รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของการใช้สารต้านอนุมูลอิสระและการออกกำลังกายในการป้องกันการเปลี่ยนแปลงการทำงานของกล้ามเนื้อหัวใจภายหลังการขาดฮอร์โมนเพศหญิง

Effects of Antioxidant and Physical Activity on Changes in Myocardial Activation after Female Sex Hormone Deprivation

โดย น.สพ. ดร.เทพมนัส บุปผาอินทร์

กรกฎาคม 2558

สัญญาเลขที่ RSA5580005

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิติกรรมประกาศ

ผลงานวิจัยฉบับนี้ได้รับการสนับสนุนจาก สำนักงานกองทุนสนับสนุนการวิจัย สำนักงานคณะกรรมการการอุดมศึกษา และมหาวิทยาลัยมหิดล คณะผู้วิจัยขอขอบคุณผู้ที่มีส่วนผลักดันให้เกิดผลงานฉบับนี้ทุกท่าน ดังรายนามต่อไปนี้

ศ. ดร.จงกลณี วัฒนาเพิ่มพูล

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ศ. ดร.ประพนธ์ วิไลรัตน์

Prof. Pieter P. de Tombe

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ดร.ไพรัตน์ ไชยนอก

นอกจากนี้คณะผู้วิจัยขอบขอบคุณผู้ที่มีส่วนสบับสนุนการวิจัยทุกท่าน ได้แก่ เจ้าหน้าที่ภาควิชาสรีรวิทยา เจ้าหน้าที่หน่วยเครื่องมือกลาง เจ้าหน้าที่หน่วยสัตว์ทดลอง และเจ้าหน้าที่หน่วยหน่วยห้องปฏิบัติการเอนกประสงค์

รศ. ดร. น.สพ. เทพมนัส บุปผาอินทร์
หัวหน้าโครงการวิจัย

Abstract

Cardiac dysfunction is a major problem in the postmenopausal women. Although, the use of hormone replacement therapy is introduced, its adverse effect is still concerned. In this project, we aim to find the mechanisms underlying the cause of myocardial dysfunction after deprivation of female sex hormones and then search for an alternative treatment based on those findings. In this study, 10-week ovariectomized rat was used as a model of female sex hormone deprivation. As hypothesized, result demonstrated that lack of female sex hormones alters cardiac mitochondrial activation by decreasing ATP production, but enhancing the production of reactive oxygen species (ROS). Based on first observation, exogenous antioxidant, Tempol, was introduced to hormone deficient rats. Tempol could prevent many cardiac changes, including myofilement activation and intracellular Ca^{2+} handling, due to lack of female sex hormones. This result strengthens our hypothesis that cardiac dysfunction after menopause is mainly induced by the increase in oxidative stress. We then introduced natural high antioxidant product, promegranate juice, to this hormone-deficient model, and found that seed-blended juice give a satisfied prevention on various observing myocardial parameters, whereas seedless juice demonstrated less effective.

Moreover, we found that lack of female sex hormones increased cardiac mast cell activation as demonstration of high mast cell number with high percentage of degranulation. This hyperactive mast cell might be a significant mediator of inducing myocardial alteration after menopause. Cardiac mast cell activity was also observed in the heart of hormone-deficit rat after eight week of regular running, one hour per day, five days per week. Interestingly, exercise training could reduce mast cell degranulation but not the number of mast cell, where estrogen supplement could suppress both parameters.

In conclusion, the findings from this project reveal that mitochondrial abnormality and oxidative stress are major mechanisms leading to cardiac dysfunction after lack of female sex hormones. The potential modulation of oxidative stress might be partly induced by cardiac mast cell hyperactivation. The supplement of antioxidant could then be one strategy of cardiac prevention instead of hormone therapy. Due to the suppressive effect regular aerobic exercise on cardiac mast cell activity, it give further hypothesis that regular exercise might associate in preventing oxidative damage in the heart of postmenopausal women.

บทคัดย่อ

ความผิดปกติของกล้ามเนื้อหัวใจเป็นปัญหาที่พบในผู้หญิงเมื่อเข้าสู่วัยหมดประจำเดือน แม้ว่าการใช้ฮอร์โมนเพศหญิงทดแทนจะถึงแนะนำแต่ความปลอดภัยในการใช้ยังเป็นที่สงสัยอยู่ เพื่อให้เข้าใจกลไกการทำงานของฮอร์โมนเพศหญิงต่อการทำงานของหัวใจ โครงการวิจัยชิ้นนี้จึงได้ทำการศึกษากลไกการเปลี่ยนแปลงทำงานของหัวใจในสภาวะที่ขาดฮอร์โมนเพศหญิงและหาวิธีป้องกันใหม่ๆ ในการทดลองเราใช้หนูขาวเพศเมียที่ผ่าตัดรังไข่ออกเป็นตัวแบบในการศึกษาภาวะขาดฮอร์โมนเพศหญิง ผลการทดลองพบว่าการขาดฮอร์โมนเพศมีผลลดการสร้างเอทีพีที่ไมโตคอนเดรีย แต่จะสร้างอนุมูลอิสระมากขึ้น จากผลการทดลองนี้เราจึงได้ทดลองให้สารเทมปอล ซึ่งเป็นสารต้านอนุมูลอิสระแก่หนูขาดตัดรังไข่ สารเทมปอลสามารถป้องกันการลดการทำงานของเส้นใยกล้ามเนื้อหัวใจ และการควบคุมแคลเซียมในเซลล์ อันเกิดจากการขาดฮอร์โมนเพศได้ ซึ่งผลการทดลองทั้งสองส่วนชี้ชัดว่าฮอร์โมนเพศหญิงมีผลต่อสภาพอนุมูลอิสระของหัวใจ โดยอนุมูลอิสระจะเพิ่มขึ้นเมื่อถึงวัยหมดประจำเดือน จากผลการทดลองดังกล่าว นำมาซึ่งสมมติฐานที่ว่าน้ำทับทิมซึ่งมีสารต้านอนุมูลอิสระสูงน่าจะช่วยป้องกันการเปลี่ยนแปลงการทำงานของหัวใจได้ อย่างไรก็ตามผลการทดลองบ่งชี้ว่าน้ำทับทิมที่คั้นทั้งเมล็ดมีประสิทธิภาพที่น่าพอใจในการป้องกันการเปลี่ยนแปลงทำงานของกล้ามเนื้อหัวใจโดยให้ผลดีกว่าน้ำทับทิมที่คั้นแบบไม่มีเมล็ด

นอกจากนี้เรายังพบว่าการขาดฮอร์โมนเพศหญิงมีผลทำให้การทำงานของแมสเซลล์ในหัวใจเพิ่มมากขึ้นทั้งในแง่จำนวนเซลล์และการปล่อยสารจากแกรนูลซึ่งอาจเป็นสาเหตุเหนี่ยวนำให้เกิดความเปลี่ยนแปลงการทำงานของหัวใจในหญิงวัยหมดประจำเดือนได้ ในขณะที่การให้เอสโตรเจนทดแทนสามารถยับยั้งการเพิ่มจำนวนเซลล์และการปล่อยสารจากแกรนูลได้ แม้ว่าหนูตัดรังไข่ที่ออกกำลังเป็นประจำคือวิ่งวันละหนึ่งชั่วโมง สัปดาห์ละห้าวัน ยังพบการเพิ่มจำนวนของแมสเซลล์ แต่การออกกำลังเป็นประจำสามารถลดการปล่อยสารจากแกรนูลจากแมสเซลล์ได้อย่างมีประสิทธิภาพ

โดยสรุป โครงการวิจัยนี้ได้พบว่าการขาดฮอร์โมนเพศหญิงมีผลทำให้เกิดความผิดปกติของไมโตคอนเดรียและก่อเกิดอนุมูลอิสระในกล้ามเนื้อหัวใจซึ่งสามารถเหนี่ยวนำให้เกิดการทำงานผิดปกติของหัวใจได้ กลไกที่ทำให้เกิดความผิดปกติเหล่านี้อาจถูกเหนี่ยวนำจากการทำงานของแมสเซลล์ที่เพิ่มขึ้นในหัวใจ ดังนั้นเมื่อให้สารต้านอนุมูลอิสระเป็นประจำจึงสามารถป้องกันการเปลี่ยนแปลงการทำงานของกล้ามเนื้อหัวใจอันเกิดจากการขาดฮอร์โมนเพศหญิงได้ และเนื่องจากการออกกำลังกายเป็นประจำสามารถลดการทำงานของแมสเซลล์ในหัวใจได้ จึงเป็นไปได้ว่าการออกกำลังกายเป็นประจำจะสามารถลดการเกิดอนุมูลอิสระในหัวใจได้

Project Code: RSA5580005

Project Title: Effects of Antioxidant and Physical Activity on Changes in Myocardial Activation after Female Sex Hormone Deprivation

(ชื่อโครงการ) ผลของการใช้สารต้านอนุมูลอิสระและการออกกำลังกายในการป้องกันการเปลี่ยนแปลงการทำงานของกล้ามเนื้อหัวใจภายหลังการขาดฮอร์โมนเพศหญิง

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บทนำ

Cardiovascular disease is epidemiologically shown to cause the highest morbidity and mortality in most countries in the world. The lower incidence of heart disease in women than men during young age but a drastic increase after menopause surely suggests a beneficial effect of female sex hormones on cardiovascular function. We have previously reported that chronic deprivation of female sex hormones leads to both systolic and diastolic dysfunctions by altering the cardiac contractile and Ca^{2+}-handling proteins in a similar outcome as those observed in failing hearts. Moreover, the use of hormone replacement therapy to prevent heart disease after menopause has not been satisfied with the additional adverse induction of breast cancer. Therefore, it is crucial to understand the signaling mechanism of the hormones as well as to search for potential cardioprotective alternatives.

