activated IKK complexes phosphorylate the IkB subunits of NF-kB/IkB to trigger the degradation of IkB, which then leads to the activation of NF-kB (Karin, 2006; Zandi et al., 1997). IKK activity is upregulated during aging by NF-kB (Kim et al., 2002), and there is further involvement of the ERK, JNK, and p38 MAPK pathways that control NF-kBdependent transcription during the inflammatory response

(Figure 5.1). Recently, the aging process was shown to strongly enhance all three ERK, JNK, and p38 MAPK activities that paralleled increases in ROS production (Kim et al., 2002).

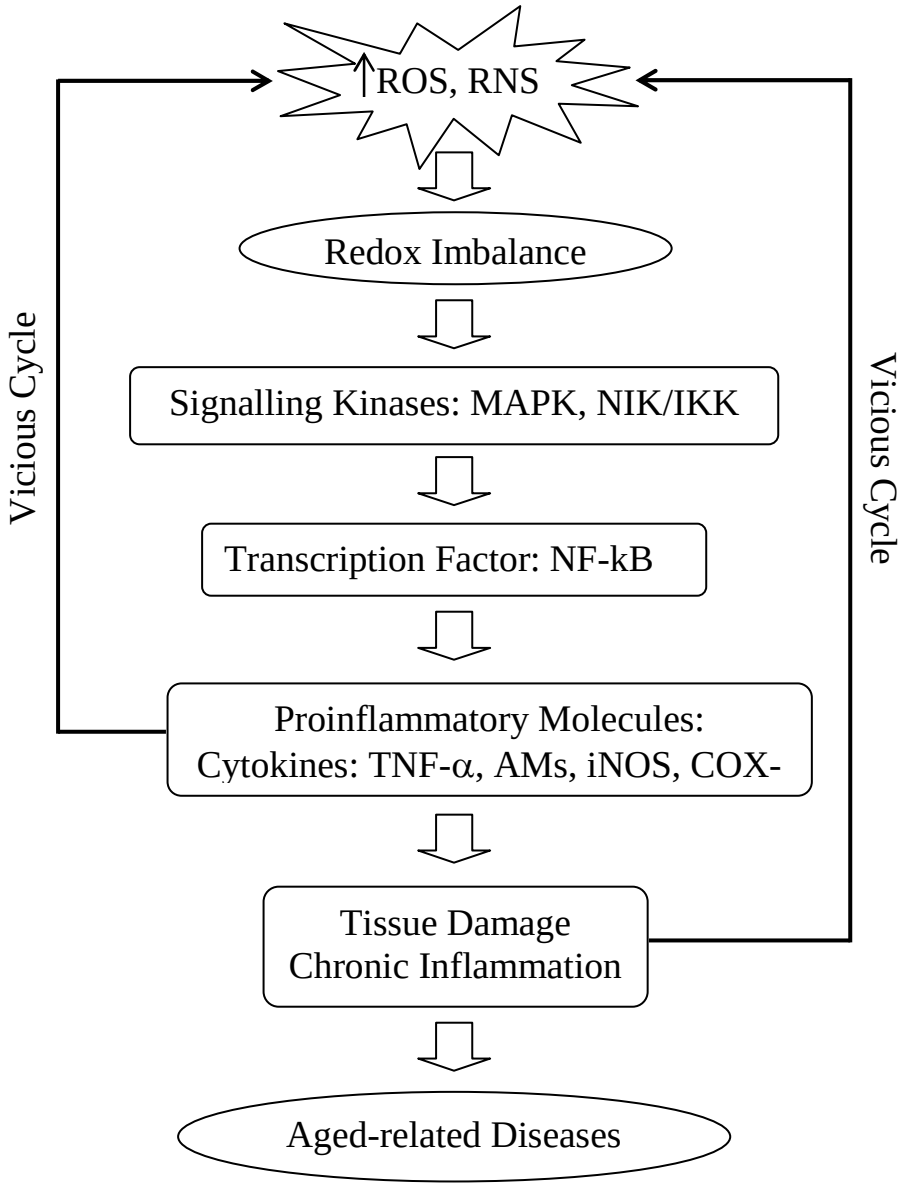


Figure 5.1 Major molecular pro-inflammatory pathways involved in aging and age related diseases. ROS, reactive oxygen species; RNS, reactive nitrogen species; MAPK, mitogen-activated protein kinases, NIK, NF-kB-induced kinase; IKK, IkB kinase; TNF-α, tumor necrosis factor-α; AMs, adhesion molecules; iNOS, inducible NO synthase; COX-2, cyclooxygenase (Modified from Chung et al., 2009).

Under normal conditions, NF-kB activation in response to oxidative stimuli is short-lived, and the reaction ceases with resolution. However, if the input signal is not well controlled as happens during aging, chronic pro-inflammatory conditions conducive to many chronic diseases ensues (Chung et al., 2006). Some of the NF-kB-induced proteins like TNF-α, IL-1, IL-6 and COX-2 themselves are potent NF-kB activators that form an auto-activating loop (Fisher et al., 1996; Handel et al., 1995). Many studies on changes in the redox sensitive transcription factor, NF-kB have consistently shown increased activity with age and in a variety of tissues, including heart, liver, kidney, and brain tissues, and high NF-kB binding activity when comparing old and young rodents (Helenius et al., 1996; Kim et al., 2002). Korhonen et al. (1997) also reported a significant upregulation of NF-kB in the rat brain (Korhonen et al., 1997). In human studies, circulating levels of proinflammatory cytokines that are well-recognized biomarkers increased during aging as shown by increased plasma levels of TNF-α and IL-6 (Bruunsgaard, 2006). Furthermore, aging is associated with increased levels of C-reactive protein (CRP), as well as high inflammatory cell (neutrophil, monocytes) counts (Bruunsgaard et al., 1999).

