EFFECTS OF FEMALE SEX HORMONES ON MYOCARDIAL STIFFNESS IN DIABETIC-OVARIECTOMIZED RATS

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Thesis
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EFFECTS OF FEMALE SEX HORMONES ON MYOCARDIAL STIFFNESS IN DIABETIC OVARIECTOMIZED RATS

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ABSTRACT

Suppression of both systolic and diastolic functions of the heart after menopause leads to the suggestion that female sex hormones might exert a protective role on cardiac function. Based on clinical reports of reduced ventricular filling velocity in postmenopausal women, whether female sex hormones play a regulatory or preventive role in the expansion ability of the myocardial tissue was examined. As the expansion or stiffness of the heart could be ultimately determined by extracellular collagen and sarcomeric structural protein titin, changes of these two proteins were detected in the left ventricle of ovariectomized (OVX), diabetic (DM) and diabetic-ovariectomized (DM-OVX) rats. Results showed no significant difference in both collagen deposition and titin isoform ratio in the heart between sham control and OVX rats. Interestingly, significant increase in compliant N2BA titin isoform was detected in the heart of DM-OVX rats while slightly increased in DM hearts. In contrast, collagen content was significantly increased in DM hearts by 63% compared to sham, but not in DM-OVX rats. In addition, estrogen supplement, but not progesterone, significantly induced an increase in collagen deposition in DM-OVX rat hearts. Increased collagen content in DM hearts was also supported by the upregulation of procollagen and its transcriptional factor Smad2. Moreover, increased matrix metalloproteinase type 2 indicated an enhanced collagen turnover in DM hearts. In conclusion, the study proved that female sex hormones exert regulatory effects on molecular structures representing stiffness of the heart under pathological insult of diabetes. It is possible that female sex hormones activate an extracellular matrix remodeling process and inhibit a shift of titin isoform toward dilated heart.

KEY WORDS: FEMALE SEX HORMONES/ DIABETES/ COLLAGEN/ TITIN

71 pages

ผลกระทบของฮอร์โมนเพศหญิงต่อความฝืดแข็งของกล้ามเนื้อหัวใจในหนูตัดรังไข่เป็นเบาหวานร่วม
EFFECTS OF FEMALE SEX HORMONES ON MYOCARDIAL STIFFNESS IN DIABETIC OVARIECTOMIZED RATS

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บทคัดย่อ

การทำงานของหัวใจที่ลดลงในหญิงวัยหมดประจำเดือนชี้ให้เห็นว่าฮอร์โมนเพศหญิงน่าจะมีบทบาทในการปกป้องการทำงานของหัวใจได้ จากรายงานทางคลีนิคถึงความเร็วในการไหลกลับของเลือดเข้าหัวใจห้องล่างที่ลดลงในหญิงวัยหมดประจำเดือนนำมาสู่แนวทางในการศึกษาบทบาทฮอร์โมนเพศหญิงต่อการควบคุมและปกป้องความสามารถในการขยายตัวของเนื้อเยื่อหัวใจ เพื่อศึกษาความฝืดแข็งของเนื้อเยื่อหัวใจ ปริมาณคอลลาเจนและโปรตีนโครงสร้างไททินได้ถูกวัดในหนูตัดรังไข่ หนูเบาหวาน และหนูตัดรังไข่เป็นเบาหวานร่วม ผลการศึกษาพบว่าไม่มีการเปลี่ยนแปลงปริมาณคอลลาเจนและชนิดของไททินในหนูตัดรังไข่เมื่อเทียบกับหนูควบคุม แต่พบการเพิ่มขึ้นของไททินชนิดที่ให้แรงหย่อนตัวสูง(เอนทูบีเอ)ในหนูตัดรังไข่เป็นเบาหวานร่วมอย่างมีนัยสำคัญ แต่มีการเพิ่มเพียงเล็กน้อยในหนูเบาหวาน ในทางกลับกันหนูเบาหวานมีปริมาณคอลลาเจนในเนื้อเยื่อหัวใจมากขึ้นประมาณร้อยละ 63 ของหนูควบคุม แต่ไม่มีการเปลี่ยนแปลงในหนูตัดรังไข่เป็นเบาหวานร่วม การเพิ่มขึ้นของคอลลาเจนยังสามารถพบได้ในหนูตัดรังไข่เป็นเบาหวานร่วมที่ได้รับเอสโตรเจนทดแทน แต่ไม่พบผลนี้ของการให้โปรเจสเตอโรนทดแทนอย่างเดียว การเพิ่มปริมาณคอลลาเจนในเนื้อเยื่อหัวใจได้รับการยืนยันจากการเพิ่มขึ้นของโปรตีนโปรคอลลาเจนและโปรตีนเหนี่ยวนำการสร้างคอลลาเจนสแมจทูในหัวใจ นอกจากนี้ยังพบว่าในหัวใจหนูเบาหวานมีปริมาณเอ็นไซม์แมทริคเมทอลโลโปรติเนสเพิ่มขึ้นด้วย ซึ่งบ่งชี้ว่าน่าจะมีอัตราการสร้างและสลายคอลลาเจนที่มากขึ้นในหัวใจ โดยสรุป การศึกษานี้ชี้ให้เห็นว่าฮอร์โมนเพศหญิงมีบทบาทในการควบคุมโครงสร้างที่ส่งผลต่อความฝืดแข็งของเนื้อเยื่อหัวใจในภาวะเบาหวาน โดยอาจเป็นไปได้ว่าฮอร์โมนเพศหญิงกระตุ้นการสร้างคอลลาเจนและชะลอการเปลี่ยนแปลงชนิดของไททินเพื่อป้องกันการเกิดภาวะห้องหัวใจขยายตัวผิดปกติ

71 หน้า

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LIST OF ABBREVIATIONS

μg	microgram
μM	micromole per liter
ACE	angiotensin converting enzyme
ANOVA	analysis of variance
ANF	atrinatriuretic factor
AngII	angiotensin II
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
BW	body weight
Ca^{2+}	calcium
Dl	deciliter
DM	diabetes mellitus
DM-OVX	diabetic-ovariectomized
E_2	17β-estradiol
ECM	extracellular matrix
ER	estrogen receptor
g	gram
Mg^{2+}	magnesium
MHC	myosin heavy chain
MI	myocardial infarction
min	minute
ml	milliliter

LIST OF ABBREVIATIONS (cont.)

mM	millimole per liter
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Na^+	sodium
OVX	ovariectomized
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error of mean
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SHAM	sham-operated
SR	sarcoplasmic reticulum
STZ	streptozotocin
TGFβ	transforming growth factor beta
TβR	transforming growth factor beta receptor
TIMP	tissue inhibitor metallproteinase
UW	uterine weight
wk	week

CHAPTER I
THEME OF THESIS

An upsurge in the incidence of cardiovascular disease in women after menopause revealed a significant protective role of female sex hormones in the hearts (Kangro *et al.*, 1995; Schillaci *et al.*, 1998; Rosamond *et al.*, 2007). In premonopausal women, the incidence of heart diseases is significantly lower than age-matched men. However, this benefit disappears after menopause which then suggests that the deprivation of female sex hormones causes risks of cardiovascular dysfunction and heart failure (Rosamond *et al.*, 2007). Suppressions of both systolic and diastolic functions after menopause compared to premenopausal women have been clearly demonstrated by echocardiographic studies (Kangro *et al.*, 1995; Schillaci *et al.*, 1998). A greater left ventricular wall thickness with a lower mid-wall fractional shortening early after menopause indicated systolic dysfunction (Schillaci *et al.*, 1998). A diastolic dysfunction was also demonstrated by a lower left ventricular filling in postmenopausal women compared to age-matched premenopausal women (Kangro *et al.*, 1995). All changes could be improved in postmenopausal women who received hormone replacement therapy (Alecrin *et al.*, 2004; Ozdemir *et al.*, 2004). In addition, many recent experimental studies demonstrated that estrogen and its derivatives could prevent cardiac changes induced by pathological insults such as pressure overload and ischemic reperfusion injury (Gardner *et al.*, 2008; Jeanes *et al.*, 2008). Unfortunately, the failure in protecting heart disease incidence by hormone replacement therapy reported by studies from clinical trails (Rossouw *et al.*, 2002) has initiated the search for more understanding regarding mechanistic signals of female sex hormones on the heart. Effect of hormones replacement therapy on cancer induction further urges for more basic studies (Rohan *et al.*, 2008). Therefore, understanding the underlying mechanism of female sex hormones on the heart will lead to approvals for novel treatment and prevention of heart disease, as well as avoiding the adverse effect of hormones.

Cardiac specific and distinctive roles of female sex hormones have been demonstrated in cardiac contractile function. Expressions of both estrogen and progesterone receptors are found in both cardiac myocytes and fibroblasts when then support the direct action of female sex hormones on cardiac function (Grohe *et al.*, 1997; Watanabe *et al.*, 2003). Using ovariectomized (OVX) model, Schaible and coworker (1994) first demonstrated a reduction of ventricular contraction in OVX rats (Schaible *et al.*, 1984). Our group has further demonstrated that the suppressed cardiac contraction is a result from a suppression of maximum myofibrillar Ca^{2+} activation and a reduction in intracellular Ca^{2+} transient amplitude (Wattanapermpool, 1998; Wattanapermpool & Reiser, 1999; Bupha-Intr *et al.*, 2007). Interestingly, we have found that chronic deprivation of female sex hormones also induced an increase in Ca^{2+} responsiveness of myofibrillar activation, which is specific only in cardiac muscle and is very exclusive in OVX condition even under condition challenged with diabetes-induced cardiomyopathy (Wattanapermpool & Reiser, 1999; Thawornkaiwong *et al.*, 2007). Our experimental series thus identified the specific effect of female sex hormones on cardiac contractile machinery in regulating systolic function. Role of female sex hormones on diastolic function of the heart that is interesting since it is consequently associated with changes in systolic function.

Diastolic dysfunction leads to a difficulty in myocardial chamber expansion during diastole and therefore affects ventricular filling and eventually end diastolic volume. Cardiac diastole can be divided into two major phases, myocardial relaxation and ventricular filling. Although the relaxation phase of myocardium is largely concerned with ventricular pressure reduction, there is passive flow of blood into the ventricles during the phase of ventricular filling. The filling capacity is mainly affected by compliance of the chamber tissue or stiffness of the heart. Our group has first demonstrated effects of female sex hormone on relaxation using ovariectomized model (Bupha-Intr & Wattanapermpool, 2006; Bupha-Intr *et al.*, 2007). We found that chronic deprivation of ovarian sex hormones caused a prolonged relaxation of cardiac myocyte by suppressing the activity, the protein and the mRNA expressions of SERCA2a. An increased Ca^{2+} responsiveness of myofibrillar activation in the heart of ovariectomized rats leads to difficulty in relaxation of cardiac muscle (Wattanapermpool, 1998). All changes during relaxation

period could be prevented by estrogen supplementation (Wattanapermpool *et al.*, 2000; Bupha-Intr & Wattanapermpool, 2006; Bupha-Intr *et al.*, 2007). Our results clearly proved that female sex hormones play a significant role in the relaxation period of cardiac diastole. Although relaxation phase of myocardium is largely dealt with ventricular pressure reduction, there is passive flow of blood into the ventricle during the phase of ventricular filling. While the filling capacity is mainly affected by compliance of the chamber or stiffness of the heart, role of female sex hormones in ventricular filling is still unclear. As mentioned that ventricular filling is determined by passive flow of blood into ventricle and the compliance of the myocardial chamber, this study was then focused on evaluating the role of female sex hormones on the compliance of the heart under both normal and pathological conditions.

In this study we challenged the heart by diabetic condition to test the preventive role of female sex hormones on structural related stiffness of the heart since diabetes has been clearly shown to induce collagen deposition in myocardial tissue (Li *et al.*, 2007; Van Linthout *et al.*, 2008). Diabetes also induced hypothyroidism in which, a shift in a titin isoform from N2B, a higher stiffness, to N2BA a lower stiffness, was detected (Wu *et al.*, 2007).

The goal of this thesis project was set to understand the cellular and subcellular adaptation of myocardial tissue after chronic female sex hormone deprivation with and without diabetic complication. Three specific objectives were raised in this study.

1. To assess the effect of female sex hormones in regulating collagen deposition and titin isoforms expression
2. To evaluate the difference in collagen deposition and titin isoforms expression in diabetic hearts in the presence and absence of ovarian sex hormones
3. To test whether estrogen and progesterone supplements could prevent the changes in collagen deposition and titin isoforms expression in diabetic-ovariectomized rats

The percent tissue fraction of collagen in the heart and the ratio of N2BA to N2B titin isoform were evaluated to determine the structural component reflecting myocardial stiffness using histochemistry and electrophoresis techniques, respectively.

Possible mechanisms activating the changes of collagen deposition including a collagen precursor procollagen, collagen gene transcriptional factor Smad2, and extracellular matrix degrading enzyme MMPs were also detected their amount of expression in the heart of ovariectomized with and without diabetic complication.