Despite the expressions of both estrogen and progesterone receptors in cardiac myocytes, no existence of sex hormone-responsive element on the genes of major cardiac contractile and Ca^{2+} handling proteins have been found. The information suggests an indirect regulation of female sex hormones on cardiac contractile function. Based on evidences showing reduction in antioxidative activity in the heart after female sex hormone deprivation, it is then feasible that deficiency of the hormones induces changes in oxidative properties of the heart leading to cardiac contractile dysfunction. Therefore, upregulation of the antioxidative activity might be able to attenuate the cardiac dysfunction and the molecular alterations induced after female sex hormone deprivation. The first objective of this study is then designed to evaluate the loss of endogenous antioxidative activity in the ovariectomized rat heart with the detailed investigation to find out the specific hormone of action. Secondly, the preventive action of exogenous antioxidant on changes in cardiac contractile function induced by female sex hormone deprivation will be further elucidated.

We have also reported the cardio-protective effects of exercise training on changes induced after deprivation of female sex hormones. However, the mechanistic signaling of prevention is still unclear. The increased activities of myocardial antioxidants after exercise training have been suggested to be responsible for attenuating the cardiac degradation induced by various pathological insults. Thus, exercise training might prevent cardiac contractile dysfunction induced after female sex hormone deprivation partly by improving cardiac antioxidative activity resulting in cardiac tolerance to oxidative stress. It is still be a question which pathological induction causes an increase in oxidative stress after deprivation of female sex hormones. In the project, we therefore focus the increase in cardiac inflammation that might be prevented by regular exercise.

The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans has been recently evaluated. Pre-supplementation with pomegranate juice could attenuate cardiac injury induced by doxorubicin and isoproterenol. Based on the very high antioxidative activity of pomegranate, we then propose that daily intake of pomegranate juice might attenuate those changes in cardiac contractile activation induced in ovariectomized rat heart.

Information from this study will verify the antioxidative action of female sex hormones in serving as a potent regulatory tool in preventing heart disease development after menopause. Our result will also provide supportive evidence for the use of physical exercise and natural antioxidant as alternative modes of cardiovascular disease prevention.

วัตถุประสงค์ของโครงการ

Hypothesis # 1 **Female sex hormones preserve the cardiac contractile activation through their antioxidant activity.**

Objective #1 To determine the loss of antioxidative status in the heart of ovariectomized rat

Objective #2 To determine the preventive effects of exogenous antioxidant on changes in cardiac contractile properties of ovariectomized rat

Hypothesis #2 **Exercise training prevents the cardiac contractile dysfunction induced after female sex hormone deprivation by enhancing cardiac antioxidative activity.**

Objective #3 To determine the mechanism of exercise training in preventing cardiac changes in ovariectomized rat

Hypothesis #3 **Natural antioxidant likes pomegranate also prevents the cardiac contractile changes from female sex hormone deprivation.**

Objective #4 To determine the preventive effects of pomegranate intake on the antioxidative status and the cardiac contractile properties of ovariectomized rat

ระเบียบวิธีทดลอง

Hypothesis # 1 "Female sex hormones preserve the cardiac contractile activation through their antioxidant activity."

Objective #1 To determine the loss of antioxidative status in the heart of ovariectomized rat

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

1. Sham-operated group (Sham)
2. Ovariectomized group (OVX)
3. OVX with estrogen supplementation (OVX + E)
4. OVX with progesterone supplementation (OVX + P)
5. OVX with testosterone supplementation (OVX+T)

Estrogen and progesterone were subcutaneously injected three day per weeks for ten weeks at 5 microgram and 1 milligram per rat, respectively. Testosterone at 2 mg/kg body weight was subcutaneously injection twice a week. Sham and OVX received the oil vehicle at the same volume of hormone supplement. Ten week after operation, rats were sacrificed, and the heart was excised for the measurement of mitochondrial ATP production, mitochondrial ROS production, mitochondrial morphology, and the expressions of mitofusin 2, SOD2, and oxidative phosphorylation protein complex.

ผลการทดลอง

Since mitochondria is the main part that product reactive oxygen species, the mitochondrial structure and function of the heart among animal groups were primarily evaluated. Using isolated mitochondrial preparation, we found a significant decrease in maximum velocity of complex I ATP production in the preparation from OVX rat heart as compared to that of SHAM (**Figure 1**). The suppression of ATP production was improved to be normal in the heart preparation in OVX rat with estrogen supplementation. However, progesterone and testosterone supplementations in OVX rat had no protective effect on this suppression. On the other hand the maximum velocity of complex II ATP production is not different among five experimental groups. This result suggests that estrogen play a regulatory role in mitochondrial ATP production.

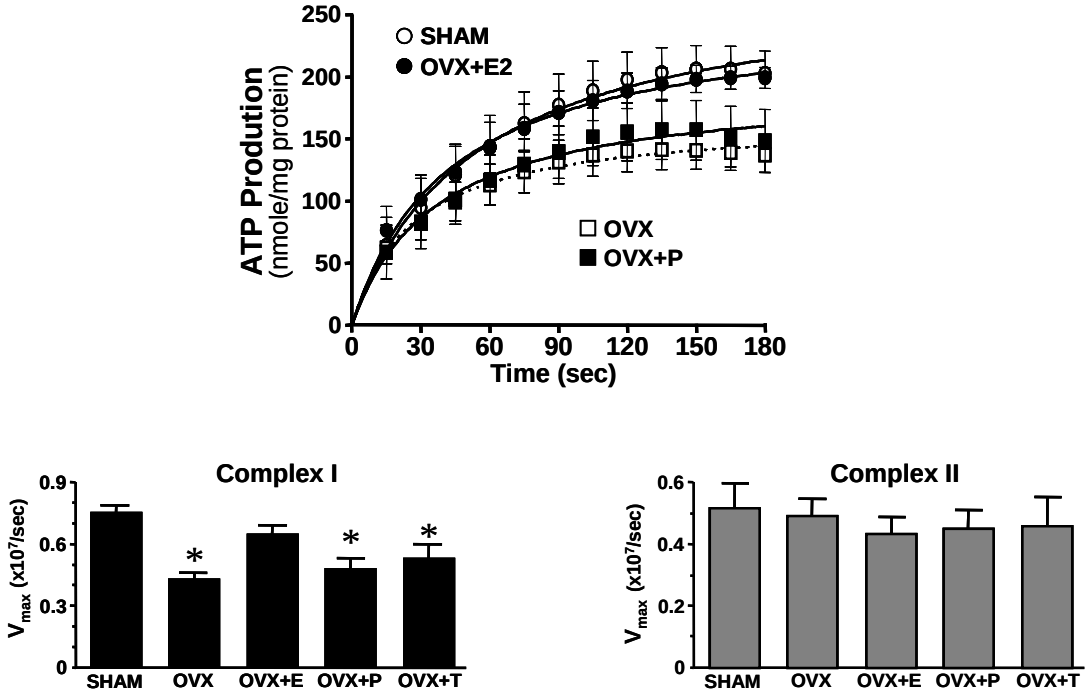

Figure 1. Effect of female sex hormones on mitochondrial ATP production. Graph curve represents rate of mitochondrial ATP production from the heart of sham-operated control (SHAM), ovariectomized rat (OVX) and OVX with estrogen supplementation (OVX+E) and OVX with progesterone supplement using Michaelis–Menten equation. Bar graphs demonstrates the maximum velocity (V_{max}) of mitochondrial ATP production by complex I and complex II of SHAM, OVX, OVX+E, OVX with progesterone supplement (OVX+P) and OVX with testosterone supplement (OVX+T). Data are mean ± SEM from 4-5 preparations in each group. * $P<0.05$ indicates significant difference to SHAM.

In addition, the effect of sex hormones on mitochondrial number and size in the heart were evaluated using electron microscope (**Figure 2**). Result shows that the number of mitochondria per area is the same in all experimental groups, but the mitochondrial size in two-dimension was significantly higher in the heart of OVX rat as compared to the heart of SHAM. Interesting that the over size of mitochondria in OVX rat heart was not observed in the heart of OVX rat with either estrogen or progesterone supplementation. While estrogen and progesterone supplementation could reduce the mitochondrial size in the heart of OVX rat, testosterone supplementation exerts no protective effect.

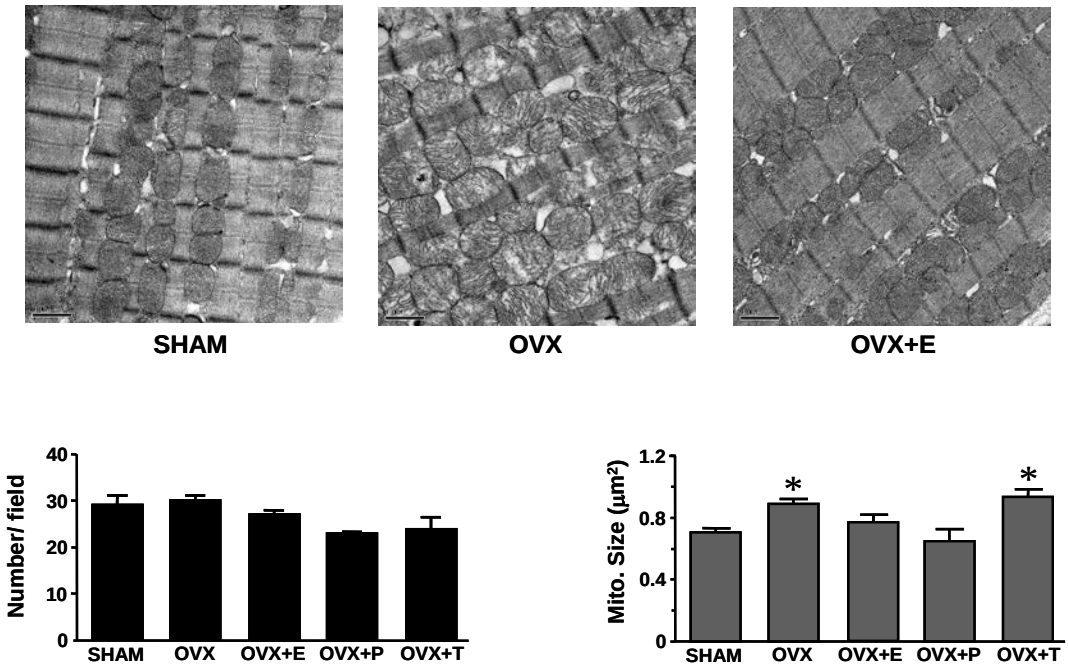

Figure 2. Effect of female sex hormones on mitochondrial number and size. Electron microscopy shows representative ultra-structure image of the cardiac muscle from SHAM, OVX and OVX+E. Bar graphs summarized the number per field and 2 dimension area of mitochondria from the heart of SHAM, OVX, OVX+E, OVX+P and OVX+T. Data are mean ± SEM from 6 hearts (10 images per heart) in each group. * $P<0.05$ indicates significant difference to SHAM.