Effects of genistein on endothelial dysfunction in aging male rats

The present study showed that, the vasodilatory responses of the arterioles to Ach significantly increased in Aged+Gen when compared to Aged+Veh group (26.87±7.94% and 12.65±4.63%, respectively). Moreover, the percentage change of NO-associated fluorescent intensity was significantly increased in Aged+Gen as compared to Aged+Veh group (74.98±17.05% and 35.15±8.69%, respectively). The TNF-α level was significantly decreased in Aged+Gen when compared to Aged+Veh

group (11.16±5.68 pg/mL and 33.97±7.66 pg/mL, respectively). The MDA levels of Aged+Gen were significantly decreased when compared to Aged+Veh group (2.53±0.59 μmol/g liver wet weight and 4.81±1.17 μmol/g liver wet weight, respectively).

In 1999, the U.S. Food and Drug Administration recommended the daily ingestion of 25 g total soy protein, primarily due to reported beneficial effects on plasma lipid levels (e.g. lowered LDL, improved LDL/HDL ratios) thought to be crucial for prevention of coronary heart disease (1999). Genistein, a major isoflavone abundant in soy, has various biological actions including a weak estrogenic effect by binding to estrogen receptors (ERs), and inhibition of protein tyrosine kinases (PTK). Genistein consists of two aromatic rings linked through a heterocyclic pyrane ring (Ross and Kasum, 2002). The chemical structure of genistein is similar to that of 17β-estradiol (E2), the endogenous estrogen primarily acting through the ERs in humans. This structural similarity indicates that genistein could potentially bind to the ERs. Actually, genistein has long been known to exert estrogenic effect. Unlike E2 however, which binds to both ERα and ERβ with nearly equal affinity, genistein shows much higher affinity to ERβ (87% of E2) than to ERα (4% of E2) (Kuiper et al., 1998).

Furthermore, many investigators are interested in genistein's mechanism and how it is able to increase NO bioavailability (Squadrito et al., 2000; Walker et al., 2001). Walker et al. showed that genistein, 17β-estradiol, can produce acute NO-dependent vasodilatation which may affect endothelial nitric oxide synthase (eNOS) activity (Walker et al., 2001). On the other hand, it has been shown that genistein has a similar affinity as estrogen because of the novel ER-β present in the vasculature (Squadrito et al., 2000). The ER-β in endothelial cell has been shown to

stimulate NO-production which known as the non-genomic effect of estrogen (Guo et al., 2005). Interestingly, it has been shown that acute in responsed to topical application of genistein could also mediated via prostacyclin (PGI_2) and NO productions (Siriviriyakul et al., 2006).

Moreover, acute action of genistein on endothelial NO production was indicated for its activation through protein kinase A (PKA) which is unrelated to the estrogenic effect (Liu et al., 2004). It is also suggested that this pathway may be crucial in protecting the cardiovascular effects seen in soy phytoestrogens. Liu et al. (2004) demonstrated that 1-μM genistein could directly activate eNOS in intact bovine aortic endothelial cells and endothelial cells from human umbilical vein over an incubation period of 10 minutes. They also proposed that these effects were mediated by PKA and were unrelated to the estrogenic effect (Liu et al., 2004). Si and Liu (2008) showed that genistein had a direct genomic effect on the vascular wall causing an increased eNOS expression and NO synthesis in spontaneously hypertensive rat model (Si and Liu, 2008).

Borras et al. (2006) suggested that genistein exerts its antioxidant effect by binding to estrogen receptor(s), leading to the rapid activation of MAPK signalling pathways and upregulation of MnSOD gene expression (Figure 5.2). Genistein has been recently reported to have the direct scavenging activity towards superoxide. It has been purposed that genistein activates the cAMP/PKA cascades, ERK1/2, Akt and PPARs, which subsequently stimulate NO production by the direct activation of eNOS and/or stimulation of eNOS expression in vascular endothelial cells (ECs). Elevated NO inhibits TNF-α, VCAM-1 and MCP-1 expression and VSMCs proliferation. Activation of PPARγ by genistein also inhibits the expression of NAPDH oxidase, thereby reducing superoxide production. Consequently, the modulation of these molecules

by genistein will preserve endothelial functions and result in preventing inflammation and atherosclerosis (Figure 5.3) (Si and Liu, 2007).