CHAPTER II
LITERATURE REVIEW

A. Effects of female sex hormones on cardiac function

Presence of estrogen and progesterone receptors in the cardiac myocytes indicates the credible direct effects of female sex hormones on cardiac contractile function (Ingegno *et al.*, 1988; Grohe *et al.*, 1997; Grohe *et al.*, 1998). Estrogen and progesterone are major female sex hormones and they can give the genomic and non-genomic effects (Babiker *et al.*, 2002; Li & O'Malley, 2003).

Impact of female sex hormones on cardiac function becomes interesting subject because epidemiologic studies demonstrated a dramatic increase of heart disease incidence in women after menopause (Rosamond *et al.*, 2007). In premenopausal women, the incidence of heart diseases is significantly lower than age-matched men. The disappearance of this benefit after menopause suggested that the deprivation of female sex hormones can cause risks of cardiovascular dysfunctions. In postmenopausal women, suppression of both systolic and diastolic functions after menopause has been clearly demonstrated as compared to age-match premenopausal women (Kangro *et al.*, 1995; Schillaci *et al.*, 1998). A greater left ventricular wall thickness with a lower mid-wall fractional shortening has been observed early after menopause (Schillaci *et al.*, 1998). In addition, the postmenopausal women have reduced peak early velocity E wave and E/A ratio compared with the premenopausal women, indicating the impairment of relaxation (Kangro *et al.*, 1995). Hormone replacement therapy could improve all these changes in postmenopausal women (Alecrin *et al.*, 2004; Ozdemir *et al.*, 2004).

As there is an adverse effect of hormone replacement therapy such as cancer induction, a tremendous amount of studies concerning with the role of female sex hormones in cardiac function was raised. Schaible and coworkers (1984) first demonstrated a reduction of ventricular contraction in OVX rats. Stroke volume and ejection fraction were reduced in both pre-pubertal and post-pubertal ovariectomized

rats (Schaible *et al.*, 1984; Scheuer *et al.*, 1987). Our group has further demonstrated that the suppressed cardiac contraction is a result of a suppression of maximum myofibrillar Ca^{2+} activation and a reduction of intracellular Ca^{2+} transient amplitude as well (Wattanapermpool, 1998; Wattanapermpool & Reiser, 1999; Bupha-Intr *et al.*, 2007). Interestingly, we have demonstrated that chronic deprivation of female sex hormones caused increase in Ca^{2+} responsiveness of myofibrillar activation which is specific only on cardiac muscle (Wattanapermpool & Reiser, 1999). It was also found that these changes were prevented by estrogen supplementation (Wattanapermpool *et al.*, 2000). These changes are very exclusive in ovarian sex hormone deficient condition even under challenging with diabetes (Thawornkaiwong *et al.*, 2007).

Although diastolic heart failure was taken into granted for just having passive function, it contributes the similar proportion to systolic heart failure, leading to hospitalization. Diastole is the process returning to its relaxed state and can be identified as the four phases; isovolumic relaxation from the moment of aortic valve closure to mitral valve opening; early rapid mitral valve opening; diastasis, a relatively constant low flow of blood at the middle of the diastole; and the late filling of the ventricles resulting from atrium contraction. Diastole can be determined, in general, by the rate of cytosolic Ca^{2+} removal, the rate of actin-crossbridge detachment, and the ability of myocardial re-expansion. Rate of cytosolic Ca^{2+} removal is dependent on a work load of sarcoplasmic reticulum Ca^{2+}/ATPase (SERCA) in re-uptaking Ca^{2+} and Na^{+}-Ca^{2+} exchanger (NCX) in extruding Ca^{2+}. SERCA contribute 70% and 92% of calcium transport in human and rat myocytes, respectively (Bers, 2000; Bassani *et al.*, 2004). Our group has demonstrated that chronic deprivation of ovarian sex hormones caused prolonged cardiac myocyte relaxation by suppressing the activity, the protein and also the mRNA expression of SERCA2a (Bupha-Intr & Wattanapermpool, 2006). In addition, we have demonstrated an increase in Na^{+}- H^{+} exchange activity in the cardiomyocytes of ovariectomized rats which can directly modulate the Ca^{2+} removal through NCX.

Altered rate of actin-crossbridge detachment after ovarian sex hormones deprivation has been proposed by our finding of increased Ca^{2+} responsiveness of myofibrillar activation (Wattanapermpool, 1998; Wattanapermpool & Reiser, 1999). All these changes during relaxation period could be prevented by estrogen

supplementation (Wattanapermpool *et al.*, 2000). Up to this point, it is clear that female sex hormones play a regulatory role in relaxation. However, the role of female sex hormones on the ability of myocardial recoil during diastole has never been proved. Ability of myocardial re-expansion, or myocardial compliance, directly determines the filling capacity of heart during diastole. Difficulty in myocardial chamber expansion during diastole reduces the rate of ventricular filling and so the end diastolic volume.

B. Myocardial Stiffness and Diastolic Function

Myocardial stiffness is inversely proportional to the compliance of the heart that is mainly influenced by two different proteins: titin, a third filament protein, and the collagen, an extracellular protein (Weber *et al.*, 1994; Granzier & Labeit, 2004). Amount of collagen accumulation reflexes on distension ability of myocardial chamber and different expression of titin isoforms defines the relengthening ability of sarcomere.

1. Titin

Titin is a structural and mechanical component of mycocardium. This is the sarcomeric protein spanning from Z disk to M-line of the sarcomere, representing about half of the sarcomere (Figure 1).

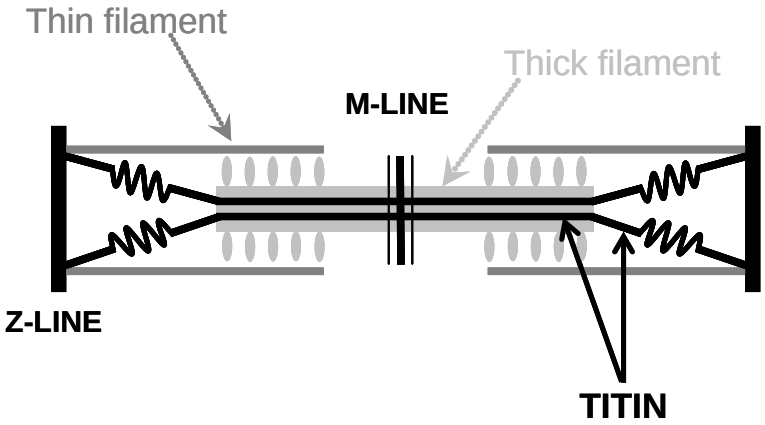

Figure 1. Sarcomeric structure

In the absence of the external force, I band region of titin is folded and so the length is nearly zero. However, when stretched, the I-band segments are lengthened and then function as the spring, giving passive tension. The passive force

is aimed to restore the overlapping of thick and thin filament at rest. For normal physiological stretch, approximately 90 percent of the elastic passive tension response is contributed by titin in cardiac myocytes (Granzier & Irving, 1995). Passive stiffness is mainly contributed by I band region of titin because it serves as the molecular spring. In fact, three sequence spring elements contribute the extensibility of I band. The first spring is composed of tandemly arranged Ig domains. The second element is the PEVK which is rich in proline (P), valine (V), glutamine (E), and lysine (K) (Labeit & Kolmerer, 1995). The last element so designated as N2B extends towards the end of normal sarcomere length. Actually, these three elements are serially arranged so that they function as the multiple spring system during stretch (Helmes *et al.*, 1999; Linke *et al.*, 1999).

The composition of these spring segments can vary according to the isoforms resulting from the splice variants. Human titin gene consists of 363 exons that are seemed to encode 38,138 amino acids (Bang *et al.*, 2001). The different splicing in I-band region of titin leads to the isoforms, contributing distinct spring compositions. Two major isoforms are expressed in heart by alternative splicing from the single titin gene (N2B=3,000 kDa and N2BA=3,200-3,700kDa) (Bang *et al.*, 2001). N2BA is longer than N2B, so it can give the more compliance compared with the later one. The fetal isoform (N2BA) is usually dominant in neonatal life but it is gradually replaced by adult isoform (N2B) with time course that can differ in accordance with the species (Opitz *et al.*, 2004). In rats, strong upregulation of N2B isoform causes the N2B isform to contribute more than 90 % of total titin shortly after birth. In fact, the N2B isoform is the predominant in the small mammals like rats although the large mammals even including human express both N2B and N2BA almost equally (Granzier & Labeit, 2004). Depending on alteration of the amount of N2B isoform, stiffness differs in different species (Cazorla *et al.*, 2000).

This switching of isoforms is a distinguished feature of contractile proteins including actin, myosin heavy chain (MHC) and other muscle regulatory proteins such as myosin light chain 1, tropomyosin and troponins (Murphy, 1996). In case of heart failure, fetal isoforms of myocardial proteins are usually reappeared. Similarly, N2BA predominant in fetal are upregulated in failing heart in which the ratio of N2BA to N2B was increased for instance in dilated cardiomyopathy (Makarenko *et al.*, 2004).

Shifting of titin isoform from N2B, which causes highest stiffness, to N2BA ,which gives lower stiffness than N2B, was also detected in the heart of hypothyroidism (Wu *et al.*, 2007). In sex hormone deprivation model, there are many isoform shiftings toward fetal stage. It is therefore not known whether there is a titin isoform shift after female sex hormone deprivation.

2. Collagen

Collagen is a major extracelullar cellular matrix component contributing the stiffness of the cardiac chamber especially under pathological condition. The shape, tensile strength and also the cellular arrangement are normally defined by the extracellular matrix (ECM) that predominately contains the collagen and proteoglycan (Weber & Brilla, 1991). Collagen is the most abundant protein in animal tissues, representing about 50% of the total body protein content. It has more than 20 types and they are rich in proline and glycine. There are five types of collagen in myocardium; type I, III, IV, V, VI (Pelouch *et al.*, 1993). Type I is produced by cardiac fibroblast, representing the 80% of total collagen and the second most abundant one is the type III, accounting for about 10% of total collagen content (Pelouch *et al.*, 1993). Type I collagen give rise the tensile strength and so its concentration is the key factor in determining the diastolic stiffness of the heart.

Collagen fibrils are synthesized as soluble precursors (pro-collagens) that contain both amino and carboxy terminals in the cell. When the procollagen is secreted into the extracellular space, the pro-peptides are cleaved by specific amino and carboxy proteinases, giving the mature fibrils. In case of cardiac myocyte necrosis, the fibrillar collagen is produced for replacing where the cell lost occurs indicating increases of myocardial stiffness with lost of contractile function (Lijnen & Petrov, 2000).

C. Collagen Deposition

1. Signaling pathway in collagen synthesis

Many cyotokines and growth factors take part in extracellular matrix assembly (Uitto & Kouba, 2000; Verrecchia & Mauviel, 2004). Among these pathways, transforming growth factor beta (TGFβ) pathway plays a major role in

extracellular matrix (ECM) construction and remodeling. TGFβ mediated transforming growth factor beta receptor I (TβRI) and TβRII, activating many cellular signals. Ligand binding causes the binding of type I and type II receptors. In this complex, type II receptor phosphorylates and activates type I receptor, leading to the activation of downstream signaling. Among many pathways, Smad pathway is the major downstream for collagen synthesis (Shi & Vesely, 2003; Schmierer & Hill, 2007). Generally, Smads can be categorized into three groups 1.regulatory Smad (Smad 2/3), 2.co-Smad (Smad 4) and 3.inhibiting Smad (Smad 6, 7). Upon activation of TGF beta receptor 1 and 2, they will phosphorylate the smad 2 and 3, which will bind with Smad 4. This heterometric complex will translocate into the nucleus and then regulate the target genes (*COL1A1, COL1A2, COL3A1 and COL5A2*) directly or binds with other DNA binding factors at times (Tsukazaki *et al.*, 1998; Feng & Derynck, 2005). Overexpression of Smad2 stimulated proliferation of basal epidermal cells (Ito *et al.*, 2001) and prevented cleft palate in TGFβ3 null mice (Cui *et al.*, 2005). Elevated Smad2 protein expression has also been demonstrated in the heart of cardiomyopathic hamster (Dixon *et al.*, 2000).

2. Collagen degradation

Collagen synthesis must be balanced with the collagen degradation so as to keep collagen within limited range. Among many collagen-cleaved proteases, matrix metalloproteinase (MMP), a zinc dependent endopeptidase, is important for the degradation of extracellular matrix (ECM). Although MMP generally expresses in fibroblast, it has also been reported that there are some MMPs inside the cardiac myocytes (Coker *et al.*, 1999). In addition, MMPs have been demonstrated to involve in cell communication, cell migration and tumor progression. Every MMPs has similar features including (1) degrade ECM components, (2) proteolytic activity is necessary for their activation from the latent form, (3) active site contain Zn^{2+}, (4) calcium is necessary for stability, (5) function at natural pH, and (6) they are inhibited by tissue inhibitor metalloproteinases (TIMPs).