Next, ROS production was also measured from isolated mitochondrial preparation by DCFDA assay. Using complex I reaction, the ROS production under normal condition was not changed in the preparation from OVX rat heart as compare to SHAM (**Figure 3**). With antimycin A incubation (ROS generator), ROS production increases in all preparations. Interestingly, the antimycin A-increased ROS production was significantly higher in mitochondria from OVX rat heart than that of SHAM. Surprisingly, estrogen supplementation could not normalize this change, but progesterone supplementation instead showed the protective effect. Testosterone supplementation, yet again, demonstrated no impact.

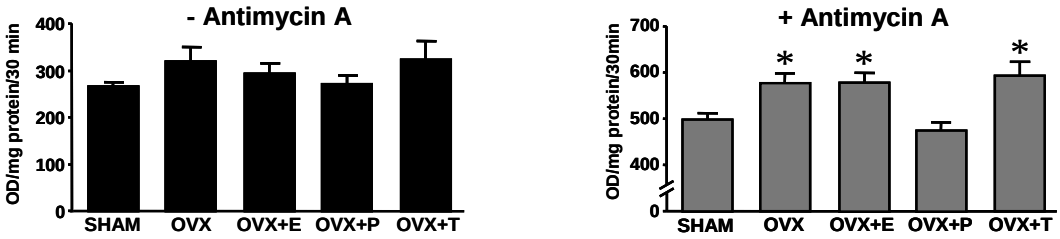

Figure 3. Effect of female sex hormones on mitochondrial ROS production. Bar graphs summarized ROS production of isolated mitochondria from the heart of SHAM, OVX, OVX+E, OVX+P and OVX+T. The reaction was measured in absence and presence of antimycin A (ROS generator). Data are mean ± SEM from 6-8 hearts in each group. * P<0.05 indicates significant difference to SHAM.

Though there were many changes in the mitochondrial activity, the expression of mitofusin 2, SOD2, and mitochondrial oxidative phosphorylation protein complex were not altered (**Figure 4**).

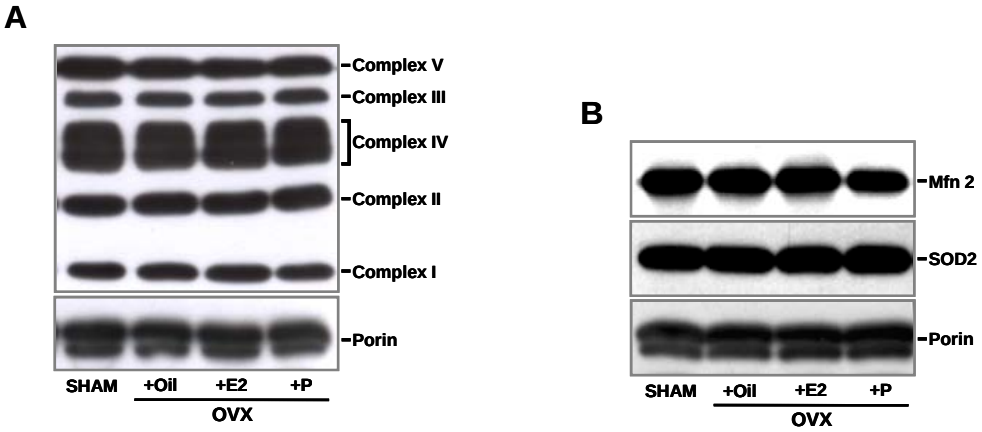

Figure 4. Effect of ovarian sex hormone deprivation on expression of mitochondrial protein complexes, mitofusin 2 (Mfn2), and SOD2. ***A.*** Representative immunoblot analysis of cardiac mitochondrial complex proteins and porin of isolated mitochondria from SHAM and OVX rats, with/without E2 or P. ***B.*** Representative immunoblot analyses of cardiac mitofusin-2 (Mfn 2), superoxide dismutase 2 (SOD2), and porin of isolated mitochondria from SHAM and OVX rats, with/without E2 or P.

Hypothesis # 1 "Female sex hormones preserve the cardiac contractile activation through their antioxidant activity."

Objective #2 To determine the preventive effects of exogenous antioxidant on changes in cardiac contractile properties of ovariectomized rat

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

1. Sham-operated group (Sham)
2. Ovariectomized group (OVX)
3. OVX with Tempol supplementation (OVX + Temp)

One week after operation, tempol was supplemented in drinking water (3 mmole/l). Ten week after operation, rats were sacrificed, and the heart was excised for the measurement of cardiomyocyte cell shortening, intracellular Ca^{2+} transients, isometric force contraction and myosin heavy chain isoform expression. Plasma was collected for the measurement of body oxidative status.

ผลการทดลอง

Lack of female sex hormones had no effect on plasma antioxidant activity, while tempol supplementation significantly increased plasma antioxidant activity (**Table I**). Tempol (SOD mimetic drug) supplementation prevented a decrease in maximum force contraction of cardiac myofilament (**Figure 5**) and myofilement Ca^{2+} hypersensitivity (**Figure 6**) induced by chronic lack of female sex hormones. But Tempol supplementation partially prevented the shift of myosin heavy chain-isoform expression from alpha to be more beta isoform) by chronic lack of female sex hormones (**Figure 8**).

Table I. Plasma antioxidant activity in sham-control, ovariectomized rat, and ovariectomized rat with tempol supplementation.

Assay	Sham-control	OVX	OVX+Tempol
FRAP assay (nmole TE/L)	1161 ± 38	1198 ± 13	1280 ± 12*
DPPH assay (nmole TE/L)	814 ± 13	833 ± 2	883 ± 24*

* $P<0.05$, significant difference from Sham and OVX. (TE = trolox equivalent)

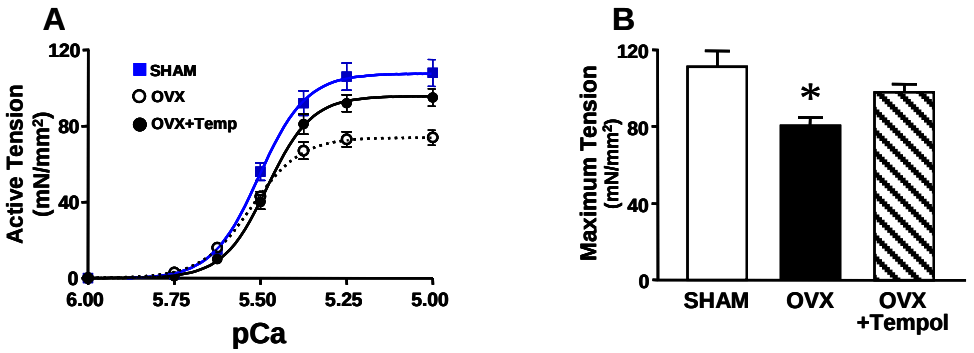

Figure 5. Effect of antioxidant (tempol) supplementation on cardiac myofilament Ca^{2+} activation in ovariectomized rat. Graph curve represents active tension-pCa (-log[Ca^{2+}]) relation of the skinned left ventricular papillary preparation comparing among SHAM, OVX and OVX with tempol supplementation (OVX+Temp). Bar graph demonstrates the maximum active tension of the preparation of the heart among three groups. Data are mean ± SEM from 16-20 preparation (5-7 hearts) in each group. * P<0.05 indicates significant difference to SHAM.

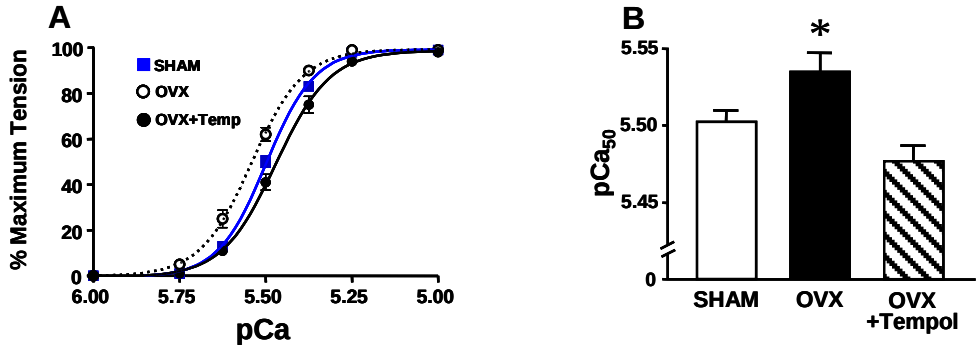

Figure 6. Effect of antioxidant (tempol) supplementation on cardiac myofilament Ca^{2+} sensitivity in ovariectomized rat. Graph curve represents percent maximum tension-pCa (-log[Ca^{2+}]) relation of the skinned left ventricular papillary preparation comparing among SHAM, OVX and OVX+Temp. Bar graph demonstrates –log Ca^{2+} concentration that provides the haft maximum tension (pCa_{50}) of the preparation from three experimental groups. Data are mean ± SEM from 16-20 preparation (5-7 hearts) in each group. * P<0.05 indicates significant difference to SHAM.

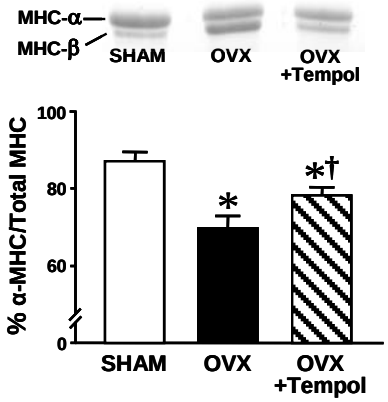

Figure 7. Effect of antioxidant (tempol) supplementation on cardiac myosin heavy chain (MHC) isoform expression in ovariectomized rat. Upper are representative bands of MHC isoforms from the heart of SHAM, OVX and OVX+Temp. Bar graph demonstrates % expression of α-MHC isoform per total expression of MHC from three experimental groups. Data are mean ± SEM from 5-6 hearts in each group. * P<0.05, indicates significant difference to SHAM. † P<0.05, indicates significant difference to OVX.