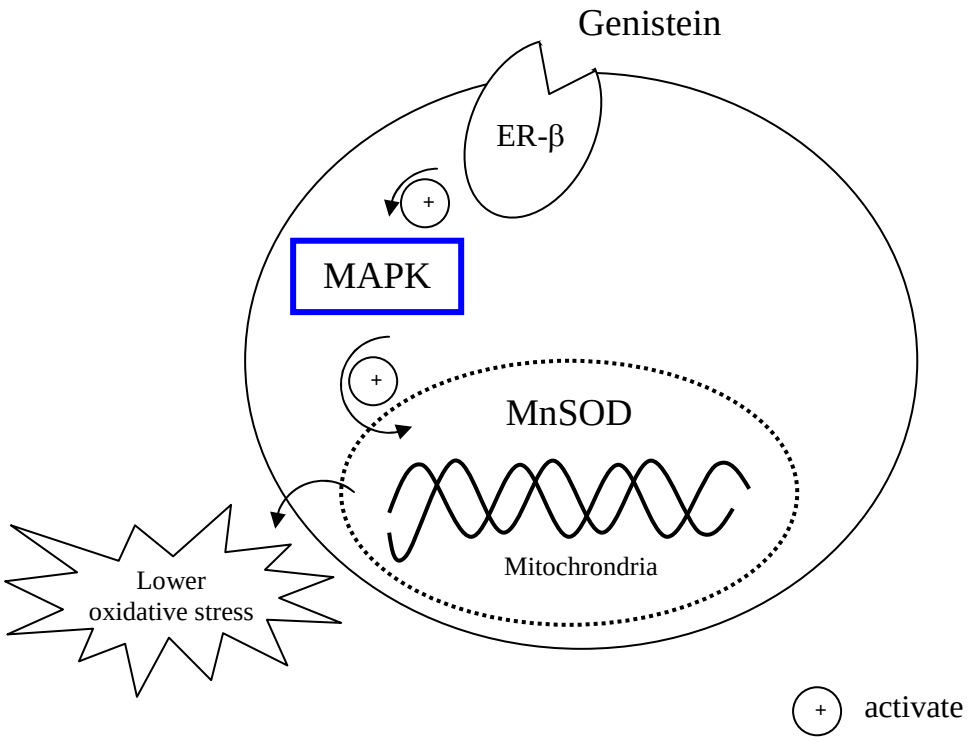


Figure 5.2 Scheme for genistein exerts its antioxidant effect by binding to estrogen receptor(s), leading to the rapid activation of MAPK signalling pathways and a delayed upregulation of MnSOD gene expression (Modified from Borras et al., 2006).

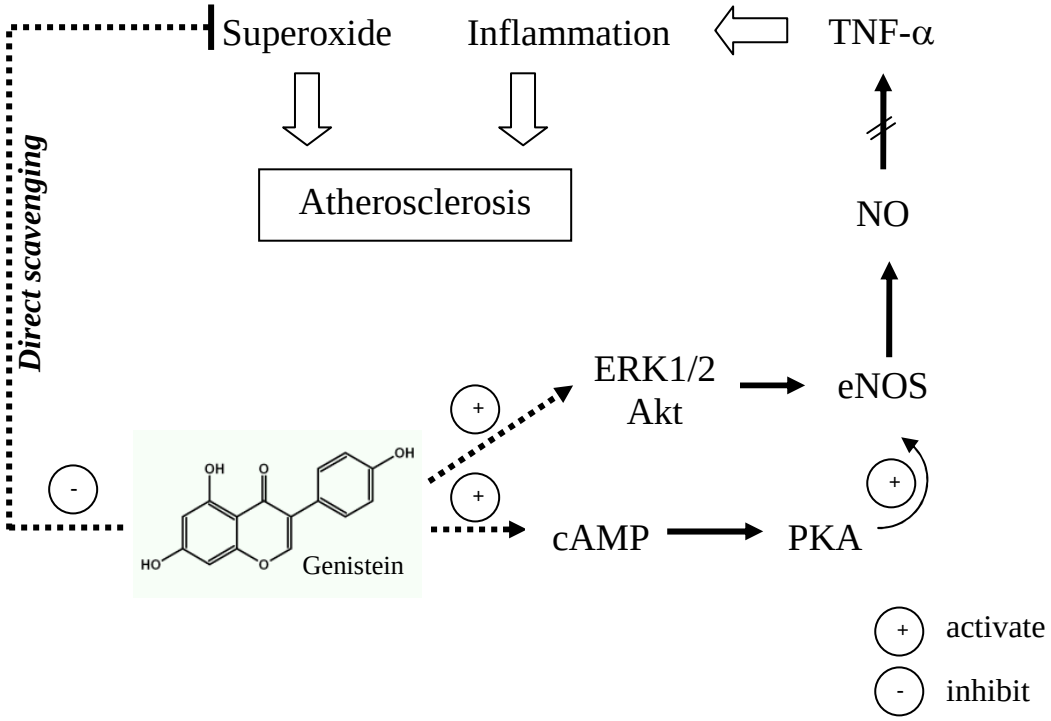


Figure 5.3 Scheme summarizing molecular targets for genistein in vasculature and consequent vasculoprotective effect (Modified from Si and Liu, 2007).

Although it is well known that NF-κB coordinates the induction of numerous proinflammatory genes, recent evidence establishes that activation of NF-κB modulates the expression of antioxidant genes, such as manganese superoxide dismutase (MnSOD) and heme oxygenase-1 (HO-1), and confers protection against apoptosis by upregulating antiapoptotic proteins (Collins and Cybulsky, 2001). In this context, induction of HO-1 by the dietary polyphenol resveratrol is abrogated by inhibitors of NF-κB activation or IκB phosphorylation (Baur et al., 2006) and deletion of NF-κB bindings sites in the HO-1 promoter reduces transcriptional activity (Juan et al., 2005). Thus, the modulation of NF-κB and Nrf2 signalling pathways by mitogen-activated protein kinases lead to transcriptional activation of antioxidant defense genes (Andreadi et al., 2006; Chen et al., 2005). According to Vina et al. (2006), it was

suggested that genistein increased the expression of MnSOD (Vina et al., 2006).

Since it is well known that age-induced oxidative stress can cause abnormal vascular functions by enhancing resistance to blood flow, hence, in this study, we measured the MDA levels in liver of the rats from the age-groups.

The results from the MDA levels showed that long-term treatment of genistein can scavenge the age-induced excess free radicals, especially superoxide anions which can directly damage molecules of protein, lipid, and DNA as which leads to cell dysfunction. Our observation of increased Ach-induced vasodilatation in Aged+Gen group confirms that anti-oxidant agent can prevent age-induced endothelial dysfunction. This ability may enhance endothelial-dependent vasodilatation by protecting NO from direct interaction with the superoxide radical. Since NO bioavailability is associated with endothelium-dependent vasodilatation, the greater NO bioavailability, therefore, a more pronounced endothelium-dependent vasodilatation effect will be observed. Interestingly, the present study showed that, in aged-treated with genistein could attenuate TNF-α level. Thus, we believed that it resulted from the increased NO bioavailability.