In the heart, MMPs are produced by fibroblast-like cells, inflammatory cells and also cardiac myocytes (Cleutjens *et al.*, 1995; Coker *et al.*, 1999). Normally, latent form is predominant but the activation is significantly increased in pathological

condition. Many studies demonstrated that the increased expression of MMP-1,-2,-3 and -9 in human, rats, and porcine hearts during remodeling after myocardial infarction (MI) (Cleutjens *et al.*, 1995; Danielsen *et al.*, 1998). The activity of MMP is increased in dilated cardiomyopathy has also been reported (Thomas *et al.*, 1998). Creemers et al., 2001 reported that inhibition of MMPs could preserve ECM, attenuating the myocardial dilatation (Creemers *et al.*, 2001). However, inhibition of MMP activity can lead to accumulation of collagen from the negative side. In heart failure, increased MMPs degraded the normal collagens turned toward loosely linked collagen, leading to ventricular dilatation (Gunja-Smith *et al.*, 1996). The direct degradation in accordance with the substrate specificity is an essential part of ECM remodeling.

3. Effects of female sex hormones on collagen deposition

Differential effects of estrogen on collagen deposition were demonstrated in different organs. Estrogen supplement prevented tubulointerstitial fibrosis concerned with diabetic nephropathy by modulating the ECM synthesis and degradation (Mankhey *et al.*, 2005; Mankhey *et al.*, 2007). Estrogen has been found to attenuate the angiotensin II-induced collagen synthesis (Zhou *et al.*, 2007) and to reduce the growth of cardiac fibroblasts as well (Dubey *et al.*, 1998; Dubey *et al.*, 2002). It was also found that estrogen could inhibit the phosphorylation of Smad proteins induced by transforming growth factor beta (TGF-β) (Malek *et al.*, 2006). On the other hand, lack of female sex hormones did not affect collagen concentration in cardiac and renal tissue (Mercier *et al.*, 2002; Lekgabe *et al.*, 2006). Estrogen stimulated collagen gene transcription in pelvic floor connective tissue has been reported (Clark *et al.*, 2005).

Effects of female sex hormones on degradation of ECM mediated through the regulation at the MMPs are still controversial. Recent finding showed that MMP-2 activity and expression were decreased by estradiol in cardiac fibroblast induced by angiotensin II (Stewart *et al.*, 2006). This finding was supported by previous observation of increases in MMP-2 and MMP-9 level in endometrium at the menstrual phase of low estradiol (Goffin *et al.*, 2003). In contrast, MMP-2 and MMP-9 activities was shown to be at the low level in the absence of estradiol using osteoporosis model

(Zecchin *et al.*, 2005). No change in MMP2 and MMP9 in the heart of ovariectomized rats as compared to sham-control has been detected, but estrogen supplement could increase MMP2 protein expression (Dai *et al.*, 2008). Another study demonstrated decreased pro-MMP2 in the heart of aged ovariectomized rats (Xu *et al.*, 2003)**.**

D. Cardiac remodeling

Cardiac remodeling can be defined as the changes in size, shape and function of the heart following the injury to the ventricles (Mihl *et al.*, 2008). This injury is typically contributed by acute myocardial infarction but, it can be caused by pressure or volume overload to the heart as well. After the onset of injury, many histological and structural changes occur in the left ventricle leading to decline in left ventricular function.

In cardiac remodeling, the cardiac myocyte is mainly concerned but the cardiac fibroblasts, collagen and interstitium take part in this process to some extent. After the myocardial infarction, a necrosis of myocardium causes thin and weakened area which is unable to stand against the pressure and volume load. Thus, it can eventually lead to dilatation of the chamber. This necrotic area is repaired initially and so the there is scar formation. This can be beneficial in maintaining the cardiac structure and function; however, ongoing remodeling leads to increase the volume and mass of myocardium which subsequently impair the heart function (Opie, 2006).

In post infarct remodeling, the activity of metalloproteinase activity is enhanced so that the collagen breakdown is increased (Gunja-Smith *et al.*, 1996). In the late state, activation of TIMP inhibits the metalloproteinases, increasing fibrosis in the already dilated heart (Sivasubramanian *et al.*, 2001). After myocardial infarction, increase in sympathetic drive stimulates brain angiotensinogen that experimentally promotes myocytes hypertrophy, interstitial fibrosis and ventricular dilatation (Lal *et al.*, 2005).

E. Role of Estrogen in cardiac function in pathological condition

It is well known that female sex hormones play the regulatory role in pathological insults (Konhilas *et al.*, 2004). After the global ischemia, the infarct size

in isolated heart of women is smaller compared with that of men (Bae & Zhang, 2005). However, other studies showed that there is no difference in infarct size between male and female using the in vivo and ex vivo reperfusion model (Li & Kloner, 1995; Schuit *et al.*, 2005).

Many epidemiological studies reported that estrogen has cardioprotective effects (Kannel *et al.*, 1976). Direct effect of estrogen on the heart is supported by that estrogen receptor α and β are expressed in cardiac myocytes and fibroblasts. It was also known that estrogen stimulates in migration and proliferation of fibroblast (Dai-Do *et al.*, 1996). High dose of estrogen can almost completely prevent the increased left ventricular systolic and diastolic chamber diameter and reduced fractional shortening after the myocardial infarction (Beer *et al.*, 2007). They also reported that such a kind of protection is even much greater than that have seen in the cases of ACE inhibitor and beta blocker. Same study also reported that increased end diastolic pressure in failing heart could be partially prevented by estrogen treatment. In addition, exogenous estrogen has been proved to have cardioprotective impact against in ischemia and reperfusion by reducing the infarct size (Li & Kloner, 1995; Booth *et al.*, 2003; Das & Sarkar, 2006). In contrast, other study reported that deficiency of estrogen and also the supplementation of estradiol at the physiological dose had no effect on fractional shortening after myocardial infarction (Hugel *et al.*, 1999).

Moreover, estrogen was proved to have an antihypertrophic effect in pressure overload hypertrophy (van Eickels *et al.*, 2001). Estrogen increases the expression of atrinatriuretic factor (ANF) which has anti-hypertrophic effect (Horio *et al.*, 2000). In addition, estrogen inhibited the upregulation of endothelin receptor type B in which associates to enhance the hypertrophy (Smith *et al.*, 2000). Other mechanisms related cardiac hypertrophy including regulation of β_1-adrenergic receptor, and intracellular Ca^{2+} handling are partly regulated by female sex hormones (Thawornkaiwong *et al.*, 2003; Bupha-Intr & Wattanapermpool, 2006).

F. Diabetic Cardiomyopathy

Cardiomyopathy is one of the leading causes of morbidity and mortality in the diabetic population. It can be characterized by the delayed cytosolic Ca^{2+} clearance, prolongation of action potential, and ventricular dysfunction, especially

diastolic function (Dhalla *et al.*, 1998). Diabetes is the possible candidate of the cardiac diseases without complication of ischemia (Dhalla *et al.*, 1998; Spector, 1998). Many mechanisms regarding diabetes-induced cardiomyopathy have been proposed. One evidence suggested that high plasma glucose causes contractile dysfunction and fibrosis by inducing reactive oxygen species (Bauters *et al.*, 2003).

Diabetes is one of the leading causes of diastolic dysfunction, progressing to heart failure in majority of the cases. Diabetes can cause the left ventricular hypertrophy and increased contents of collagen, compromising of cardiac compliance (Lorell & Carabello, 2000). For cardiac fibrosis, tremendous amount of studies showed that the protein synthesis increase in culture fibroblast cell by giving the high glucose level and profibrotic activity is increased in human fibroblast cell when it is exposed to high level of glucose (Jones *et al.*, 1999; Lam *et al.*, 2003). Reduction of matrix degradation process by the enzyme matrix metalloproteinase was also reported in diabetic hearts (Van Linthout *et al.*, 2008). However, there are some studies demonstrated increases in matrix metalloproteinase in hyperglycemic diabetes (Camp *et al.*, 2003; Sung *et al.*, 2009).

It is not known at present whether diabetes could affect titin isoform expression. Only evidence of hypothyroidism induces swift of titin isoform, suggests a possibility that diabetes which induces hypothyroid might also have a change in titin isoform expression (Wu *et al.*, 2007).

CHAPTER III
MATERIALS AND METHODS

A. Animals

The animal proposal was approved by the Experimental Animal Committee, Faculty of Science, Mahidol University in accordance with National Laboratory Animal Centre, THAILAND. Female Sprague Dawley rats at the age of 10 weeks with the weight between 160-180 g were obtained from the National Laboratory Animal Center at Salaya Campus, Mahidol University. The rats were housed in hanging stainless steel cage in temperature and humidity controlled room with 12 hour, dark-light cycle. Water and rat chow (C.P., Thailand) were fed *ad libitum*. Ovariectomy was performed on bilateral incision under anesthetic condition of 50 mg/kg body weight of thiopental sodium, intraperitoneal injection as previously described (Thawornkaiwong *et al.*, 2007). The incision was closed with silk and the rats were allowed to recover with observation. The same procedure was performed in sham-operated control without removing of the ovaries. The uterine weight was measured on the date of sacrifice to indicate the success of ovariectomy and therefore verify the condition of ovarian sex hormone deficiency.

B. Experimental protocols

Model of study

The ovareiectomy was performed to induce the ovarian sex hormones deficiency on post pubertal female Sprague-Dawley rats weighting between 180-200 g (approximately 8 weeks of age). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ), a toxin to the pancreatic beta cell. This study used a dose of 65 mg/kg body weight for diabetic groups and a fixed volume of 0.2 ml of citrate buffer (Sigma), two weeks after the ovariectomy. The rats were sacrificed 10 weeks after ovariectomy. The uterus weight was measured to confirm the ovarian sex hormones are deficient. To check the diabetic state, the urinary glucose was tested 24 hr after

diabetic induction and before sacrifice using the glucose strip. For sham operation, the animals were operated as the same procedure as ovariectomy except that both ovaries were kept intact. For non-diabetic groups, only the citrate buffer was injected instead of streptozotocin.

Protocol 1: To assess the effect of female sex hormones in regulating collagen deposition and titin isoforms expression

Eight-weeks female Sprague-Dawley rats weighing between 180-200 g (8-9 weeks old) were randomly divided into two experimental groups as follow:

1. Sham-operated group (SHAM)
2. Ovariectomized group (OVX)

Figure 2. Diagram of experimental protocol I

Protocol 2*:* To evaluate any difference of collagen deposition and titin isoforms expression in diabetic hearts in the presence and absence of ovarian sex hormones

Eight-weeks female Sprague-Dawley rats weighing between 180-200 g (8-9 weeks old) were randomly divided into four experimental groups as follow:

1. Sham-operated group (SHAM)
2. Ovariectomized group (OVX)
3. SHAM with diabetic induction (DM)
4. OVX with diabetic induction (OVX-DM)

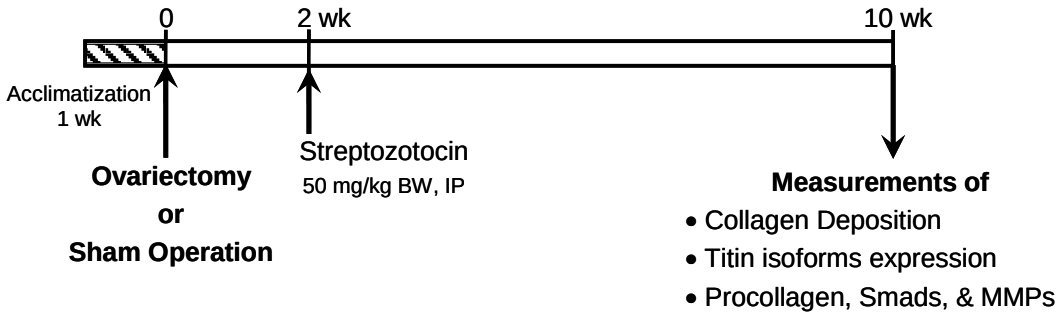

Figure 3. Diagram of experimental protocol II

Two weeks after surgery, diabetes was induced in DM, and OVX-DM groups by intraperitoneal injection with freshly prepared streptozotocin (50 mg/kg body weight). In SHAM and OVX groups (objective II), citrate buffer was injected as control condition. Verification of diabetic condition was performed one day after induction by determining urinary glucose using a glucose strip (Roche, Indianapolis, IN). Diabetic condition was checked again on the day before sacrifice.