Tempol supplementation prevented a decrease in % cell shortening and an increase in re-lengthening duration of cardiomyocyte isolated from OVX rats (**Table II**). Tempol supplementation also prevented a decrease in Ca^{2+} transient amplitude and a prolonged Ca^{2+} decay time of cardiomyocyte isolated from OVX rats (**Table II**).

Table II. Effect of Tempol supplement on contraction and relaxation properties of isolated cardiomyocytes

Parameters	SHAM	OVX	OVX+Tempol
Sarcomere shortening			
% shortening	8.282 ± 0.314	7.359 ± 0.245*	8.150 ± 0.224
Time to peak (sec)	0.220 ± 0.006	0.227 ± 0.004	0.238 ± 0.005
T_{50} (sec)	0.378 ± 0.012	0.402 ± 0.009	0.430 ± 0.010*
T_{90} (sec)	0.480 ± 0.014	0.525 ± 0.0131*	0.543 ± 0.013*
(+)dl/dt	2.144 ± 0.115	2.115 ± 0.130	2.171 ± 0.143
(-)dl/dt	1.537 ± 0.097	1.255 ± 0.061*	1.256 ± 0.059*

Intracellular Ca^{2+} Transients			
Baseline (F_1/F_0)	1.837 ± 0.127	1.972 ± 0.102	2.379 ± 0.065*
Amplitude (F_1/F_0)	0.324 ± 0.021	0.268 ± 0.018*	0.328 ± 0.014
Time to peak (sec)	0.066 ± 0.003	0.060 ± 0.002	0.068 ± 0.002
T_{50} (sec)	0.213 ± 0.006	0.202 ± 0.0047	0.209 ± 0.006
T_{90} (sec)	0.402 ± 0.012	0.442 ± 0.014*	0.398 ± 0.012

*Data are mean □ SE. * P < 0.05, significantly different from control using ANOVA following by Student Newman Keuls Test.*

Hypothesis #2 "Exercise training prevents the cardiac contractile dysfunction induced after female sex hormone deprivation by enhancing cardiac antioxidative activity."

Objective #3 To determine the mechanism of exercise training in preventing cardiac changes in ovariectomized rat

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. Sedentary sham-operated group (Sham)
2. Sedentary ovariectomized group (OVX)
3. OVX with estrogen supplementation (OVX + E)
4. OVX with exercise training (OVX + Ex)

Estrogen was subcutaneously injected three day per weeks for ten weeks at 5 microgram per rat. Regular exercise program including one week acclimatization following by ten weeks of moderate intensity running (65-75% maximum oxygen consumption) was introduced to the rat. Exercised rat was run two intervals of 30 minutes per day, 5 days per week. They were rested 10-15 minutes between each interval. Adequacy of running program was verified by echocardiography at the end of study. Eleven week after operation, echocardiography was performed under anesthesia. Then, rats were sacrificed, and the heart was excised for the measurement of mast cell density and % mast cell degranulation. The expressions of chymase, interleukin 6 (IL-6) and interleukin 10 (IL-10) were examined.

ผลการทดลอง

Chronic deprivation of ovarian sex hormones induced a significant decrease in left ventricular contraction demonstrated by fractional shortening and ejection fraction (**Table III**). Both exercise training and estrogen supplement could prevent these changes. Although lack of female sex hormones and exercise training increased left ventricular mass, only exercise rats demonstrated cardiac hypertrophy by increasing left ventricular mass index.

Table 2 Echocardiographic parameters of sham-operated (SHAM) and ovariectomized (OVX) rats and OVX with estrogen supplementation or exercise training.

Parameters	SHAM	OVARIECTOMY		
		Oil	Estrogen	Exercise
IVS_s (cm)	0.233 ± 0.007	0.240 ± 0.011	0.258 ± 0.014	0.261 ± 0.006
IVS_D (cm)	0.175 ± 0.007	0.190 ± 0.014	0.193 ± 0.017	0.208 ± 0.007
$LVPW_S$ (cm)	0.232 ± 0.009	0.282 ± 0.024	0.280 ± 0.026	0.302 ± 0.026
$LVPW_D$ (cm)	0.143 ± 0.014	0.201 ± 0.018	0.186 ± 0.016	0.183 ± 0.013
$LVID_S$ (cm)	0.373 ± 0.012	0.414 ± 0.022	0.314 ± 0.025$^{\#}$	0.367 ± 0.015
$LVID_D$ (cm)	0.588 ± 0.020	0.602 ± 0.037	0.523 ± 0.030	0.615 ± 0.015
LV FS (%)	36.6 ± 0.3	30.9 ± 0.6*	39.9 ± 1.0*$^{\#}$	40.5 ± 1.2*$^{\#}$
LV EF (%)	72.5 ± 0.4	64.8 ± 0.8*	76.6 ± 1.2*$^{\#}$	76.7 ± 1.3*$^{\#}$
RWT	0.463 ± 0.052	0.680 ± 0.092	0.728 ± 0.097	0.603 ± 0.058
Estimated LV mass (g)	0.541 ± 0.023	0.789 ± 0.063*	0.614 ± 0.045$^{\#}$	0.817 ± 0.016*
LV mass index	2.08 ± 0.11	2.04 ± 0.12	2.25 ± 0.08	2.51 ± 0.11*$^{\#}$

Data are mean ± SEM. *, $^{\#}$ $P < 0.05$, significantly different from SHAM and OVX, respectively, using Student-Newman-Keuls test after ANOVA. Data includes systolic ($_S$) and diastolic parameters ($_D$) of interventricular septum, *IVS*; left ventricular posterior wall, *LVPW*; left ventricular internal diameter, *LVID*; fractional shortening, *FS*; ejection fraction, *EF*; relative wall thickness, *RWT*; and left ventricular mass.

Increase in mast cell density in cardiac tissue was demonstrated in ovariectomized rat heart as compared to sham-controls (**Figure 8, table IV**). Estrogen supplement, but not exercise training, prevented increased mast cell density in ovariectomized rat heart. Although female sex hormone deficiency did not affect mast cell degranulation, exercise training significantly suppressed the percent mast cell degranulation (**table IV**). Expression of chymase protein in the heart was not affected by either female sex hormones or exercise training (**Figure 9**). Although ovariectomy did not alter the expression of both IL-6 and IL-10, estrogen supplementation suppressed the IL-6 expression in the heart tissue

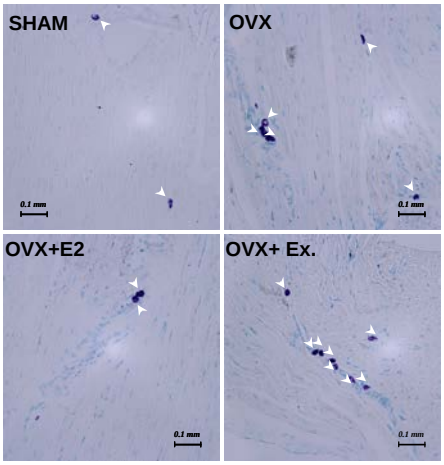

Figure 8. Effect of regular exercise on the density and % degranulation of cardiac mast cells in ovariectomized rats. Images represent the transverse section of ventricle at 40X from SHAM; sham-operated controls, OVX; ovariectomized rats, OVX+E2; ovariectomized rats with estrogen supplementation, and OVX+Ex; exercise trained ovariectomized rats. By using toluidine blue stain, cardiac mast cells are identified in dark-purple (arrow head).

Table IV. Effect of exercise training on cardiac mast cell activation in ovariectomized rat.

Mast Cell Activities	SHAM	Ovariectomized		
		+ Oil	Estrogen	Exercise
Density (cells/mm^2)	1.89 ± 0.12	3.13 ± 0.18*	2.32 ± 0.24$^{\#}$	3.03 ± 0.31*
% Degranulation (%)	48.3 ± 2.9	61.8 ± 1.3*	52.5 ± 3.9	40.8 ± 4.9$^{\#}$

*Data are mean □ SE. *, $^{\#}$ P < 0.05, significantly different from Sham and OVX+Oil, respectively, using ANOVA following by Student Newman Keuls Test.*

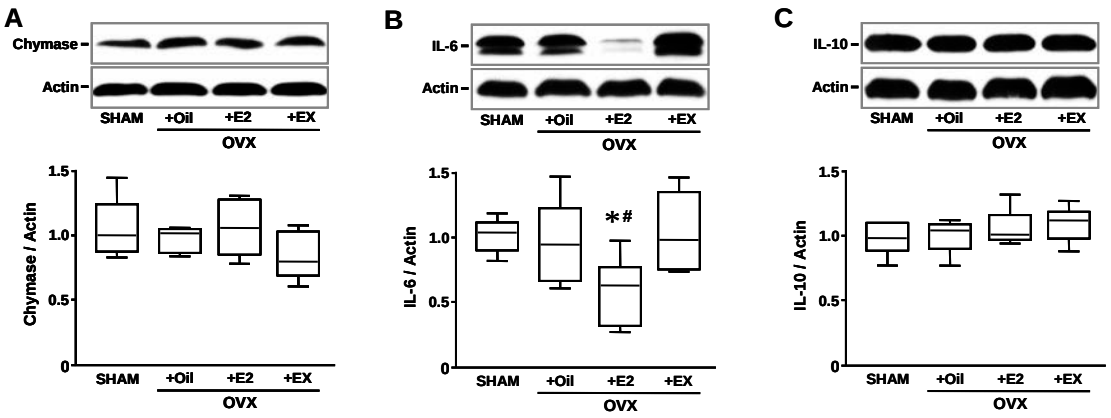

Figure 9. Effect of regular exercise on the expression of inflammatory cytokines in the heart of ovariectomized rats. Representative immunoblots of chymase (***A***), interleukin-6 (***B***), and interleukin-10 (***C***) in comparable to β-actin expression are shown above box plots. Data are the ratio of each protein band density to actin's band density from 6 hearts in each group. *, # indicates significant difference ($P < 0.05$) from SHAM and OVX, respectively, using Student-Newman-Keuls test after ANOVA.