Effects of exercise training on endothelial dysfunction in aging male rats

The present study showed that, the dilatory responses of the arterioles to Ach significantly increased in Aged+Ex when compared to Aged+Without-Ex group (31.02±4.11% and 14.41±4.13%, respectively). The percentage change of NO-associated fluorescent intensity was significantly increased in Aged+Ex as compared to Aged+Without-Ex

group (76.34±17.82% and 39.68±7.15%, respectively). The TNF-α level was significantly decreased in Aged+Ex when compared to Aged+Without-Ex group (14.40±11.03 pg/mL and 36.29±14.41 pg/mL, respectively). The MDA level of Aged+Ex was significantly decreased when compare to Aged+Without-Ex group (2.51±0.49 μmol/g liver wet weight and 4.33±2.00 μmol/g liver wet weight, respectively).

Exercise training is well known to have beneficial effects across a broad spectrum of biological processes, including the favourable modulation of inflammatory signalling (Wannamethee et al., 2002). However, the response of the immune system to physical activity varies, depending on the frequency, intensity, volume of exercise, and on the subject's endurance capacity. Indeed, strenuous exercise has been shown to increase local and systemic production of proinflammatory cytokines, possibly as a consequence of muscle damage and subsequent inflammation (Chung et al., 2009).

On the other hand, moderate regular physical activity has been associated with reduced levels of TNF-α, IL-6, and CRP in a population of healthy older adults (Colbert et al., 2004). In addition, a recent study reported that aerobic exercise is associated with decrease serum levels of IL-6 and increased levels of IL-10, a potent anti-inflammatory cytokine, in healthy older men (Jankord and Jemiolo, 2004). Furthermore, recently demonstrated that life-long, voluntary wheel running reduces plasma levels of CRP, but not those of IL-6, in old rats (Kalani et al., 2006). The mechanisms underlying the anti-inflammatory effects of physical exercise are complex and not fully elucidated. However, it has been hypothesized that the reduced production of proinflammatory cytokines observed in response to regular physical exercise may stem at least partly from a reduction of adiposity (Colbert et al., 2004). Indeed, adipocytes are an

important source of TNF-α and IL-6 (Coppack, 2001). However, other mechanisms, independent of body composition, are thought to be involved in the modulation of inflammation by exercise training.

In 2005, Gomez-Cabrera et al., reported that ROS generated during exercise activate MAPKs (p38 and ERK1/ERK2), which in turn activate NF-κB, and resulted in increased expression of important enzymes associated with cell defense (MnSOD and glutathione peroxidase (GPx)) (Gomez-Cabrera et al., 2005). In addition, they also showed that the prevention of ROS formation by inhibition of xanthine oxidase (XO) could abolish these effects. To highlighting the role of ROS generated in moderate exercise training in the upregulation of antioxidant enzymes and, thus, the fact that moderate exercise training can behave as an antioxidant, the idea was shown by the diagram in Figure 5.4 (Gomez-Cabrera et al., 2008).

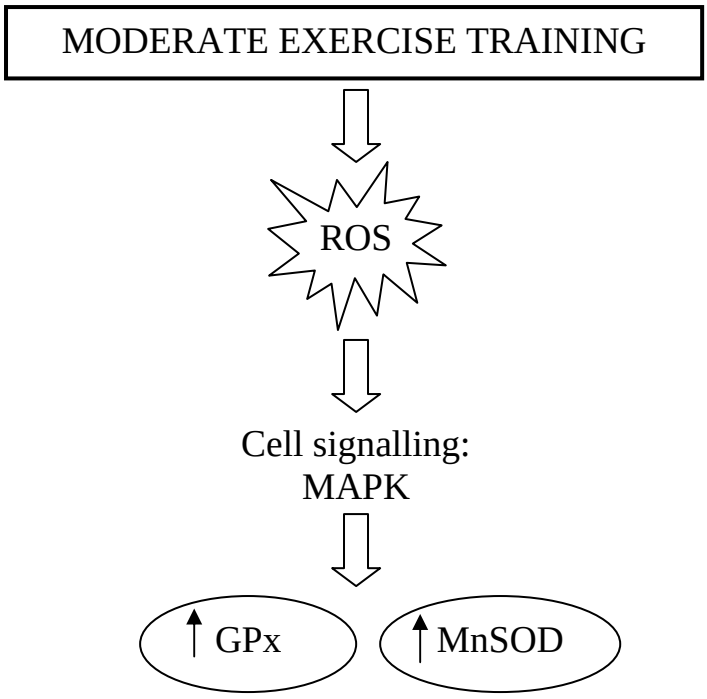


Figure 5.4 Mechanisms by which ROS activate the expression and activity of antioxidant enzymes. Exercise is an antioxidant (Modified from Gomez-Cabrera et al., 2008).

Aside from that, our findings indicated that genistein and exercise training could protect endothelial functions from age-induced dysfunction. A significant reduction in MDA levels in Aged+Ex group was observed when compared to the Aged+Without-Ex group. These results confirmed the effectiveness of our training protocol, 40 min/day, 5 day/week for a total of 8 weeks, in reducing peripheral vascular resistance in aged male rat. The following might help explain the benefits of long-term exercise training: 1) reduction of age-induced oxidative stress by increasing the enzymatic anti-oxidants expression, and 2) an increase in shear-stress mediated eNOS activity (Maiorana et al., 2003). Also Ahmadiasl and colleagues (2007) showed that long-term endurance training could increase superoxide dismutase (SOD) activities in rat myocardium (Ahmadiasl et al., 2007).

Nevertheless, in our study, a moderate exercise training program consisting of 8 weeks is sufficient enough to reduce age-induced endothelium dysfunction. It appears to us that the outcome of exercise training has the same effects seen in genistein. Exercise training could protect NO bioavailability in the Aged group and enhance endothelium-dependent vasodilatation. The reason for this may be that exercise training improves endothelial function and provides cardioprotective benefits.