Protocol 3: To test whether estrogen and progesterone supplements could prevent the changes in collagen deposition and titin isoforms expression in diabetic-ovariectomized rats

Eight-weeks female Sprague-Dawley rats weighing between 180-200 g (8-9 weeks old) were randomly divided into four experimental groups as follow:

1. OVX-DM with oil injection (OVX-DM-OIL)
2. OVX-DM with estrogen supplement (OVX-DM-E)
3. OVX-DM with progesterone supplement (OVX-DM-P)
4. OVX-DM with estrogen plus progesterone supplement (OVX-DM-EP)

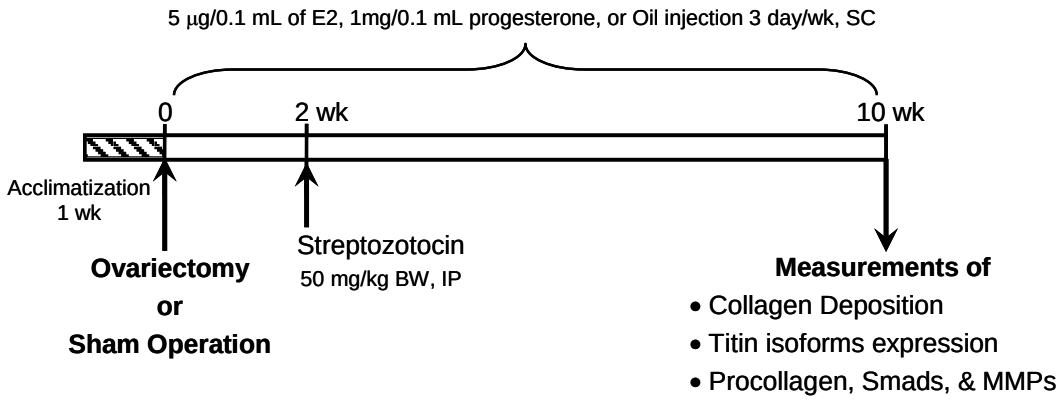

Figure 4. Diagram of experimental protocol III

For ovariectomy and sham operation, rats was anesthetized by intraperitoneal injection of thiopental (50 mg/kg body weight) as previously described (Thawornkaiwong *et al.*, 2007). Hormone supplements are estrogen 0.5 μg/rat in 0.1 ml of corn oil, progesterone 1 mg/rat in 0.1 ml of corn oil, or 0.5 μg estrogen and 1 mg progesterone in 0.1 ml of corn oil injecting subcutaneously three days per week. In control groups, 0.1 ml of corn oil were injected as placebo (Wattanapermpool *et al.*, 2000). Diabetic induction was performed as mentioned above.

C. Materials

Most of the chemicals were purchased from Sigma, St Lousis, MO, and Fisher Scientific, Pittsburgh, PA. Chemicals for SDS-PAGE and immunoblotting were obtained from Bio-Rad, Hercules, CA. Monoclonal and polyclonal antibodies of Smad2, MMP-2 and -9, and procollagen were purchased from Cell Signaling Technology or Santa Cruz.

D. Methods

1. Histochemical analysis of collagen deposition

For interstitial collagen deposition analysis, the whole heart was horizontally cut in half and only the lower half was frozen in the optimum temperature compound (OTC, Sakura tissue tek). Sections of 10 μmeter were prepared using cryotome (Frozen section). Some sections from each sample were stained with Sirius Red (Sigma) to quantify the total collagen deposition under bright-field microscope. After taking the pictures, the amount of collagen was analyzed by using image J soft ware.

2. Titin separation

Portion of the ventricular muscle was homogenized in urea sample buffer and then protein concentration was determined using BCA protein assay (Sigma). Firstly, it is necessary to set up slab gel apparatus. The next step is to prepare 0.5% agarose gel/2% acrylamide (minivertical gel electrophoresis system, Bio-Rad) as previously described (Tatsumi & Hattori, 1995). As soon as the gel is prepared and poured, the 10-well comb needs to be inserted. After the preparation step is done, the gel is allowed to polymerize at 4^{o} C (over night). When the gel is polymerized, it is to remove the comb and clean the well with distilled water 2 or 3 times so as to make the proper shape of the well. Then, the chamber is set up and filled the running buffer until it covers the top of the gel surface. One hundred milligram of the homogenized sample is loaded in this experiment. For the gel running, the pre running is 30 minutes at 8mA. Then, gel is run continuously at cool temperature and constant current at 15 mA for 6 hours. After running for 6 hrs, the gel is stained by silver stain kid (Bio-

Rad). Quantities of the two titin bands (N2B & N2BA) is analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

3. Immunoblot analysis for Procollagen, MMPs, and Smad2

Expression of procollagen, MMP2, MMP-9, were measured using immunoblot analysis. The frozen tissue was homogenized in extracting buffer with protease and phosphatase inhibitors (Sigma) at $4^{o}C$ followed by ultra-sonication for 1 hour at $4^{o}C$. Protein concentration was measured using BCA protein assay kit (Sigma) before adding dithiothreitol. The sample was run in 10 or 12% (depends on size of interesting protein) SDS-PAGE at a constant voltage of 100 volts for 2 hours. Proteins were transferred to the nitrocellulose membrane by running at 100 volts for 1 hour. The membrane was blocked for 1 hour in 5% milk TBST, and incubated with specific primary antibody (Procollagen, MMP-2 & MMP-9 from Santa Cruz; Smad2 from Cell Signaling) overnight. After washing with TBST, the membrane was incubated with secondary antibody conjugated HRP for 1 hour. After washing with TBST, the protein band on the membrane was developed using ECL reagent and detected by exposing to hyperfilm (Amersham Pharmacia Biotech). The amount of protein bands was calculated using Image Master Labscan and Image Master Totallab.

5. General methods and statistics

Amount of Smad2, MMP-2, MMP-9 and procollagen from left ventricular homogenate were determined by general immunoblot method. Dilutions for antibodies of Smad2, were 1:5000. For procollagen, MMP-2 and MMP-9, dilution of 1:1000 was used. Secondary anti-rabbit antibody was used at dilution 1:20000 for Smad 2, procollagen, MMP-2 and MMP-9. For Smad2, secondary anti- mouse antibody was used at the dilution of 1:5000.

Data are presented as means ± SE. One way analysis of variance (ANOVA) was used for determination of the difference among groups with the significance set at $P<0.05$. Student Newman-Keuls multiple comparison was used for determining the significant difference between two means.

CHAPTER IV
RESULTS

A. General observations

Deprivation of ovarian sex hormones increased the body weight significantly from the sham-operated (SHAM) control group (358 ± 2 g versus 278 ± 2 g). In contrast, the body weight of diabetes (DM) group was significantly decreased as compared with the SHAM group (237 ± 5 g and 278 ± 2 g, respectively). As expected, diabetic ovariectomized (DM-OVX) had a significant lower body weight than ovariectomized (OVX) group (281 ± 5 g versus 358 ± 2 g). Results revealed that diabetes decreased the body weight while deprivation of female sex hormones increased the body weight. This fact was also confirmed in DM-OVX rats with various supplementations. Supplementation of estrogen (215 ± 4 g) and estrogen plus progesterone (215 ± 11 g) could decrease the body weight significantly from the DM-OVX group plus vehicle (260 ± 8 g). On the other hand, progesterone supplementation had no effect (304 ± 2 g).

Uterine weight was measured for the estimation of plasma ovarian sex hormone levels. There is no difference in uterine weight between SHAM and DM groups (0.56 ± 0.02 g and 0.53 ± 0.02 g, respectively). The uterine weight was significantly reduced in both OVX and DM-OVX groups compared with the SHAM group (0.14 ± 0.01 g and 0.12 ± 0.01 g, respectively). Only estrogen and estrogen plus progesterone supplementations could reverse these changes (0.53 ± 0.02 g and 0.52 ± 0.01 g, respectively) but not progesterone supplementation as compared to DM-OVX-OIL group (0.12 ± 0.01 g).

All diabetes animals showed the positive sign of diabetic induction (urine glucose is over 250 mg/dl).

B. Regulation of female sex hormones on collagen deposition

Effect of female sex hormones on myocardial stiffness was indirectly determined by the amount of collagen deposit in the ventricle of both diabetes and non-diabetic rats. Sirius red staining is the most reliable method to determine the collagen deposition of the heart (Figure 5). Sham group is the control group to make comparison among four groups (SHAM, OVX, DM, and DM-OVX). The collagen deposition between SHAM and OVX groups didn't show any significant difference (3.10 ± 0.23 % of tissue fraction and 2.95 ± 0.09 %, respectively). In contrast, in DM group the collagen deposition was increased significantly compared with SHAM group (3.10 ± 0.23 % and 5.06 ± 0.27 %, respectively). In the absence of female sex hormones, diabetes induced slightly increase in collagen deposition in the heart (3.95 ± 0.23 % of tissue fraction in DM-OVX). Thus, results indicated that female sex hormones had no impact on collagen accumulation in the heart under normal condition, but exerted a regulatory effect on increasing collagen deposition under diabetic complication.

In order to compare the differential activities between estrogen and progesterone, ventricular tissues from DM-OVX rats supplemented with 17β-estradiol, progesterone, or 17β-estradiol plus progesterone were quantified for collagen deposition (Figure 6). For the supplementation groups, result of DM-OVX plus oil injection (DM-OVX-OIL) group was used as control to compare with other three DM-OVX groups supplemented with estrogen alone (DM-OVX-E), progesterone alone (DM-OVX-P) and estrogen plus progesterone (DM-OVX-EP). The collagen deposition in the heart between DM-OVX-OIL group and DM-OVX-P group did not show any significant difference (3.80 ± 0.12 % and 4.00 ± 0.31 %, respectively). However, in the heart of DM-OVX-E group, the collagen deposition was significantly higher than that of DM-OVX-OIL group (4.80 ± 0.13 % and 3.80 ± 0.12 %, respectively). Similarly, the collagen deposition was also significantly higher in DM-OVX-EP group than DM-OVX-OIL group (5.20 ± 0.27 %, and 3.80 ± 0.12 %, respectively). The results interestingly indicated that estrogen but not progesterone played a role in regulating collagen remodeling in the diabetic hearts insult.

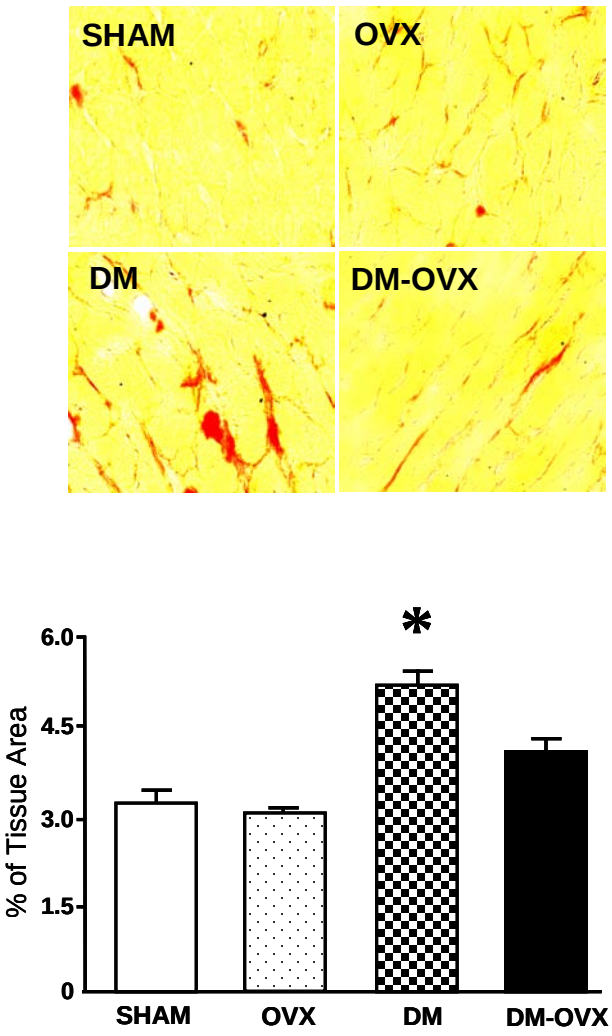

Figure 5. Percentage of collagen deposition in the heart from sham-operated (SHAM), ovariectomized (OVX), diabetes (DM) and diabetic ovariectomized (DM-OVX) rats. Data are mean ± SE from 480 tissue areas from four hearts. ***P* < 0.05, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

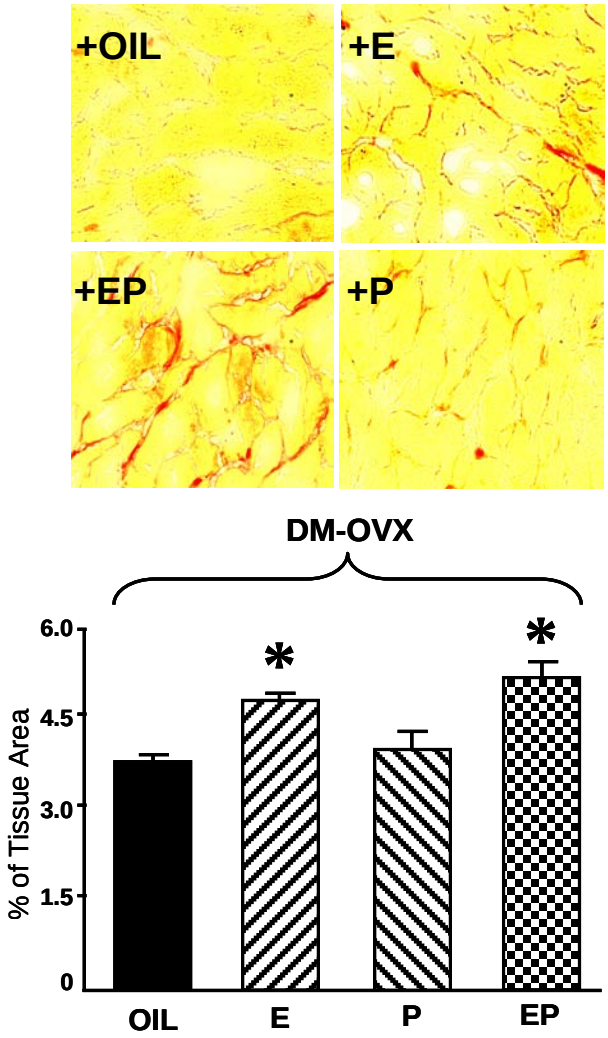

Figure 6. Percentage of collagen deposition in the heart from DM-OVX rats with supplementation with oil (OIL), estrogen (E), progesterone (P) and estrogen plus progesterone (EP). Data are means ± SE from around 480 tissue areas from four hearts. **P* < 0.05, significant difference from DM-OVX-OIL groups using Student Newman-Keuls test after ANOVA.