Hypothesis #3 "Natural antioxidant likes pomegranate also prevents the cardiac contractile changes from female sex hormone deprivation."

Objective #4 To determine the preventive effects of pomegranate intake on the antioxidative status and the cardiac contractile properties of ovariectomized rat

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. OVX supplemented with seed-blended pomegranate juice (Pom-I)
4. OVX supplemented with seedless pomegranate juice (Pom-II)

In this study, ovariectomized rats randomly received 50 mL of 30% seed-blended pomegranate juice (Pom-I) or seedless juice (Pom-II) everyday for 10 weeks. OVX control rats also received sucrose by adding 8% in drinking water.

ผลการทดลอง

Decrease in cardiac cell shortening in the cardiomyocyte of ovariectomized rat could be prevented by supplementation with both pomegranate juice formulas (**Table V**). However, pomegranate juice could not prevent the prolongation of time to peak shortening and relengthening time in this ovariectomized model. Suppressed amplitude of intracellular Ca^{2+} transients in the cardiomyocyte from ovariectomized rat could be attenuated by seed-blended pomegranate juice (Pom-I), but not by seed-free pomegranate juice (Pom-II). Prolongations of cardiac Ca^{2+} reuptake as demonstrated by time to 50% of Ca^{2+} decay (T_{50}) in female sex hormone deficient model could not be improved by both formulas of pomegranate juice supplement.

In addition, seed-blended pomegranate juice significantly reversed the isoform expression of cardiac myosin heavy chain (MHC) in the heart of ovariectomized rat (**Figure 10**). Amount of alpha-MHC was increased by pomegranate juice (Pom-I). However, there was no protective action of seed-free juice on MHC isoform expression.

Table V. Effect of daily pomegranate intake on contraction and relaxation properties of isolated cardiomyocytes

Parameters	SHAM	OVX	OVX+Pom-I	OVX+Pom-II
Sarcomere shortening				
% shortening	10.87 ± 0.11	9.74 ± 0.12*	10.15 ± 0.12	10.23 ± 0.13
Time to peak (msec)	205 ± 2	225 ± 2*	241 ± 2*	239 ± 3*
T_{50} (msec)	330 ± 3	371 ± 4*	411 ± 5*	403 ± 5*
T_{90} (msec)	403 ± 5	452 ± 6*	499 ± 7*	486 ± 7*
Intracellular Ca^{2+} Transients				
Baseline (F_1/F_0)	1.68 ± 0.03	1.63 ± 0.04	1.63 ± 0.02	1.50 ± 0.02
Amplitude (F_1/F_0)	0.204 ± 0.007	0.173 ± 0.005*	0.195 ± 0.006	0.183 ± 0.005*
Time to peak (msec)	62.0 ± 2.2	73.9 ± 4.9	63.6 ± 1.6	63.6 ± 2.1
T_{50} (msec)	193 ± 3	218 ± 4*	206 ± 3*	205 ± 3*
T_{90} (msec)	371 ± 10	364 ± 9	365 ± 8	347 ± 9

*Data are mean ± SE from five hearts each group (30 cells/heart were recorded). * P < 0.05, significantly different from control using ANOVA following by Student Newman Keuls Test.*

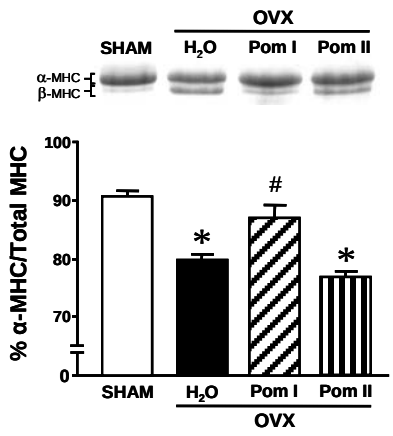

Figure 10. Effect of daily pomegranate juice inatake on cardiac myosin heavy chain (MHC) isoform expression in ovariectomized rat. Upper are representative bands of MHC isoforms from the heart of SHAM, OVX, OVX+Pom I, and OVX+Pom II. Bar graph demonstrates % expression of α-MHC isoform per total expression of MHC from three experimental groups. Data are mean ± SEM from 5 hearts in each group. * $P<0.05$, indicates significant difference to SHAM. # $P<0.05$, indicates significant difference to OVX.

บทสรุปและวิจารณ์

The first objective is to determine the role of female sex hormones on the oxidative activity in the heart. Role of sex hormones in mitochondrial function, which is a major organelle regulating cellular free radical was evaluated. Result demonstrated that the mitochondria isolated from ovariectomized (OVX) rat heart had a lower ATP production activity of complex I than that of sham-operated control (SHAM). The significant suppression of ATP production in OVX group was prevented by estrogen supplement, but not progesterone. Electron microscopy demonstrated that the heart of OVX rat has a significant larger mitochondrial size than that in SHAM heart. This mitochondrial swelling could be normalized by either estrogen or progesterone supplement. For ROS production, there is no effect of female sex hormones on mitochondrial ROS production under normal stage; however, rate of ROS production under *in vitro* stress by either Ca^{2+}-overload or antimycin A was significantly higher in mitochondrial isolated from OVX rat heart. This alteration in OVX rat could completely prevent by either estrogen or progesterone. In this study, we observed no change in the expression of mitochondrial complex proteins, mitofusin-2, and superoxide dismutase-2 in the mitochondria of OVX rat heart as compared to those of SHAM.

The second experiment aims to test whether changes in cardiac contractile function after female sex hormone deficiency can be prevented by exogenous antioxidant. Tempol, a cell-permeable superoxide dismutase mimetic drug, was added in the drinking water. Rat received Tempol after ovariectomy for 10 weeks. Using skinned fiber preparation of papillary muscle, myofilament from the heart of OVX rat has a decrease in maximum active tension, but an increase in myofilement Ca^{2+} sensitivity as compared to SHAM similar to our previous report. These changes of myofilament from the heart of OVX could not be observed in the preparation from OVX rat with Tempol treatment. However, shift of myosin heavy chain isoform from predominant alpha-isoform to more beta-isoform in OVX rat heart could partially reverse by Tempol. By using isolated cardiomyocyte preparation, cells from OVX rats has a significant lower % cell shortening, longer time of cell relengthening, lower amplitude of intracellular Ca^{2+} transients, longer time of cytosolic Ca^{2+} decay than that of cells from SHAM. Tempol supplementation could prevent changes in % cell shortening, amplitude of intracellular Ca^{2+} transients, and time of cytosolic Ca^{2+} decay. However, it could not normalize the prolonged time of cell relengthening in OVX rat cardiomyocytes. Results from these experiments imply that antioxidative property could be one cardioprotective mechanism of female sex hormones, but not all.

The third experiment aims to prove whether regular exercise can prevent cardiac changes induced after deprivation of female sex hormones by suppressing free radical

production. Since the ROS could induce pathological cardiac development by stimulating mast cell activation, we then first prove the effect of regular exercise on cardiac mast call activity in the ovariectomized rat heart. Ten weeks after ovariectomy, result demonstrated an increase in mast cell number in the ventricular tissue in which estrogen supplementation could attenuate this change. Although regular exercise could not inhibit the increase in mast cell number in the heart, the % mast cell degranulation significantly decreased in the heart of OVX rat with regular running. This result indicated that regular exercise could also attenuate the mast cell activity but in difference of estrogen mechanism.

The last experiment aims to test whether pomegranate juice, which has a high antioxidant activity, could replacing estrogen supplementation for preventing cardiac change after female sex hormone deficiency. We demonstrated that seed-blended pomegranate juice exerts more effective in preventing cardiac changes due to deprivation of female sex hormones than seed free juice. It is possible that the cardio-preventive effect of pomegranate is due to phytoestrogen in the seed, but may be not phenolic compound in the aril.

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

1. ศึกษากลไกที่เหนี่ยวนำให้เกิดความผิดปกติของ mitochondria ในภาวะที่ขาดฮอร์โมนเพศ
2. ศึกษาความสัมพันธ์ระหว่างสารที่หลั่งออกมาจาก cardiac mast cell กับการทำงานของกล้ามเนื้อหัวใจ รวมทั้งการเปลี่ยนแปลงที่ mitochondria
3. ศึกษาสารประกอบใน เมล็ดทับทิมที่สามารถนำมาพัฒนาเพื่อเป็นยาที่ใช้ป้องกันความผิดปกติของหัวใจ

Keywords : Female sex hormones, Mitochondria, Antioxidant, Regular exercise, Pomegranate juice

(คำหลัก) ฮอร์โมนเพศหญิง ไมโตคอนเดรีย สารต้านอนุมูลอิสระ การออกกำลังกายเป็นประจำ น้ำทับทิม

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   1.1 Rattanasopa C, Phungphong S, Wattanapermpool J, **Bupha-Intr T.**
   Significant role of estrogen in maintaining cardiac mitochondrial functions.
   J Steroid Biochem Mol Biol. 2015;147:1-9.