Effects of combined treatment of genistein and exercise training on endothelial dysfunction in aging male rats

The result showed that, the dilatory responses of the arterioles to Ach significantly increased in Aged+Gen+Ex when compared to their age-matched control groups (30.02±12.14%, 12.65±4.63% and 14.41±4.13%, respectively). The percentage change of NO-associated fluorescent intensity was significantly increased in Aged+Gen+Ex as

compared to their age-matched control groups (70.22±16.05%, 35.15±8.69% and 39.68±7.15%, respectively). The TNF-α level was significantly decreased in Aged+Gen+Ex when compared to their age-matched control groups (10.25±5.91 pg/mL, 33.97±7.66 pg/mL and 36.29±14.41 pg/mL, respectively). The MDA level of Aged+Gen+Ex was significantly decreased when compare to their age-matched control groups (2.51±0.49 µmol/g liver wet weight, 4.81±1.17 µmol/g liver wet weight and 4.33±2.00 µmol/g liver wet weight, respectively). From these results could summarized as shown in Table 5.1.

	Aged+Veh/ Aged+Without-Ex	Aged+Gen	Aged+Ex	Aged+Gen+Ex
MAP	⇧	⇩	⇩	⇩
Testosterone	⇩	— (No effects)	— (No effects)	— (No effects)
Vascular response to Ach	⇩	⇧	⇧	⇧
NO production	⇩	⇧	⇧	⇧
Oxidative stress	⇧	⇩	⇩	⇩
TNF-α level	⇧	⇩	⇩	⇩

Table 5.1 Summary of the results from Aged+Sedentary and aged treated with genistein, exercise training and combination with genistein and exercise training.

In the present study, we have shown that genistein, 8-week exercise training, and combined treatments of genistein and exercise training could reduce the age-induced abnormalities, including hypertension, age-induced endothelial dysfunction, age-induced oxidative stress and age-induced TNF-α expression. Moreover, our finding also demonstrated the linear correlation between endothelial-dependent vasodilatation improvement and NO bioavailability increment. Therefore, it implied that beneficial effects of genistein and exercise training on age-induced endothelial dysfunction resulting from increase NO bioavailability.

Our result confirmed that both testosterone levels and ratios of seminal weight/BW were significantly decreased by aging. Genistein supplementation and exercise training did not affect those parameters. Other studies have reported similar findings that low doses of phytoestrogens do not have any effects on reproductive functions of either males or females (Anthony et al., 1996; Mitchell et al., 2001). However, the increased seminal vesicle/BW ratio in Aged+Ex and Aged+Gen+Ex groups may be resulted by the metabolic effect of exercise training on fat composition leading to weight loss in these group.

Kashiwagi et al. (2002) showed a linear relationship between the gray levels of fluorescence intensities and DAF-2T concentrations (between 10 nmol/L and 1 μmol/L) (Kashiwagi et al., 2002). From our results demonstrated that aging resulting in increased oxidative stress, decreased Ach-induced arteriolar response and decreased NO bioavailability. Therefore, we investigated whether there are any correlation between NO-associated fluorescent intensity and the Ach-evoked vasodilatation in all five groups. Our correlation results between the means percent changes of NO-associated fluorescent intensity and the

mean percent of arteriolar diameter changes from all 5 groups were significantly correlated and could be fitted by a linear line: y = 0.4276x – 2.0966 (Figure 4.15) (Pearson's correlation = 0.82, $P<0.01$). These findings suggested that aged-induced reduction of Ach-activated arteriolar dilatation could be restored by using genistein and exercise training in relation with endothelial NO bioavailability.

The results obtained showed an increase of NO bioavailability after utilizing two interventions. Both of these interventions have multiple functions effecting eNOS expression, eNOS activity, and eNOS cofactor; tetrahydrobiopterin (BH_4). Both interventions can increase eNOS expression through both direct and indirect pathways (Liu et al., 2004; Si and Liu, 2008; Sindler et al., 2009; Tanabe et al., 2003). Similarly, Tanabe and his colleagues (2003) reported that the 8-week exercise swimming could up-regulate eNOS expression in the aorta in aged rats (Tanabe et al., 2003). Along the same lines, Sindler et al. (2009) suggested that exercise training can restore the important eNOS cofactor, tetrahydrobiopterin (BH_4), content and then leads to enhance flow-stimulated NO availability in old rats (Sindler et al., 2009).

Our results may be the first *in vivo* evidence of genistein and exercise training in protecting endothelial cells against age-induced oxidative stress by detecting *in situ* NO released using a fluorescent indicator-diaminofluorescein (DAFs). The *in situ* release of NO from cremasteric endothelial cells after acetylcholine activation was examined in both genistein and exercise training groups. The effect of genistein and exercise training on increased NO bioavailability may be associated with multiple direct and indirect pathways, therefore, additional studies are needed to confirm these molecular mechanisms.

However, the present study does not show the synergistic effects of genistein and exercise training, Figard et al., demonstrated that swimming

exercise and dietary supplementation with soy proteins did not have synergistic effects in ovariectomized rats (Figard et al., 2006).

Based on the summary data shown in Table 5.1, it implied that genistein and exercise training could protect endothelial function against age-induced oxidative stress via similar mechanisms. Our findings showed that genistein and exercise training improved age-induced endothelial dysfunction related to the increased NO bioavailability, decreased oxidative stress and TNF-α production. Therefore, the proposed mechanisms from our study could be summarized as shown in Figure 5.5.