C. Effects of female sex hormones on titin isoforms expression

Titin is the intracellular protein and its isoforms changes can alter the stiffness of the heart. Therefore, titin isoforms were analyzed using agarose-acrylamide gel electrophoresis (Figure 7 & 8). The N2BA/N2B titin ratio was not significantly different between SHAM and OVX group (5.2 ± 0.2 and 7.7 ± 1.9). Although the ratio was higher in both DM and DM-OVX groups, only the later one showed significant difference compared with SHAM group (18.4 ± 2.3 and 27.9 ± 5.9 respectively). Therefore, it is possible that female sex hormones exert an impact on titin isoforms expression.

To figure out the differential effect of female sex hormones on titin isoforms, left ventricular homogenate from DM-OVX rats supplemented with vehicle, estrogen, progesterone and estrogen plus progesterone were prepared and electrophoresis was done. N2BA/N2B titin ratio was not significantly different in all supplemented groups compared to DM-OVX-OIL group (44.0 ± 1.1; 38.0 ± 0.5; 43.0 ± 1.5; 40.0 ± 1.1, respectively).

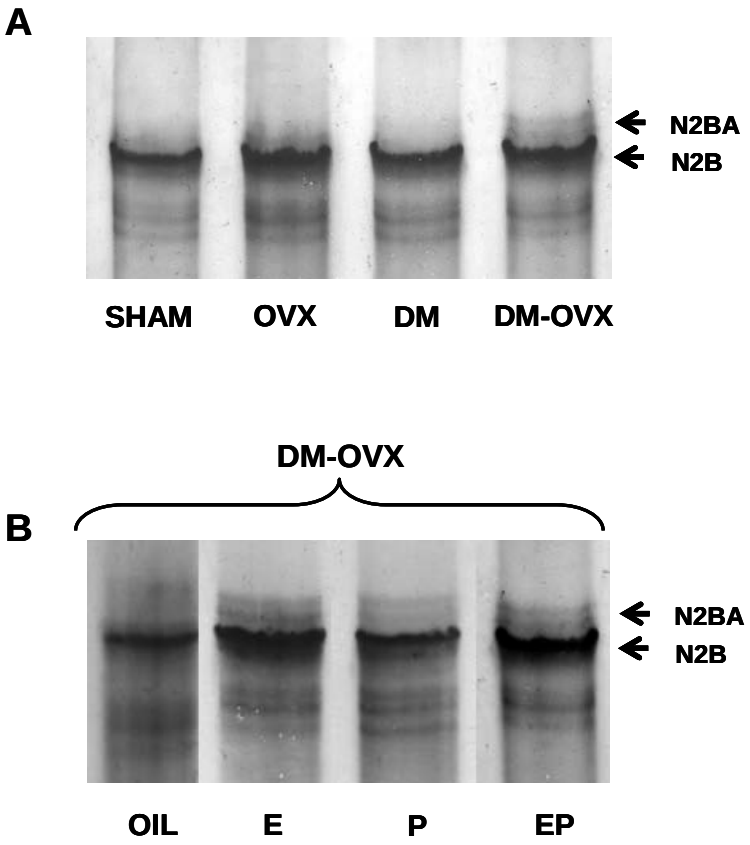

Figure 7. Separation of N2BA and N2B titin isoforms from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with oil, estrogen, progesterone and estrogen plus progesterone supplementation (B).

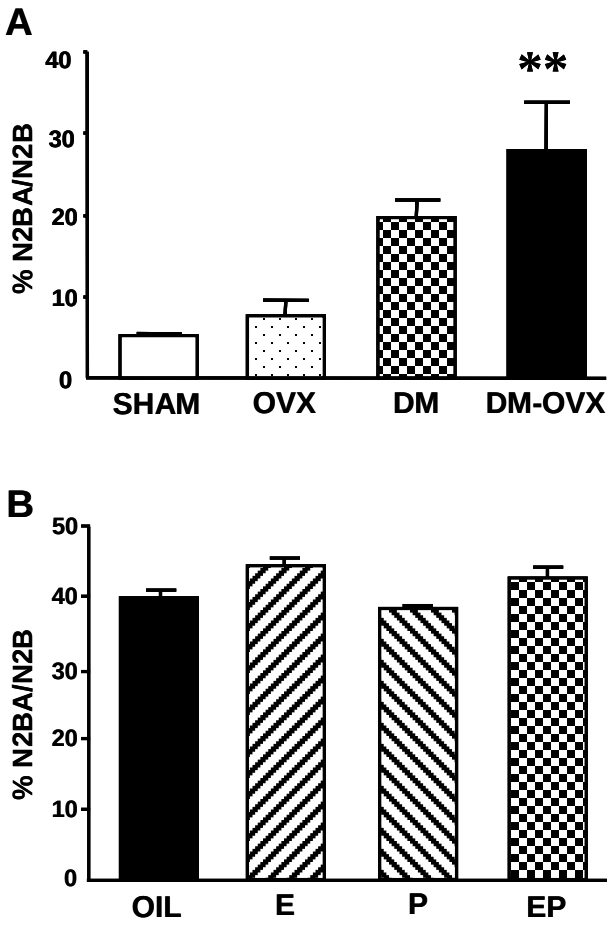

Figure 8. N2BA/N2B ratio from SHAM, OVX, DM and OVX rats (A), and DM-OVX rats with oil, estrogen, progesterone and progesterone plus estrogen supplementation (B). Data are means ± SE from 4 to 6 preparations. *P* <0.05, significant difference from SHAM group using Student Newman-Keuls test after ANOVA.

D. Female sex hormones and procollagen expression in the heart

It is well accepted that degree of collagen deposition is dependent on two mechanistic processes: collagen synthesis and degradation. In this experiment, we investigated to know whether increased collagen deposition in diabetic rat hearts is due to increase in collagen synthesis by measuring protein procollagen expression of the heart from various animal groups (Figure 9.). The procollagen expression between SHAM and OVX groups did not show any significant difference (0.95 ± 0.10 and 1.16 ± 0.20, respectively). In DM group, the procollagen expression was increased compared with SHAM group (1.61 ± 0.10 and 0.95 ± 0.10, respectively). No significant difference in procollagen expression was found in the heart of SHAM and DM-OVX groups (0.95 ± 0.10 and 1.53 ± 0.21, respectively). This similar change of procollagen expression to those collagen deposition indicated that increased collagen in the heart of DM rat is due to increase in collagen synthesis. It is still questionable whether estrogen or progesterone contributes to this collagen synthesis.

In order to answer this question, we also measured the expression of procollagen in cardiac tissues from DM-OVX-E, DM-OVX-P, and DM-OVX-EP rats respectively. Interestingly, there was no significant difference in procollagen expression in the heart among supplementation groups. An increased in procollagen expression was detected only in DM-OVX-EP (1.50 ± 0.37 folds compared to DM-OVX-OIL group. The argument about the role of estrogen alone in collagen deposition and procollagen expression in DM-OVX rat heart points to another possible effect of female sex hormones on regulating the developmental process of procollagen to mature collagen.

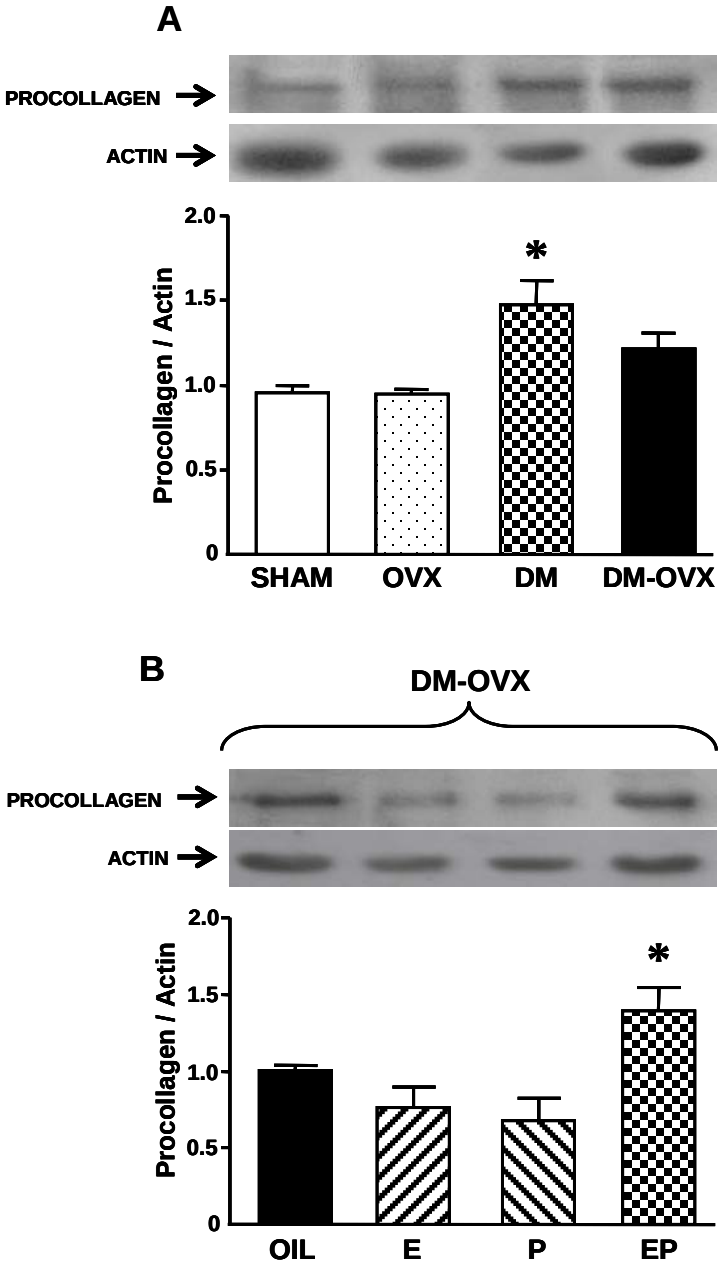

Figure 9. Procollagen region on immunoblot membrane and the relative amount of procollagen in left ventricular homogenates form SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with hormone supplements (B). Data are means ± SE from 4 to 6 preparations.*$P < 0.05$, significant difference from SHAM (A) and DM-OVX-OIL (B), using Student Newman-Keuls test after ANOVA.

E. Female sex hormones and Smad2 expression in the heart

Smad signaling pathway is a major signaling transcription factor in regulating collagen gene transcription in many organs including heart. We then evaluated whether female sex hormones play a role in Smad pathway in the heart of diabetes ovariectomized rats by measuring Smad2 protein expression. As expected, immunoblot analysis demonstrated no significant difference in Smad2 expressions between SHAM and OVX groups (1.03 ± 0.08 and 1.03 ± 0.14, respectively) (Figure 10.). However, Smad2 expression was significantly higher in the heart of DM rats compared with SHAM (1.46 ± 0.15 and 1.03 ± 0.08, respectively). No significant difference in Smad2 expression in the heart between DM-OVX and SHAM was also detected (1.20 ± 0.07 and 1.03 ± 0.08). Up to this point, it is possible to say that female sex hormones are associated with diabetes-induced collagen synthesis in the hearts partly via the Smad2 expression.

Therefore, to study the differential effect of progesterone and estrogen in Smad2 expression under diabetes condition, cardiac homogenate from DM-OVX groups supplemented with different hormones were used. Significant increase in Smad2 expression was detected only in the DM-OVX-EP when compared to DM-OVX-OIL (1.32 ± 0.11 versus 0.91 ± 0.11). No effect of estrogen or progesterone supplement alone induced Smad2 expression in DM-OVX rat heart. The similar changes in procollagen and Smad2 expression observed in our studies could imply a possibility that both estrogen and progesterone are working together in regulating collagen synthesis in the heart.