   1.2 Phungphong S, Kijtawornrat A, Wattanapermpool J, **Bupha-Intr T.**
   Regular exercise modulates cardiac mast cell activation in ovariectomized rats.
   J Physiol Sci. 2015 Oct 14. [Epub ahead of print]

2. การนำผลงานวิจัยไปใช้ประโยชน์

   2.1 เชิงวิชาการ

      2.1.1 งานวิจัยนี้เป็นหนึ่งในวิทยานิพนธ์ ของ นส.ชุติมา รัตนโสภา นักศึกษาหลักสูตรปริญญาเอก สาขาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

      2.1.2 งานวิจัยนี้เป็นหนึ่งในวิทยานิพนธ์ ของ นส.สุกัญญา พึ่งพงษ์ นักศึกษาหลักสูตรปริญญาโท สาขาสรีรวิทยาของการออกกำลังกาย คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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Significant role of estrogen in maintaining cardiac mitochondrial functions

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ABSTRACT

Increased susceptibility to stress-induced myocardial damage is a significant concern in addition to decreased cardiac performance in postmenopausal females. To determine the potential mechanisms underlying myocardial vulnerability after deprivation of female sex hormones, cardiac mitochondrial function is determined in 10-week ovariectomized rats (OVX). Significant mitochondrial swelling in the heart of OVX rats is observed. This structural alteration can be prevented with either estrogen or progesterone supplementation. Using an isolated mitochondrial preparation, a decrease in ATP synthesis by complex I activation in an OVX rat is completely restored by estrogen, but not progesterone. At basal activation, reactive oxygen species (ROS) production from the mitochondria is not affected by the ovariectomy. However, after incubated in the presence of either high Ca^{2+} or antimycin-A, there is a significantly higher mitochondrial ROS production in the OVX sample compared to the control. This increased stress-induced ROS production is not observed in the preparation isolated from the hearts of OVX rats with estrogen or progesterone supplementation. However, deprivation of female sex hormones has no effect on the protein expression of electron transport chain complexes, mitofusin 2, or superoxide dismutase 2. Taken together, these findings suggest that female sex hormones, estrogen and progesterone, play significant regulatory roles in maintaining normal mitochondrial properties by stabilizing the structural assembly of mitochondria as well as attenuating mitochondrial ROS production. Estrogen, but not progesterone, also plays an important role in modulating mitochondrial ATP synthesis.

1. Introduction

The roles of female sex hormones in the regulation of cardiovascular function have been recently re-evaluated due to disputation in the benefits of hormone replacement therapy in postmenopausal women. [1]. Increased ventricular wall thickness but decreased fractional shortening in the hearts of postmenopausal females compared to age-matched premenopausal controls independent of hypertension suggested potential direct actions of female sex hormones on cardiac muscle [2,3]. Many clinical trials have previously demonstrated an improvement in cardiac performance in postmenopausal females who received hormone replacement therapy [4–6]. In addition, postmenopausal females who received hormone replacement therapy had a significantly improved in-hospital survival after coronary bypass [7], indicating the protective effects of female sex hormones against cellular damage. However, some studies found a small advantage in reducing heart disease incidence using hormone replacement therapy [8,9]. These inconclusive reports further challenged the benefit of hormone replacement therapy in cardiovascular medicine and indicated the need for more direct evidence of the regulatory roles of sex hormones in cardiac function.

Mitochondria may be one potential target of female sex hormone actions in the heart. It is well known that mitochondria play a fundamental role in regulating cell survival processes involved in ATP production, reactive oxygen species (ROS) generation, intracellular calcium mobilization, and the regulation of cell apoptosis [10–12]. Many previous studies have revealed the effects of estrogen and progesterone on mitochondria protein expression and respiratory function in various tissues [13–15]. However, only a few studies have determined the effect of female sex hormones on the mitochondria of cardiomyocytes [16], in which estrogen could prevent damage of the mitochondrial structure and a decrease in mitochondrial respiratory function induced after ischemia-reperfusion. Interestingly, the rate of ATP production in the heart of female rats was significantly lower compared to male hearts [17], whereas mitochondrial ROS production of male rat hearts was higher compared to female

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0960-0760/

hearts [18]. A decrease in the rate of mitochondrial oxygen consumption with a reduction of mitochondrial complex IV protein was observed in ovariectomized rats, [19]. An acute study showed that estrogen preincubation decreased the sensitivity of mitochondria to Ca^{2+}-induced permeability transition pore opening and the release of cytochrome *c* from cardiac mitochondria [20]. Another study demonstrated that estrogen treatment protected mitochondrial ATP production against trauma-hemorrhagic stress [21]. Importantly, all previous studies had indicated the cardioprotective effect of estrogen on mitochondrial function and did not highlight the cellular effect of progesterone. On the basis of the results of the Women's Health Initiative study [22], a significant contribution of progesterone to the increased risk of cardiovascular disease during hormone replacement therapy in postmenopausal females was indicated.

In the present study, we aim to demonstrate changes in the function of mitochondria in female rat hearts after chronic deprivation of ovarian sex hormones after estrogen and progesterone supplementation. Ten-week ovariectomized rats are used in this study, as previous reports had demonstrated significant suppression in cardiac contraction and relaxation after 10 weeks of sex hormone deprivation [23,24]. Two major functions of mitochondria, i.e., ATP production and ROS generation, are assessed using isolated a mitochondrial preparation. Our results demonstrate that chronic deprivation of ovarian sex hormones results in a decrease in mitochondrial ATP, but an increase in ROS production. Estrogen plays a regulatory role in ATP synthesis whereas progesterone contributes to suppressed ROS generation.

2. Materials and methods

2.1. Animal preparations

Female Sprague-Dawley rats weighing between 180 and 200 g (8–9 weeks old) were sham-operated or ovariectomized. All animals were fed *ad libitum* (C.P., Thailand) and had access to tap water during the entire experiment. Rats were individually housed in a shoe box under a 12:12 light-dark cycle with controlled temperature and humidity. Two days after the operation, ovariectomized rats were randomly divided into control and hormone-supplemented groups. Hormone supplementation was performed by subcutaneous injection of estrogen (5 μg/rat) and progesterone (1 mg/rat), three times a week as previously described [25]. Sham-operated and ovariectomized control rats were injected with corn oil (vehicle). Ten weeks after the operation, the rats were anesthetized and the hearts were quickly excised and placed in ice cold Krebs Henseleit (KH) buffer. Deficiency of ovarian sex hormones was confirmed by reduced uterine weight. Serum hormones were analyzed using ELISA test kit (Calbiotech) for estradiol and ECLIA system (IMMULITE 2000 XPi) for progesterone and testosterone levels. The animal protocol was approved by the Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with the National Animal Laboratory Centre, Thailand.

2.2. Isolation of cardiac mitochondria

Mitochondria were isolated from the left ventricle (LV) as previously described [26]. Briefly, the LV was weighed and minced in 8 mL of ice-cold homogenizing buffer containing 50 mM sucrose, 200 mM mannitol, 5 mM KH_2PO_4, 5 mM MOPS, 2 mM taurine, 1 mM EGTA, and 1% w/v BSA. The sample was transferred into a Glass–Teflon homogenizer for tissue homogenization by 12 strokes at speed 4 (800 rpm). The homogenate was centrifuged at 500 × *g* (1500 rpm) and 4 °C for 5 min, and the supernatant was kept at 4 °C. The total mitochondrion-containing supernatant was centrifuged at 15,000 × *g* (11,000 rpm), 4 °C for 5 min. The pellet was resuspended in 8 mL of homogenizing buffer, blended, and centrifuged at 10,000 × *g* (9100 rpm) and 4 °C for 5 min. The final pellet was washed with 1 mL of suspension buffer (50 mM sucrose, 200 mM mannitol, 5 mM KH_2PO_4, 5 mM MOPS, 2 mM taurine) and suspended in 400 μL of suspension buffer [26]. The protein concentration of the isolated mitochondria was determined using the Bradford assay [27]. The protein dilution was prepared for the ROS and ATP production assay.

2.3. Measurement of mitochondrial swelling

Cardiac mitochondrial swelling was determined by a decrease in light absorption as previously described [28], with modification. Isolated mitochondria were diluted with suspension buffer to a final concentration of 1 mg/ml. The optical density was continuously measured in 96-well microplate using a microplate reader at 540 nm wavelength.

2.4. Measurement of mitochondrial ATP synthesis

Measurement of mitochondrial ATP production was performed as previously described [26]. Complex I activity was determined in reaction buffer A (150 mM KCl, 25 mM Tris, 10 mM KH_2PO_4, 2 mM EDTA, 0.1 mM MgCl, and 0.1% BSA (pH 7.4)) with the addition of 1 mM pyruvate and 1 mM malate as substrates, 0.8 mM luciferin and 20 μg/ml luciferase (Promega), 0.15 mM di-adenosine pentaphosphate (to inhibit adenylate cyclase) and 30 μg mitochondria. Complex II activity was also determined in reaction buffer A using 5 mM succinate as the substrate and 40 μM rotenone as complex I inhibitor in 30 μg of mitochondria. In every reaction, ADP was added to a final concentration of 0.1 mM to initiate ATP synthesis

Table 1
Characteristics of sham-operated (SHAM) and ovariectomized (OVX) rats with and without estrogen or progesterone supplementation.

	SHAM	Ovariectomy		
		Oil	Estrogen	Progesterone
Body weight (g)	274 ± 3	351 ± 5*	242 ± 3**	343 ± 5*
Heart weight (g)	1.16 ± 0.02	1.39 ± 0.02*	1.06 ± 0.02**	1.34 ± 0.02*
Heart/body weight (×100)	425 ± 8	396 ± 7*	439 ± 6**	398 ± 7*
Uterine weight (g)	0.66 ± 0.03	0.14 ± 0.01*	0.51 ± 0.03**	0.18 ± 0.02*
Serum estradiol (pg/mL)	6.21 ± 0.73	2.68 ± 0.30*	6.65 ± 0.99**	2.59 ± 0.62*
Serum progesterone (ng/mL)	20.8 ± 1.1	6.4 ± 1.7*	12.4 ± 1.3*,**	12.6 ± 0.9*,**
Serum testosterone (ng/mL)	44.0 ± 4.9	34.3 ± 3.3	<20[NA]	<20[NA]

Data are mean ± SE from 10–12 rats of each group. [NA] indicates no statistical analysis.
* $P < 0.05$, significantly different from SHAM, using Student–Newman–Keuls test after ANOVA.
** $P < 0.05$, significantly different from OVX, using Student–Newman–Keuls test after ANOVA.

and the luminescence signals were simultaneously recorded every 15 s (Lumat, Berthold) for an approximate duration of 4–5 min. Oligomycin (2 μg/ml) was added to determine the non-mitochondrial rate of ATP synthesis under the same conditions. The luminescence recordings (relative light units, RLU) were plotted against time and the linear part of the curve was used to calculate the maximum rate of ATP synthesis. The rate of ATP synthesis attributable to mitochondria was calculated by subtracting the rate of ATP synthesis in the presence of oligomycin. The rate of ATP production was fitted with one phase association equation ($Y = Y0 + (\text{Plateau} - Y0)^*(1 - \exp(-K^*x))$, GraphPad Prism).