Findings of the present study could be summarized and brought to the proposed mechanisms shown in Figure 5.5. There are three major factors which involved age-induced endothelial dysfunction in male rats, including decreased testosterone level, increased oxidative stress, and increased TNF-α level. The ROS and TNF-α decreased the bioavailability of NO and resuting in endothelial dysfunction. However, genistein, exercise training and their combination have potential to decrease oxidative stress and increase NO bioavailability through partially similar mechanisms. In the present study, genistein and exercise training could increase antioxidant expression and NO bioavailability in equally manner. However, genistein could directly scavenge superoxide, increase MnSOD expression via MAPK pathway, bind the selective ER-β and activate eNOS expression via ERK1/2, Akt and PKA pathways. Even though, exercise training could increase MnSOD expression via MAPK pathway, but exercise training could activate eNOS expression by different pathway of shear-stress activated AKt signalling. With these signalling processes, it implied that both genistein and exercise training have shared their benefit actions by using the same mediators including

MAPK, Akt and eNOS. Therefore, the combination of genistein and exercise training did not show more effects on protecting endothelial function against aging when compared to each intervention. We believe that this is the reason to simply explain our finding of the present study. However, it is noted that the advance aging process may cause the increase in oxidative stress more and more. Genistein or exercise training may be not enough for protecting endothelial function. In case of this situation, the combination of genistein and exercise training may give the better beneficial effect against age-induced endothelial dysfunction.

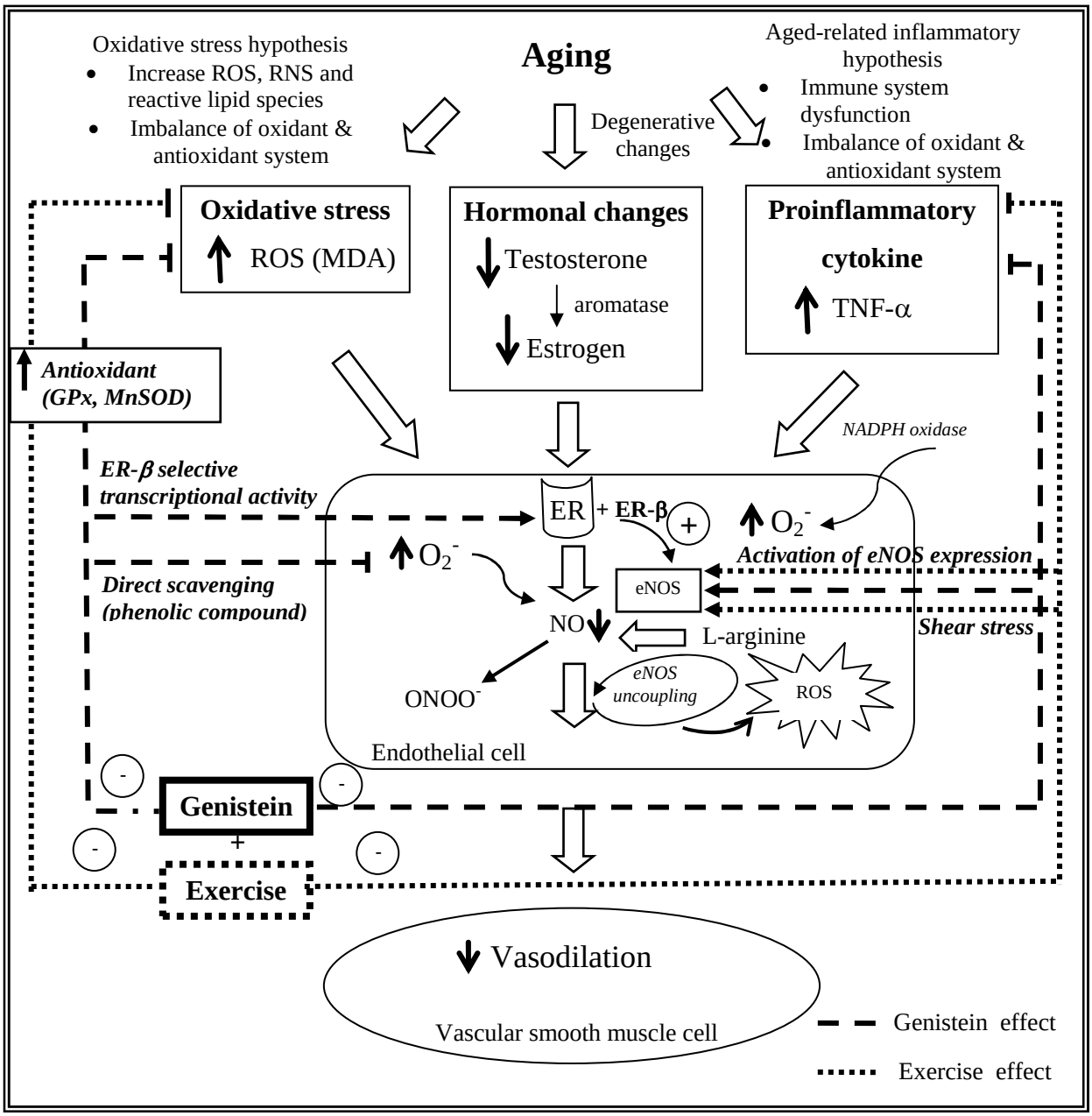


Figure 5.5 The proposed mechanisms from the present study is that genistein and exercise training could ameliorate age-induced endotheial dysfunction by increasing NO bioavailability, decreasing oxidative stress and TNF-α production.

CHAPTER VI

CONCLUSION

In the present study, the effects of genistein and exercise training on endothelial dysfunction in aging male rats were studied. The significant findings could be summarized as follows:

1. Mean arterial blood pressure (MAP) was significantly elevated in aged rat as compared to young rat.
2. Testosterone level was significantly decreased in aged rat as compared to young rat.
3. Oxidative stress, represented by liver malondialdehyde (MDA), was significantly increased in aged rat as compare to young rat.
4. Tumor necrosis factor-α (TNF-α) level was significantly increased in aged rat as compared to young rat.
5. Age-induced endothelial dysfunction could be demonstrated by decreased Ach-activated vascular dilation and NO production.
6. Genistein supplementation for 8 weeks has significantly not only improved MAP, endothelium-dependent vasodilation and NO production, but decreased oxidative stress and TNF-α also.
7. A moderate exercise training program consisting of 8-week swimming could reduce age-induced endothelium dysfunction.
8. Exercise training could significantly improve MAP, decrease oxidative stress and serum TNF-α levels.
9. It appeared that both genistein and exercise training gave the same effects on protecting endothelial function via their antioxidant and anti-inflammation against aging process.