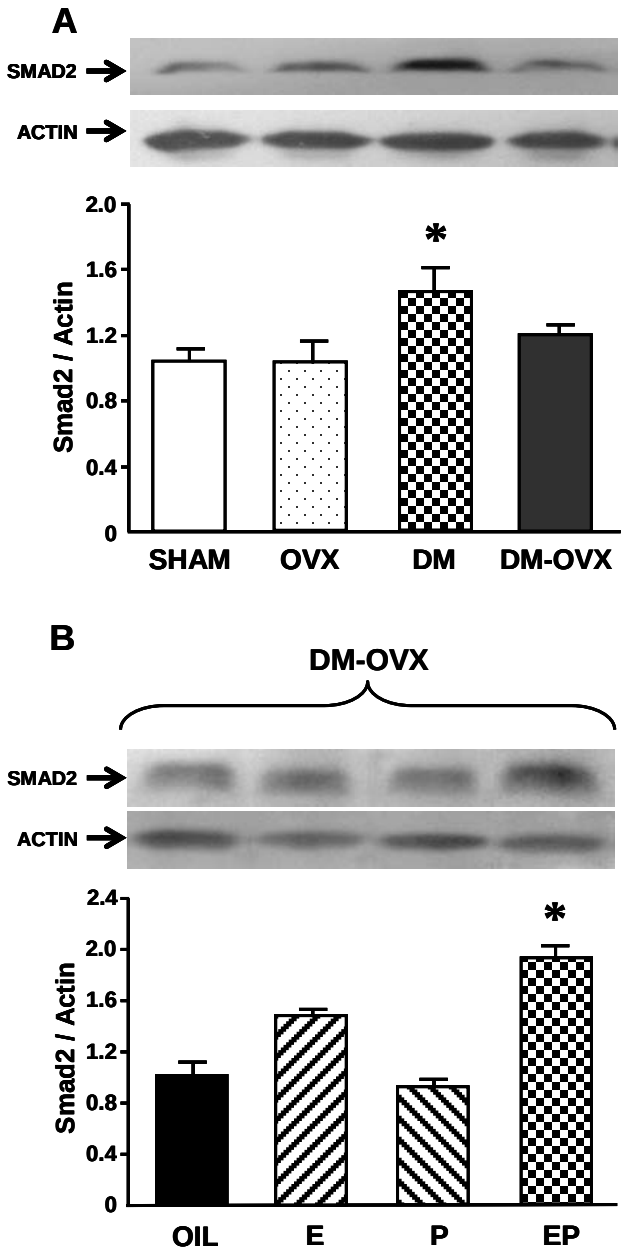

Figure 10. Smad2 region on immunoblot membrane and the relative amount of Smad2 in left ventricular homogenates form SHAM, OVX, DM and DM-OVX (A), and DM-OVX rats with hormone supplements (B). Data are means ± SE from 4 to 6 preparations. *$P < 0.05$, significant difference from SHAM (A) and DM-OVX-OIL (B), using Student Newman-Keuls test after ANOVA.

F. Female sex hormones and expression of matrix metalloproteinase

Collagen degradation is another mechanism determining collagen deposition. We then proved a possible role of female sex hormones on collagen degradation by measuring the expression of matrix metalloproteinases (MMPs) in the heart of diabetes and diabetes ovariectomized rats (Figure 11-13). Immunoblot analysis demonstrated no change in expression of total MMP-2 and MMP-9, two major MMP proteins expressed in the heart, between SHAM and OVX rats (1.00 ± 0.07 and 1.10 ± 0.11 for MMP-2 and 1.00 ± 0.14 and 1.20 ± 0.18 for MMP-9, respectively). Interestingly, the expression of total MMP-2 was significantly higher in DM but not DM-OVX compared to SHAM group (1.70 ± 0.14, 1.30 ± 0.19 and 1.00 ± 0.17, respectively) without any change in MMP-9. In addition, the ratio of active to total form of MMP-2 was significantly higher in DM group than that in SHAM group (1.27 ± 0.06 versus 1.00 ± 0.06, respectively). These results indicated that female sex hormones possess an effect on MMPs protein expression under diabetic conditions indicated a collagen remodeling role of the hormones.

Differential effects of progesterone and estrogen supplements on MMPs expression in the heart were tested in diabetic ovariectomized rats. No significant difference in the expression of MMP-9 among the DM-OVX rats with and without hormone supplements (Figure 12.). DM-OVX-EP is the only group that showed a significant increase in total MMP-2 (1.37 ± 0.12 versus 1.00 ± 0.13 in DM-OVX-OIL). These results indicated that both estrogen and progesterone are required for regulating the expression of MMP-2. At the end, impacts of female sex hormones on both collagen synthesis and extracellular matrix remodeling were summarized.

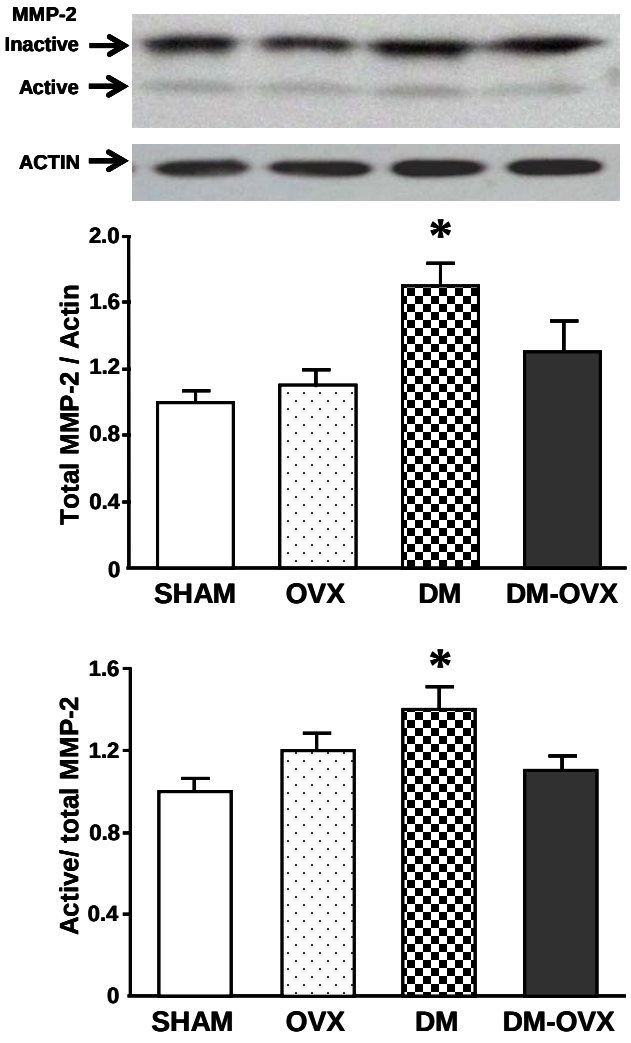

Figure 11. MMP-2 active and inactive regions on immunoblot membrane and the relative amount of total MMP-2 to actin (A) and active to total MMP-2 (B) of left ventricle homogenate from SHAM, OVX, DM and DM-OVX. Data are means ± SE from 4 to 6 preparations. *$P < 0.05$, significant difference from SHAM, using Student Newman-Keuls test after ANOVA.

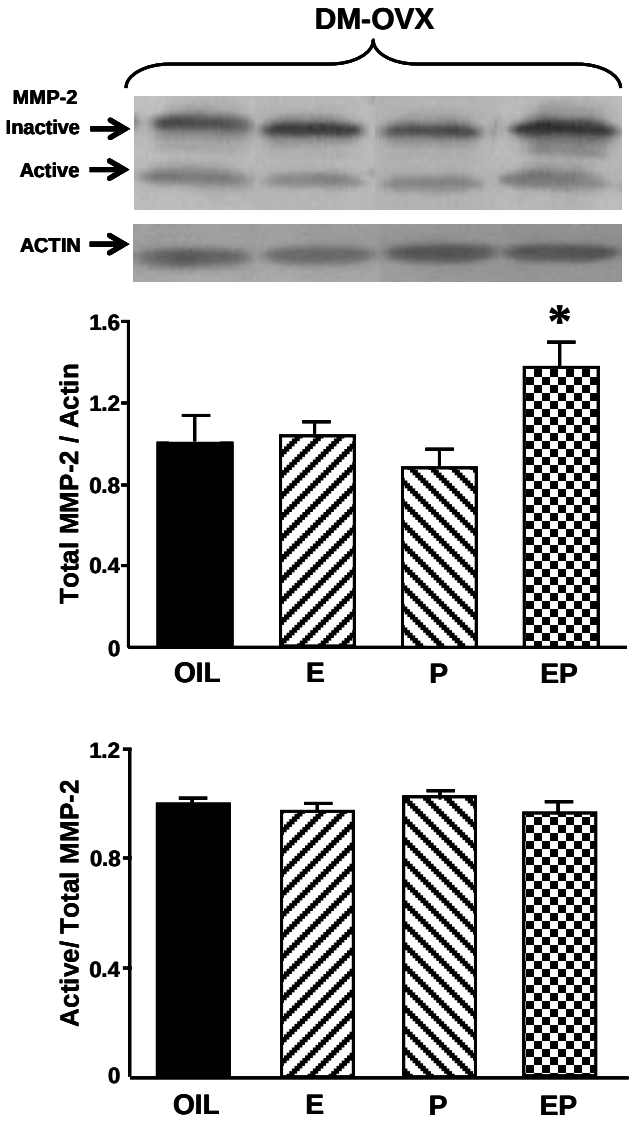

Figure 12. MMP-2 active and inactive regions on immunoblot membrane and the relative amount of total MMP-2 to actin (A) and active to total MMP-2 (B) in left ventricular homogenates form DM-OVX plus oil and hormone supplements. Data are means ± SE from 4 to 6 preparations. *$P < 0.05$, significant difference from SHAM using Student Newman-Keuls test after ANOVA.

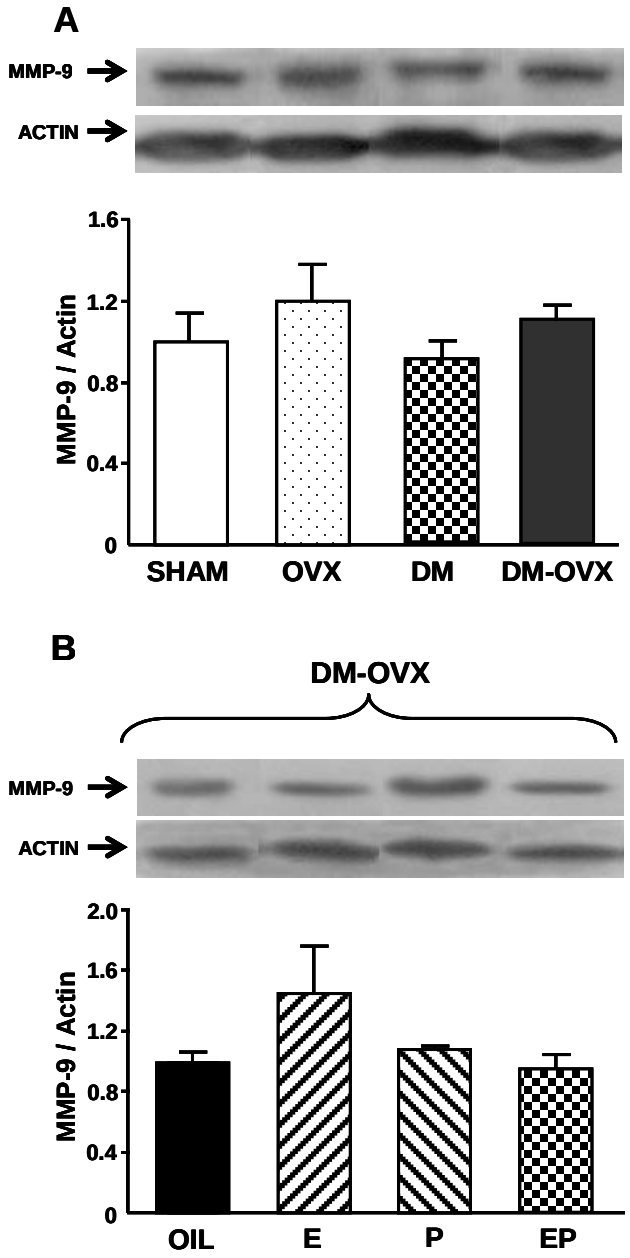

Figure 13. MMP-9 region on immunoblot membrane and the relative amount of MMP-9 in left ventricle homogenate from SHAM, OVX, DM and DM-OVX rats (A), and DM-OVX rats with hormone supplements (B). Data are means ± SE from 4 to 6 preparations. No significant difference among groups was detected using ANOVA.