2.5. Measurement of mitochondrial ROS production

Mitochondrial ROS production was determined using isolated mitochondria. Five microlitre of dichloro-dihydro-fluorescein diacetate (DCFH-DA) (10 mM dissolved in DMSO or DMF, Sigma) and 45 μL of ethanol (96% v/v) were hydrolyzed in the presence of 200 μL of NaOH (10 mM stock) and subsequently neutralized with 1000 μL of phosphate buffer (25 mM stock). Mitochondrial ROS production was measured by incubating the isolated mitochondria with reaction buffer A, and substrates including 1 mM pyruvate, 1 mM malate, and 2 μM $DCFH_2$ for 30 min at 37 °C in the dark. $DCFH_2$ was subsequently oxidized by ROS into 2′, 7′-dichlorofluorescein (DCF), a highly fluorescent compound, which can be detected using fluorescence spectroscopy with a maximum excitation and emission spectra of 500 nm and 530 nm, respectively. In a separate tube, 50 μM $CaCl_2$ or 100 μM actimycin A was added to stimulate mitochondrial ROS production [29].

2.6. Mitochondrial structure using transmission electron microscopy

The heart was rapidly excised and immediately perfused with Krebs Henseleit (KH) solution containing (in mM) 137 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 10 glucose, and 0.25 $CaCl_2$. A concentration of 20 mM 2,3-butanedione monoxime (BDM) was added to the perfusate to minimize the cutting damage and to arrest the heart. The left papillary muscle was dissected and cut into 3 rod-shaped pieces (2 mm in diameter and 4 mm in length). The pieces were pre-fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (electron microscopy sciences) at 4 °C, overnight. Following post-fixation in 1% OsO_4 for 1 h, the pieces were then dehydrated through an ethanol series followed by infiltration with araldite resin. The sample was embedded in Epon plastic and allowed to polymerize at 45 °C for 2 days and 60 °C for 2 more days. The sample was then sectioned to a thickness of approximately 0.5 μm with a glass knife using an ultramicrotome (Leica EM UC6). Semi-thin sections were stained by 0.5% methylene blue and observed under a light microscope to evaluate the orientations of the sections and to select the areas containing predominantly longitudinal myofibers. The selected area on the resin was sectioned to a thickness of 90 nm with a glass knife using an Ultramicrotome (Leica EM UC6). The ribbon of section was picked up by a 200-mesh copper grid, stained with uranyl acetate and lead citrate and the grid was dried overnight. Structural observation was performed using transmission electron microscopy (FEI, TECNAI T20). Ten fields containing longitudinally arrayed myofibrils excluding nuclei per section were imaged at 2500 magnification. A mask was drawn over the micrograph and the

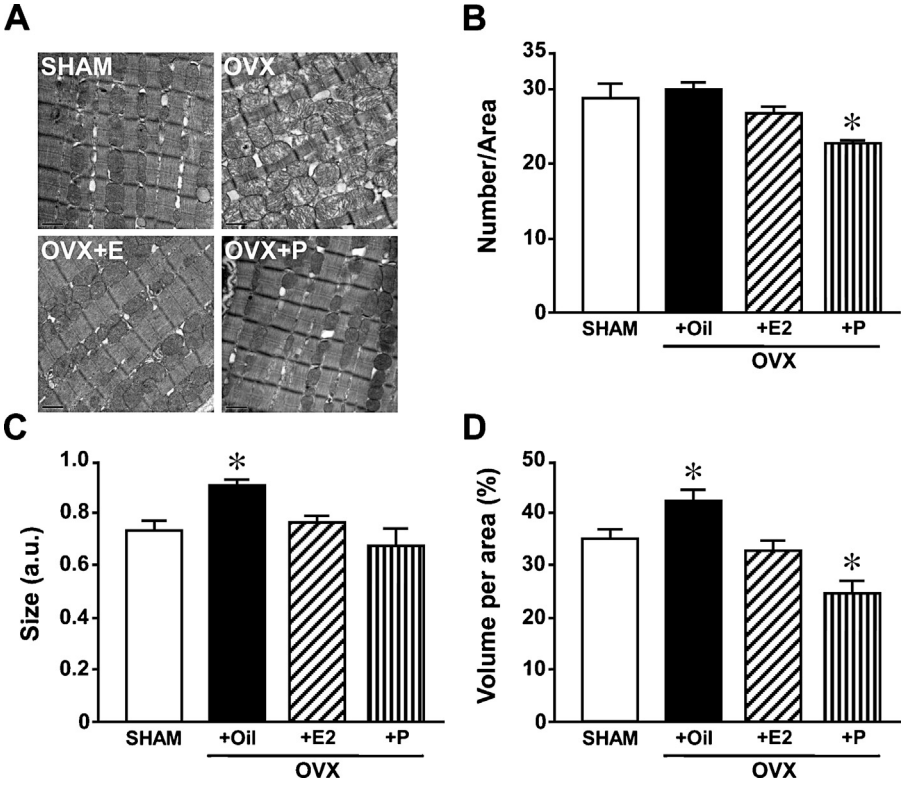

Fig. 1. Effect of ovarian sex hormone deprivation on cardiac mitochondrial assembly. (A) Transmission electron microscopic images of representative mitochondrial from left ventricular papillary muscle section from sham control (SHAM) and ovariectomized (OVX) rats, with/without estrogen ($+E_2$) or progesterone (+P) supplementation. Bar graph compares: number of mitochondrial per field area (B), mitochondrial two dimensional size (C), and mitochondrial volume fraction (D) among four experimental groups. Data are mean ± SE from 6 hearts from each group (average of 10 TEM field per heart). *Significantly different ($P < 0.05$) from SHAM using Student–Newman–Keuls test after ANOVA.

mitochondrial size of individual mitochondria, number, and volume density were assessed directly from the micrographs using ImageJ (Wayne Rasband, NIH) [30].

2.7. Immunoblot analyses

The frozen left ventricular tissue was homogenized in the extracting buffer containing 150 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, 0.1% SDS, 50 mM NaF, 40 mM β-glycerophosphate, 2 mM Na_3VO_4, and protease inhibitors, including leupeptin, pepstatin-A, aprotinin, and PMSF. Proteins were size-separated by 10% SDS-PAGE, transferred onto PVDF membranes, and blocked with phosphate-buffered saline (PBS) and 0.1% Tween-20 (PBS-T) plus 5% non-fat dry milk prior to incubation with primary antibodies: (rabbit polyclonal anti-Mfn2, Aviva System Biology, 1:5000 dilution; rabbit polyclonal anti-SOD2, Aviva System Biology, 1:10,000; mouse WB antibody cocktail total OXPHOS, Abcam, 1:2,500 and rabbit monoclonal anti-β-porin, Abcam,1:10,000 dilution). The secondary antibody used was goat anti-rabbit immunoglobulin G (Zymed) or donkey anti-mouse immunoglobulin G (Fitsgerald). The relative amount of porin was used to determine the total mitochondria load. The band density was analyzed using Image Master Labscan version 3.01 and Image Master Totallab version 1.0 (Amersham Pharmacia Biotech).

2.8. Statistical analysis

All values shown are expressed as the mean ± standard error of the mean (SEM) unless otherwise specified. When the two groups were compared, Student's two-tailed *t*-test was performed (unpaired). For comparisons of 3 or more groups, one-way analysis of variance (1-way ANOVA) was used, and if statistically significant differences were detected, then the Student–Newman–Keuls test was applied to further identify groups with different means. Differences were considered statistically significant at $P \leq 0.05$.

3. Results

Similar to our previous finding [31], 10 week-deficiency of female sex hormones after ovariectomy (OVX) results in an increase in body weight (351 ± 5.4) but a decrease in uterine weight (0.14 ± 0.01) compared to sham controls (274 ± 2.9, 0.66 ± 0.04, respectively). Estrogen but not progesterone supplementation prevents these changes in weight (Table 1). Sufficiency of estrogen supplement is indicated by a significant increase in uterine weight, while a decrease in serum progesterone level in the OVX rat can be rescued by either estrogen or progesterone treatment. Serum testosterone levels are not changed by the ovariectomy, but the levels become absent in an OVX rat with either estrogen or progesterone supplement.

An ultrastructural study using a transmission electron microscope demonstrates the swelling of myocardial mitochondria in the OVX rat heart with crista deconstruction (Fig. 1). Although the number of mitochondria per area is not different between the OVX and sham groups (Fig. 1B), the average dimension (μm^2) of mitochondria in the left ventricle of the OVX rat is significantly greater than that of the sham (Fig. 1C). The mitochondrial volume per myofilament fraction is also increased after female sex hormone deprivation (Fig. 1D). In addition, these changes in the OVX group can be prevented by estrogen supplementation. Interestingly, the number of mitochondria per myocyte area is significantly reduced in the heart of the OVX rat with progesterone supplementation compared to either the sham or OVX rats. Progesterone supplementation also prevents the increase in