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APPENDICES

APPENDIX A

LIST OF PUBLICATIONS

- **Eksakulkla S**, Suksom D, Siriviriyakul P, Patumraj S. (2008) Genistein and exercise training could protect endothelial functions against aging process. *Poster presentation*, The 7th Asian Congress for Microcirculation in corporation the 6th Chinese National Congress for Microcirculation. (Abstract)
- **Eksakulkla S**, Suksom D, Siriviriyakul P, Patumraj S. (2008) Genistein and exercise training could protect endothelial functions against aging process. *Oral presentation*, The 11th Annual Meeting of Thai Society of Microcirculation (TSM). (Abstract)
- **Eksakulkla S**, Suksom D, Patumraj S, Siriviriyakul P**. (**2009) Effects of genistein and exercise training on endothelial dysfunction in aging male rats. *Oral and Poster presentation*, Joint Conference in Medical Sciences (JCMS'09). (Abstract)
- **Eksakulkla S**, Suksom D, Siriviriyakul P, Patumraj S. Increased NO bioavailability in aging male rats by genistein and exercise training: using 4, 5-diaminofluorescein diacetate. Reprod Biol Endocrinol. 2009 Sep 7; 7(1): 93. [Epub ahead of print]
- Viboolvorakul S, Niimi H, Wongeak-In N, **Eksakulkla S**, Patumraj S. Increased capillary vascularity in the femur of aged rats by exercise training. Microvasc Res. 2009 Aug 6. [Epub ahead of print]

APPENDIX B

FURTHER INFORMATIONS FOR METHODOLOGY

Exercise protocol

Week	Stage
1	Day 1: swim 10 min Day 2: swim 10 min Day 3: rest Day 4: swim 20 min Day 5: swim 30 min Day 6: swim 40 min Day 7: rest
2-8	Day 1: swim 40 min Day 2: swim 40 min Day 3: rest Day 4: swim 40 min Day 5: swim 40 min Day 6: swim 40 min Day 7: rest

Tumor necrosis factor (TNF)-α assay

Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α has been pre-coated onto a microplate. Standard and samples are pipetted into the wells and any rat TNF-α present is bound by the immobilized specific for rat TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of rat TNF-α bound in the initial step. The sample values are the read off the standard curve.

List of reagents

- Rat TNF-α conjugate
- Rat TNF-α standard
- Assay diluent RD1-41
- Calibrator diluent RD5-17
- Wash buffer
- Color reagent A
- Color reagent B
- Stop solution

Reagent preparation

- Wash buffer

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough wash buffer for one plate, add 25 mL wash buffer concentrate into deionized or distilled water to prepare 625 mL of wash buffer.

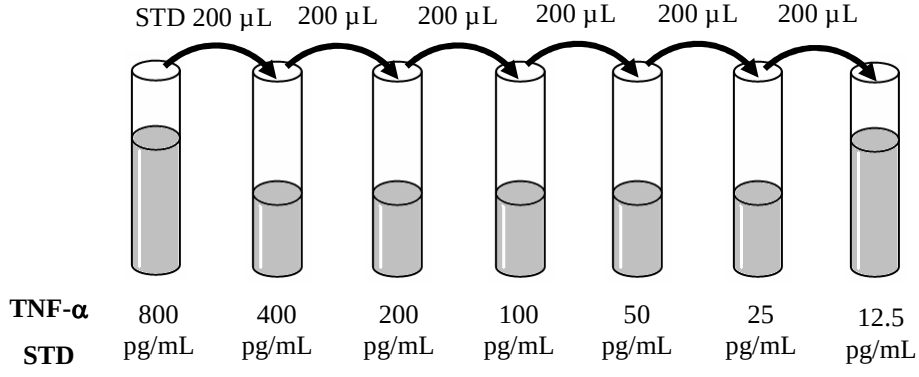
- Substrate solution

Color reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of resultant mixture is required per well.

- TNF-α standard

Reconstitute the rat TNF-α standard with 2.0 mL of Calibrator diluent RD5-17. Do not substitute other diluents. This reconstitution produces a stock solution of 800 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator diluent RD5-17 into each tube. Use stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted rat TNF-α standard serves as the high standard (800 pg/mL). Calibrator diluent RD5-17 serves as the zero standard (0 pg/mL).