CHAPTER V
DISCUSSION

The present study extends our understanding on the tissue, cellular, subcellular and molecular impacts of female sex hormones on the cardiac diastolic function regarding on expansion ability of myocardial chamber. In this thesis, it was hypothesized that female sex hormones plays a significant role in myocardial stiffness under both physiological and pathological conditions. The main objective of my thesis is to elucidate the adaptations of myocardial tissue after the chronic deprivation of female sex hormones with and without complication of diabetes. Under physiological condition, no effect of female sex hormones on collagen deposition and titin expression was concluded. The myocardial tissue tends to be dilated in ovariectomized rats under diabetes condition that is supported by the increased expression of N2BA titin isoform. The presence of female sex hormones under diabetes condition induced the compensatory changes to prevent the formation of dilated heart by increasing collagen deposition. Under diabetes condition, female sex hormones increased collagen deposition by enhancing the collagen synthesis possibly through the upregulation of collagen gene transcriptional factor Smad2. The increased in collagen synthesis in diabetes was partly supported by increased procollagen expression as well. Interestingly, the MMP-2 expression was also increased in diabetic heart in the presence of female sex hormones. The answer could be that female sex hormones modulate the collagen remodeling via both synthesis and degradation as well. The net increased in collagen deposition studied by histochemical analysis implied that female sex hormones enhanced the synthesis more than degradation. Although female sex hormones did not play a role in maintaining the myocardial stiffness under physiological condition, female sex hormones modulate the myocardial stiffness by regulating the collagen deposition and titin expression under pathological condition of diabetes.

A. Female sex hormones and myocardial stiffness under physiological condition

Results from this study demonstrated no changes in every measured variables, including titin isoforms and collagen deposition in the heart of ovarian sex hormones-deprived rats from SHAM controls. Despite the absence of female sex hormones effect on these factors which implies the unaltered diastolic function of the heart, estrogen has been demonstrated to stimulate collagen gene transcription in bone connective tissue (Clark *et al.*, 2005). Similar to cardiac tissue, lack of female sex hormones did not affect collagen concentration in renal tissue (Mercier *et al.*, 2002; Lekgabe *et al.*, 2006).

So far, there is no clear evidence concerning with the effect of female sex hormones on the collagen production in the heart. On the other hand, there are far more information on collagen degradation in the heart even though they are still controversial. Studies on collagen degradation in the heart were mostly concentrated on evaluating the MMP enzymes which play a major role in extracelluar matrix degradation or remodeling process. Previous studies found that MMP-2 expression in the heart was firstly decreased after ovarian sex hormone deprivation and then gradually increased overtime until reaching the same level to that in sham-control after eight weeks of hormone deficiency (Lam *et al.*, 2003; Xu *et al.*, 2003). The sudden decrease in MMP-2 expression within two weeks after ovariectomy has also been suggested by studies in other organs (Zecchin *et al.*, 2005; Lam *et al.*, 2009). In the presence of estrogen, MMP-2 activity and expression have been demonstrated to increase in mesenteric artery, culture mesengial cell, and kidney tissue (Zhang *et al.*, 2000; Guccione *et al.*, 2002; Mankhey *et al.*, 2007). It is, however, unknown for mechanism involving in the gradual increased of MMP-2 level to reach the normal level in 8 or 10 weeks after ovariectomy as shown in previous and our present study, respectively. One possible explanation is that female sex hormones may indirectly regulate the expression of MMP.

My result clearly showed that deprivation of female sex hormones had no effect on the myocardial stiffness under physiological condition. Therefore, suppressed ventricular filling in postmenopausal women (Kangro *et al.*, 1995) might be due to the impaired myocardial relaxation after chronic deprivation of female sex

hormones (Bupha-Intr & Wattanapermpool, 2006; Bupha-Intr *et al.*, 2007). Moreover, the diastolic dysfunction could be resulted from the additional factors of post menopausal women such as aging, hypertension and diabetes and so on. The parallel study from our laboratory (Figure 14) demonstrated no significant difference in a sarcomere length-passive tension relationship of myocardial tissue of ovariectomized rats from sham controls (Bupha-Intr T, 2009). This result and our present finding of no change in collagen deposition and titin isoform expression in the heart of ovariectomized rats thus indicate that lack of female sex hormones alone did not affect myocardial compliance.

B. Female sex hormones and myocardial stiffness under pathological condition

Although female sex hormones had no regulatory effect on the myocardial stiffness, the hormones seem to play significant influences on cardiac stiffness under pathological stress to the heart. It is possible that female sex hormones activate collagen accumulation in the heart of diabetic rats in compensation to the reduced myocardial stiffness from high expression of compliant N2BA titin isoform.

Titin expression was demonstrated to change in some pathological conditions. N2BA/N2B ratio was increased in dilated cardiomyopathy, leading to decreased stroke volume (Makarenko *et al.*, 2004). In addition, hypothyroidism increased the N2BA expression (Wu *et al.*, 2007) which might also be the cause of increased N2BA titin isoform in the heart of diabetes. In the present study, a shift in titin isoform towards the higher compliant one in diabetes might imply the pathological development of dilated cardiomyopathy when combined absence of female sex hormones. Thus, female sex hormones may protect the heart by inducing more collagen deposition.

Collagen contributes to the stiffness of the myocardial chamber and therefore changes in its amount can affect the diastolic function of the heart. Female sex hormones have been suggested to play significant role in collagen deposition in various tissues. The effect of female sex hormones on collagen deposition in the heart is still controversial. Smad signaling pathway, a major downstream pathway

regulating collagen gene transcription is a parameter used to estimate the collagen synthesis activity (Schmierer & Hill, 2007). Among the Smad proteins, Smad2 is the regulatory Smad which plays a crucial role in collagen synthesis. Increases in Smad2 expression and activity will activate collagen synthesis (Sysa *et al.*, 2009) and attenuate cardiac myocyte hypertrophy induced by pressure overload (Xu *et al.*, 2006). Activation of Smad2 signal was also found in condition of myocardial infarction and diabetes (Hao *et al.*, 1999; Dixon & Maric, 2007). Significant increased Smad2 expression in diabetic heart in a presence of female sex hormones (Figure 10.) but not in diabetic ovariectomized rats indicated that the preventive action of female sex hormones on activating the collagen synthesis will only operate under pathological stress. This present finding, interestingly, reveal against those reports regarding the inhibitory effect of estrogen on collagen synthesis in culture cells (Malek *et al.*, 2006). This differential effect of female sex hormones might be due to more factors contributing collagen synthesis in complex tissue.

Differential effect of female sex hormones on collagen deposition was also found in the collagen degradation signal. Our results demonstrated that presence of female sex hormones only affect MMP-2 but not MMP-9 expression in the diabetic heart. The difference between the two MMPs is that MMP-2 can degrade collagen type I(major form in our heart), IV and V but MMP-9 degrade collagen type IV and V (Pelouch *et al.*, 1993; Creemers *et al.*, 2001). Increased expression of MMPs has been reported in several heart diseases (Tziakas *et al.*, 2005; Morita *et al.*, 2006; Martos *et al.*, 2007; Sivakumar *et al.*, 2008). However, it is still controversial about the changes in the expression of MMP-2 and MMP-9 in pathological condition. Increases in MMP-2 and MMP-9 in the heart and in serum were mostly reported in dilated cardiomyopathy, but less in ischemic cardiomyopathy (Tziakas *et al.*, 2005; Martos *et al.*, 2007). Interestingly, evidences of MMPs in diabetic condition are rather varied. Increased MMP-9 mRNA level in the heart of diabetic rats after 4 weeks induction has been reported (Sung *et al.*, 2009). Eight weeks after introducing sucrose enriched diet induced increases in MMP-2 and MMP-9 expression in the heart compared to that taking standard chow diet (Monti *et al.*, 2008). MMP-9 activity in plasma and left ventricle was increased in alloxan-induced diabetes (Camp *et al.*, 2003) but not changed in another study (Bollano *et al.*, 2006; Westermann *et al.*, 2007b; Van

Linthout *et al.*, 2008). After diabetic induction for 8 weeks, decreased MMP-2 activity was found in the heart (Westermann *et al.*, 2007a). Coronary heart disease patients who have diabetes also showed higher arterial MMP-2 and MMP-9 activities than those who have no diabetes (Chung *et al.*, 2009). Many studies reported decreased MMP-2 expression and activity in the heart of diabetic rats (Bollano *et al.*, 2006; Westermann *et al.*, 2007b; Van Linthout *et al.*, 2008). Based on the function of MMP, increases in MMP expression and activity could be expected in the remodeling activation of tissue. Thus, differences in the expression and activity of these enzymes in the heart under diabetic condition may represent differential pathophysiological status of cardiac injury. At present, it is not clear for the exact mechanistic signal in regulating MMPs expression especially the subtypes of MMP-2 and MMP-9.

The implication from our results of increased collagen deposition under diabetic complication is that female sex hormones should have involved in the remodeling process of the formation of dilated cardiomyopathy. Results from a parallel study from our laboratory (Figure 14) supported (Bupha-Intr T, 2009) that passive stiffness was significantly increased in diabetic but not diabetic ovariectomized group.

C. Female sex hormones supplementations and myocardial stiffness under pathological condition

Supplementation of either estrogen or progesterone has no effect on titin isoform ratio in the heart of diabetic-ovariectomized rats. However, collagen deposition was significantly increased in DM-OVX-E and DM-OVX-EP. Again, the procollagen and Smad2 were found to be increased only in the group of estrogen plus progesterone supplement. Therefore, we can conclude that estrogen and progesterone work together in regulating the procollagen and Smad2 under diabetes condition. Interestingly, estrogen supplementation alone could increase collagen accumulation without any effect on Smad2 and procollagen. This could imply that estrogen mediates collagen deposition by other signaling processes besides Smad2 activation. One proposed signal might be TNFα that has been reported to be expressed in cardiac cells and can activate collagen deposition (Bitzer *et al.*, 2000). However, action of female sex hormones on this cytokine in the heart has never been reported.

Estrogen might activate other types of procollagen since the procollagen measured in this study is specific for the procollagen gene COL1A1. Estrogen has been demonstrated to increase the mRNA level of procollagen type III (Clark *et al.*, 2005). In any case, our results from supplementation studies reveal that many other mechanisms could have participated in regulation of myocardial stiffness.

D. Female sex hormones and diastolic function

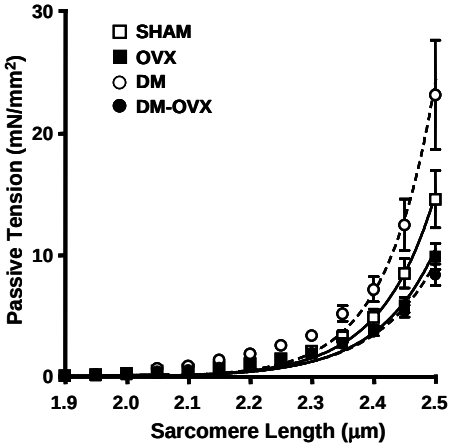

Figure 14. Relation of passive tension at various sacomere lengths of myocardial tissue from sham-operated (SHAM), ovariectomized (OVX), diabetes (DM) and diabetic-ovariectomized (DM-OVX) rats. Values are means ± SEM from 15 to 18 muscle fiber isolated from 5 to 8 hearts. (Bupha-Intr *et. al.*, 2009)

Among the rate limiting steps of cardiac relaxation including the rate of cytosolic Ca^{2+} removal, rate of actin-crossbridge detachment, and the ability of titin in retracting thick filament, ability of titin to pull back the thick filament is the process that works passively. Titin is the third myofilament system that connects thick filament to the Z-line and acts as a recoil spring during myofilament relengthening (Figure 1). Based on its stoichiometry, shorter spring gives more retracted tension. Between the two isoforms of titin, the ratio of N2BA and N2B in human heart is around 50 to 50 percentage (Granzier & Labeit, 2004) which could be changed in heart disease. Higher expression of the compliant N2BA titin isoform has been

observed in dilated cardiomyopathy with eccentric left ventricular remodeling (Nagueh *et al.*, 2004). On the other hand, patients with concentric heart failure had lower N2BA/N2B titin ratio despite the similar myocardial fibrosis (van Heerebeek *et al.*, 2006). Although many investigators proposed that the increased compliant N2BA is a compensatory mechanism against cardiac fibrosis, it is not clear whether this compensation gives advantage to cardiac function.

Protective effect of female sex hormones on protecting a development of dilated cardiomyopathy has been proposed. Epidemiologic study in European population has demonstrated that women had less frequent coronary heart disease and dilated cardiomyopathy incidences compared to men (Nieminen *et al.*, 2008). It is interesting that the presence of female sex hormones turns the heart toward concentric hypertrophy, whereas the heart of male or ovariectomized animal hearts develops eccentric remodeling under similar pathological insult (Jain *et al.*, 2002; Brower *et al.*, 2003). The shift of titin from stiff N2B toward compliant N2BA titin in diabetic ovariectomized rat heart in this present study partly supportes the possibility. However, signaling mechanisms underling the action of hormones in regulating titin isoform expression needs further investigations. One possible signal in stimulating N2BA expression is triiodo-thyronine (T3). However, previous study by our laboratory demonstrated no significant difference in serum T3 level between diabetes and diabetic ovariectomized rats (Thawornkaiwong, 2008).