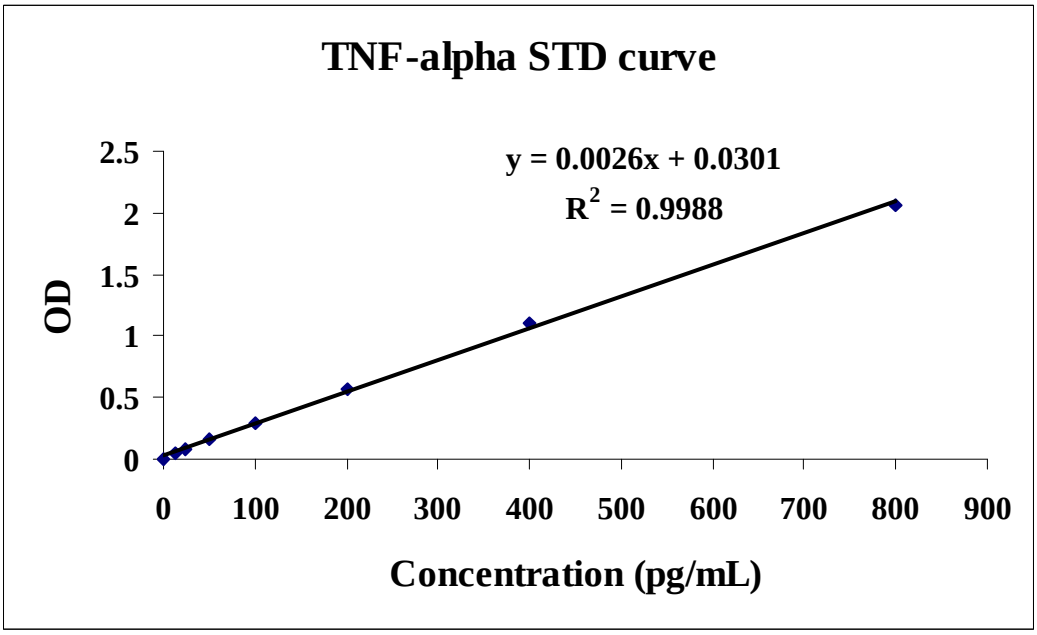
Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 50 µL of assay diluent RD1-41 to each well.
4. Add 50 µL of standard, or sample to each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with wash buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
6. Add 100 µL of rat TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of substrate solution to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 100 µL of stop solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available,

subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate.

Readings made directly at 450 nm without correction may be higher and less accurate.



The sample of standard curve for assay TNF-alpha level

Malondialdehyde (MDA) assay

Principle of the assay

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

Reagents

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

Dissolve SDS 8.1 g in distilled water and allow to stand overnight at room temperature until it is dissolved. Then make up to 100 ml. Do not shake because this solution will produce a lot of bubbles.

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

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

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

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

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

TMP is used as an external standard. The level of lipid peroxide is expressed as nmole of MDA. Prepared stock 10^3 nmole TMP with distilled water, then pipette 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 ml of this stock TMP solution and add distilled water to 10 ml in each concentration. These will give the following concentration of standand TMP: 2, 4, 6, 8, 10 and 12 nmole/ml. Prepare stock TMP fresh.

5. 1.15% (w/v) KCl

Dissolved KCl 11.50 g in 1,000 ml of distilled water and mix throughly.

Procedure

1. After washing the isolated tissues in ice-cold 0.9% (w/v) NaCl, the liver is prepared by homogenizing each gram of wet tissue in 9 ml of 1.15% KCl.
2. Pipette the following solutions into a series of glass tubes with screw caps:

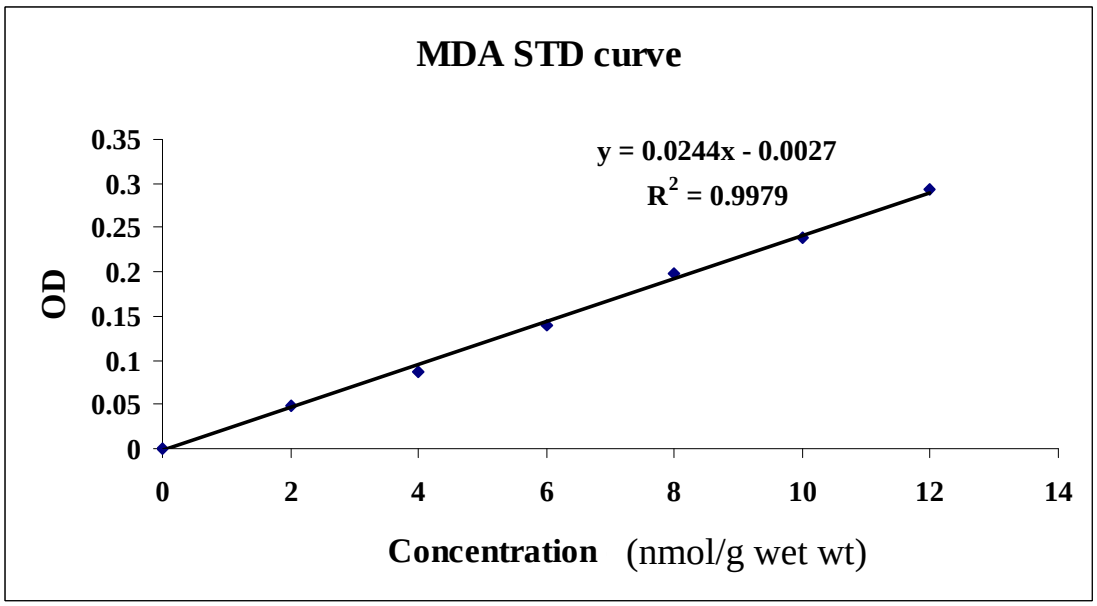
Solution	Blank (ml)	Standard (ml)	Unknown (ml)
Sample	-	-	0.2
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH 3.5)	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5
TMP stock standard	-	0.2	-
Distilled water	0.8	0.6	0.6

3. Heated the tubes in the water-bath at 95°C for 60 min.

4. After cooling the tubes by immersion with tab water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 (v/v)) are added and shaken vigorously (at least 1 min).
5. After centrifugation at 4,000 rpm for 10 min, the organic layer is removed and its absorbance at 532 nm is measured
6. The content of lipid peroxide is expressed in terms of nmole MDA/g wet wt., converting the O.D. reading using the data generated as the calibration curve, below.

Calibration curve

1. Prepare a series of tube containing TMP stock standard in water in the following concentrations: 2, 4, 6, 8, 10 and 12 nmole/ml. Perform the procedure as in step 2, above. Determine the absorbance at 532 nm. Then plot the optical density versus nmole of MDA of tissue homogenate.



The sample of standard curve for assay MDA level

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