At the end, we concluded that lack of female sex hormones causes potential changes of cardiac performance toward dilatation under diabetic complication. Although, most studies showed an increase in the compliant N2BA titin is the compensatory mechanism to the increased myocardial fibrosis, the higher stiffness could inversely benefit for the faster relaxation. The latter implication is also based on the high expression of stiff N2B titin in animal with high heart rate (Cazorla *et al.*, 2000).

CHAPTER VI
CONCLUSION

The main objective of this study is to evaluate the effects of female sex hormones on myocardial stiffness under normal and pathological conditions. Diabetes was used as the pathological insult in this study. Collagen deposition was measured by histochemical analysis using Sirius Red staining. Protein level of the procollagen, Smad2, MMP-2, and MMP-9 were determined using immunoblot analysis. Changes in titin isoforms were determined using agarose-acrylamide gel electrophoresis.

1. Deprivation of female sex hormones had no effect on the collagen deposition in the heart under normal condition.
2. Female sex hormone deprivation did not induce significant changes in the procollagen, Smad2, MMP-2, and MMP-9 protein levels of the heart.
3. There was no significant difference in the ratio of N2BA/N2B titin in the heart of ovariectomized rats compared to sham control.
4. The presence of female sex hormones in the condition with diabetes complication leaded to an increased collagen deposition in the heart.
5. Upregulation of procollagen protein in the heart of diabetic rats with intact sex hormones supported the increased in collagen synthesis activity.
6. An increased protein expression of Smad2 in diabetic heart further supported the impact of female sex hormones on collagen synthesis.
7. Increases in total protein expression and active form of MMP-2 in diabetic hearts implied the impact of female sex hormones on extracellular matrix degradation.
8. Supplementation of either estrogen alone or in combination with progesterone in diabetic ovariectomized rats significantly increased the collagen deposition in the heart.

9. Supplementation of estrogen alone or in combination with progesterone in diabetic ovariectomized rats also increased protein expressions of procollagen and MMP-2 but not MMP-9 in the heart.

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APPENDICES

APPENDIX A
COLLAGEN DEPOSTION BY HISTOCHEMICAL ANALYSIS
(Yiming Wu et al., 2006)

Instrument

Cryostat machine

Procedures

1. The whole heart was horizontally cut in half.
2. The lower half was frozen in the optimum temperature compound (OTC, sakura tissue tek).
3. Sections of 10 μ meter were prepared using cryotome (Frozen section).
4. Some sections from each sample were stained with Sirius Red (Sigma) for the quantification of total collagen deposition under bright-field microscope.
5. The pictures were taken using the light microscope.
6. The amount of collagen was analyzed using Image J software.

Reference:

Wu Y, Peng J, Campbell KB, Labeit S & Granzier H (2007). Hypothyroidism leads to increased collagen-based stiffness and re-expression of large cardiac titin isoforms with high compliance. *J Mol Cell Cardiol* **42,** 186-195.

APPENDIX B

TITIN ISOFORMS SEPARATION ANALYSIS
(Martina et al., 2006)

Reagent

Reagent A	38.5% acrylamide
Reagent B	1.5MTris P_H(8.6)
Reagent C	50%glycerol
Reagent D	10% SDS
Reagent E	Ammonium persulfate (10%)
Reagent F	TEMED

Procedures

1. Portion of the ventricular muscle was homogenized in urea sample buffer and then protein concentration was determined using BCA protein assay (Sigma).
2. Firstly, it is necessary to set up slab gel apparatus.
3. The next step is to prepare 0.5% agarose gel and 2% acrylamide (minivertical gel electrophoresis system, Bio-Rad) as previously described (Tatsumi & Hattori, 1995).
4. As soon as the gel is prepared and poured, the 10-well comb needs to be inserted. After the preparation step is done, the gel is allowed to polymerize at 4^o C (over night).
5. When the gel is polymerized, it is to remove the comb and clean the well with distilled water 2 or 3 times so as to make the proper shape of the well. Then, the chamber is set up and is filled the running buffer until it covers the top of the gel surface.

6. One hundred milligram of the homogenized sample is loaded in this experiment. For the gel running, the pre running is 30 minutes at 8mA.
7. Then, gel is run continuously at cool temperature and constant current at 15 mA for 6 hours.
8. After running for 6 hrs, the gel is stained by Silver Stain method. Quantities of the two titin bands (N2B & N2BA) is analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

Reference:

Kruger M, Kohl T & Linke WA(2006). Developmental changes in passive stiffness and myofilament Ca2+ sensitivity due to titin and troponin-I isoform switching are not critically triggered by birth. *Am J Physiol Heart Circ Physiol* **291,** H496-H506.

APPENDIX C

PROTEIN DETERMINATION WITH BCA REAGENT

(Hill and Straka, 1998)

Reagents

Reagent A	BCA Stocking solution Composed of 1.0% Bicinochoninic acid, 2.0%, NaCo3, 0.16%, NaK-tartrate, 0.4 %NaOH, and 0.95% NaHCO3, pH11.25
Reagent B	2% $CuSO_4$+$5H_2O$
Reagent C	BCA working solution Mixed reagent A and reagent B at ratio 50:1 freshly prepared

Standard

Protein standard solution was prepared by dissolving 0.01 g of bovine serum albumin (BSA) from Sigma Chemical, MO in100 ml of distilled water.

Procedures

1. The sample and standard proteins were diluted to appropriate concentration with distilled water to obtain a total volume of 50µl.
2. Added 1 ml of mixed reagent C and then mixed by vortex immediately.
3. Incubated for at least 30 minutes at 37ºC.
4. Read optical density at 562 nm by spectrophotometer.
5. Protein concentration of sample was calculated using intercept and slope factors of standard curve.

Reference:

Hill HD and Straka JG. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal Biochem 1998;170(1):203-208.

APPENDIX D

Table I Body weight (gram) of rats from sham operated (SHAM), ovariectomized (OVX), diabetic (DM), diabetic-ovariectomized (DM-OVX), and diabetic-ovariectomized rats with supplementation of oil (OIL), estrogen (E), progesterone (P) and estrogen plus progesterone (EP).

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	299	345	210	360	301	237	306	211
2	273	366	290	201	295	230	278	181
3	275	330	262	254	306	225	228	242
4	290	387	287	308	318	222	203	238
5	313	345	202	301	293	235	286	205
6	274	407	225	310	311	163		
7	266	354	174	218		194		
8	305	352	247	280				
9	290	274		300				
10	257	380						
11		380						
12		361						
13		387						
14		363						
15		342						
MEAN	278	358	237	281	304	215	260	215
SE	2	2	5	6	2	4	9	11
N	10	15	8	9	6	7	5	5

APPENDIX E

Table II Uterine weight (gram) of rats from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	0.43	0.16	0.44	0.12	0.01	0.47	0.12	0.57
2	0.52	0.19	0.59	0.16	0.11	0.47	0.12	0.45
3	0.41	0.18	0.72	0.11	0.01	0.54	0.13	0.50
4	0.59	0.17	0.69	0.17	0.12	0.51	0.15	0.52
5	0.72	0.16	0.43	0.14	0.10	0.63	0.12	0.59
6	0.35	0.15	0.62	0.21	0.14	0.42		
7	0.66	0.12	0.27	0.10		0.57		
8	0.81	0.11	0.46	0.11				
9	0.73	0.14		0.12				
10	0.33	0.17						
11		0.11						
12		0.12						
13		0.15						
14		0.93						
15		0.14						
MEAN	0.56	0.14	0.53	0.12	0.11	0.52	0.13	0.53
SE	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.02
N	10	15	8	9	6	7	5	5

APPENDIX F

Table III collagen percentage of rats from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	3.25	2.85	5.33	3.78	3.60	4.42	3.78	5.50
2	3.27	2.70	5.55	4.10	3.85	5.03	4.22	5.10
3	3.02	2.80	4.82	3.95	3.94	4.75	4.01	5.00
4	2.81	3.47	4.55	4.01		5.00		
Average	3.09	2.96	5.06	3.96	3.80	4.80	4.00	5.20
SE	0.11	0.17	0.23	0.07	0.10	0.14	0.13	0.15
n	4	4	4	4	3	4	3	3

APPENDIX G

Table IV N2BA/N2B ratio of rats from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	5.73	9.69	9.88	45.10	39.79	41.15	40.10	41.34
2	4.75	3.48	31.20	12.54	40.93	44.30	37.37	46.98
3	5.19	3.30	17.11	28.83	36.74	46.39	37.21	37.90
4	5.31	12.89	17.16	45.40	41.96	45.45	37.99	42.15
5		8.94	12.68	19.69			38.48	44.45
6			18.59	15.96				
7			17.98					
8			22.29					
MEAN	5.24	7.66	18.36	27.93	39.86	44.32	38.23	42.56
SE	0.20	1.87	2.27	5.92	1.13	1.14	0.52	1.52
N	4	5	8	6	4	4	5	5

APPENDIX H

Table V Amount of procollagen from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	0.61	0.89	1.40	0.92	0.84	0.76	0.55	0.92
2	1.11	0.98	2.02	2.03	1.17	0.15	0.59	0.78
3	1.30	1.88	1.59	2.17	1.25	0.60	0.89	1.92
4	0.70	0.90	1.45	1.66	0.72	1.70	1.23	2.28
5	0.99		1.58	1.37				
6	1.00			0.99				
MEAN	0.95	1.16	1.61	1.53	0.99	1.05	0.80	1.50
SEM	0.10	0.20	0.10	0.21	0.13	0.24	0.16	0.37
N	6	4	5	6	4	4	4	4

APPENDIX I

Table VI Amount of Smad2 from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	1.14	0.47	1.66	1.37	1.13	0.85	0.71	1.17
2	1.09	1.34	2.09	0.99	0.90	0.90	0.61	1.22
3	0.84	0.84	1.20	0.98	0.69	1.09	0.94	1.57
4	0.77	1.19	1.23	1.21				
5	1.04	1.01	1.18	1.32				
6	1.33	1.34	1.38	1.31				
MEAN	1.03	1.03	1.46	1.20	0.91	0.94	0.75	1.32
SEM	0.08	0.14	0.15	0.07	0.11	0.06	0.08	0.11
N	6	6	6	6	3	3	3	3

APPENDIX J

Table VII Amount of MMP-2 active from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	0.96	1.2	1.11	1.05	0.99	0.91	1.00	0.98
2	0.95	1.40	1.40	1.05	1.02	1.00	1.03	1.06
3	0.82	1.01	1.17	1.08	1.05	0.93	0.97	0.98
4	1.29	1.02	1.45	1.09	0.95	1.04	0.96	0.82
5	1.00	1.32	1.20	1.43				
6	1.00							
MEAN	1.00	1.20	1.27	1.10	1.00	0.97	1.03	0.96
SEM	0.06	0.08	0.06	0.07	0.02	0.03	0.03	0.05
N	6	4	5	5	4	4	4	4

Table VIII Amount of MMP-2 total from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E2	P	EP
1	1.23	1.39	1.14	0.90	0.69	0.89	0.67	1.51
2	1.14	1.15	1.96	1.01	1.32	1.08	1.09	1.27
3	0.89	0.95	1.66	1.30	1.01	1.22	0.91	1.10
4	0.78	0.76	1.91	1.25	0.99	0.95	0.85	1.61
5	1.02		1.91	2.07				
6	1.01							
MEAN	1.00	1.10	1.70	1.30	1.00	1.03	0.88	1.37
SEM	0.07	0.11	0.14	0.19	0.13	0.07	0.09	0.12
n	6	4	5	5	4	4	4	4

APPENDIX K

Table IX Amount of MMP-9 from SHAM, OVX, DM, DM-OVX, DM-OVX-OIL, DM-OVX-E, DM-OVX-P and DM-OVX-EP

NO	SHAM	OVX	DM	DM-OVX	DM-OVX			
					OIL	E	P	EP
1	1.48	1.82	1.29	1.10	0.85	1.10	1.14	0.95
2	1.00	1.21	0.55	1.33	1.13	2.34	1.09	0.81
3	0.65	1.46	0.84	0.99	0.88	1.35	1.05	1.27
4	0.97	1.11	0.69	1.12	1.09	1.00	1.02	0.95
5		0.68	0.84	0.73				
6		0.66	0.97	1.11				
MEAN	1.00	1.20	0.90	1.10	0.99	1.45	1.08	0.95
SE	0.14	0.18	0.10	0.08	0.07	0.31	0.03	0.10
n	4	6	6	6	4	4	4	4

BIOGRAPHY

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