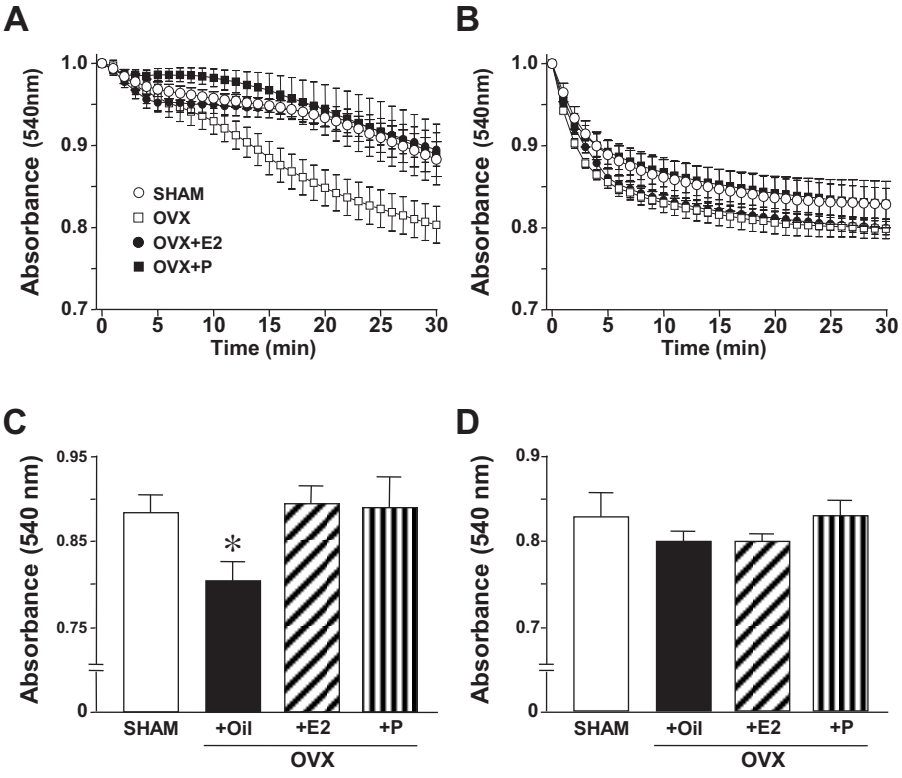

Fig. 2. Effect of ovarian sex hormone deprivation on mitochondrial swelling. Kinetic study of mitochondrial light absorbance at 540 nm without Ca^{2+} (*A*) and with 100 μM Ca^{2+} (*B*) using mitochondrial preparation from SHAM and OVX with/without E_2 or P supplementation. Mitochondrial swellings at 30 min are compared in an absence and presence of 100 μM Ca^{2+} among four experimental groups (*C*). Data are mean ± SE from six mitochondrial preparations from each group. *Significantly different ($P < 0.05$) from SHAM using Student-Newman-Keuls test after ANOVA.

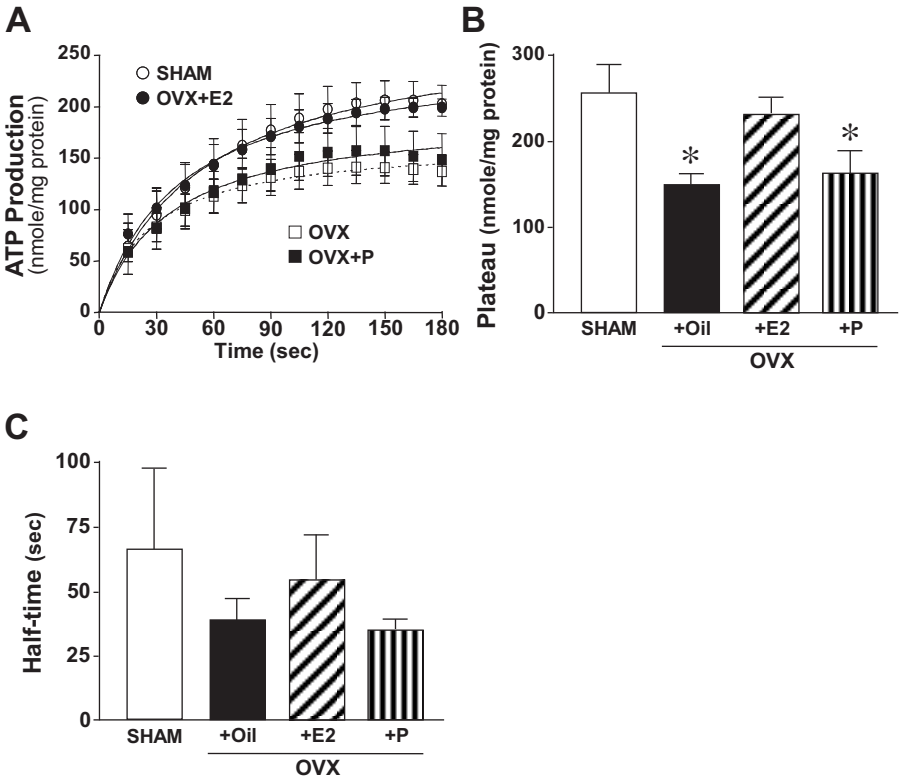

Fig. 3. Effect of ovarian sex hormone deprivation on mitochondrial ATP synthesis. (A) Mitochondrial ATP production by activation of pyruvate and malate (complex I). Bar graph compares: maximum ATP production (plateau) (B), and time to 50% of maximum production (half time) (C) among four experimental groups. Data are mean ± SE from 5–7 heart preparations from each group. *Significantly different ($P < 0.05$) from SHAM using Student–Newman–Keuls test after ANOVA.

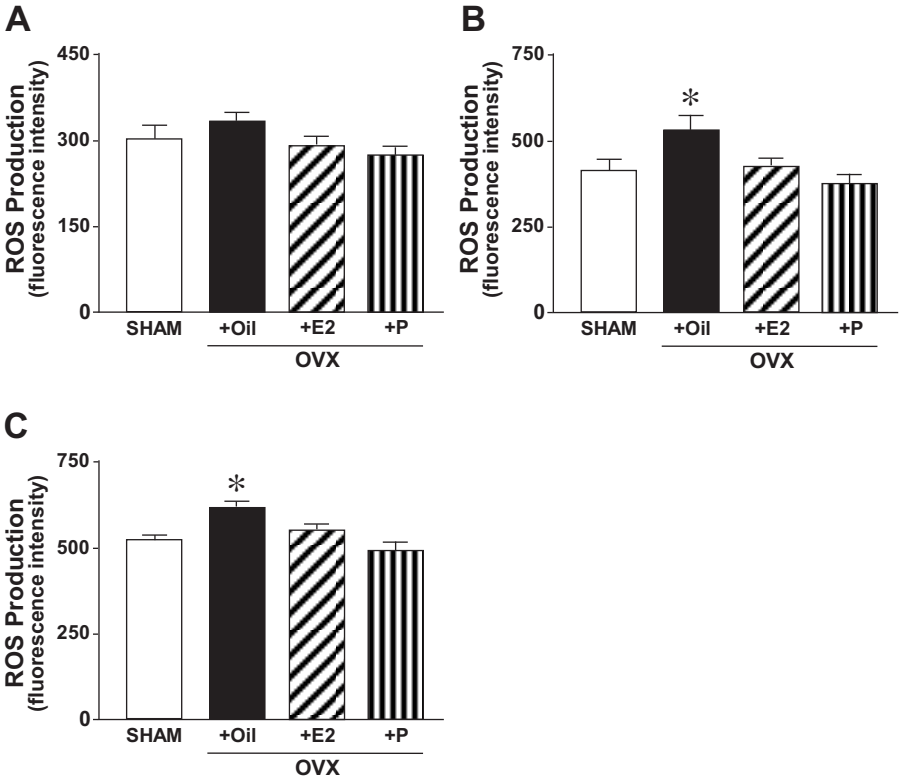

Fig. 4. Effect of ovarian sex hormone deprivation on mitochondrial ROS production. Mitochondrial ROS production was compared between SHAM and OVX rats, with/without E_2 or P supplementation at basal level (A), in a presence of high Ca^{2+} (B), and in a presence of antimycin A (C). Data are mean ± SE from 8–11 heart preparations from each group. *Significantly different ($P < 0.05$) from SHAM using Student–Newman–Keuls test after ANOVA.

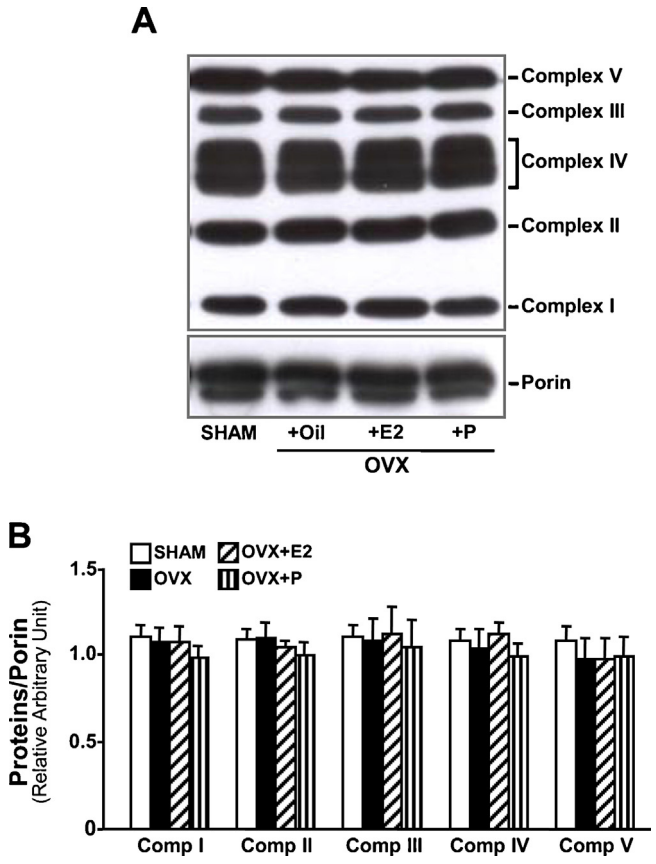

Fig. 5. Effect of ovarian sex hormone deprivation on expression of mitochondrial protein complexes. Representative immunoblot analysis of cardiac mitochondrial complex proteins and porin of isolated mitochondria from SHAM and OVX rats, with/without E_2 or P. Bar graphs demonstrate densitometric analysis of each complex/porin expression ratio (B). No significant difference in each complex protein to porin ratio was found using one-way ANOVA.

mitochondrial size in the OVX rat heart, whereas the mitochondrial fraction is significantly lower compared to the other groups. Mitochondrial swelling in the heart of OVX rat is also confirmed by a marked decrease in optical density absorbance at 540 nm compared to the mitochondria of the sham control (Fig. 2A). Either estrogen or progesterone supplementation can prevent this change. However, when mitochondria exposed with 100 μM Ca^{2+}, a decrease in light absorbance is similarly observed among all four groups (Fig. 2B), which suggested a potential previous Ca^{2+} overload in the mitochondria of the OVX rat myocardium.

To further examine the effects of female sex hormones on cardiac mitochondrial function, ATP and ROS production were accessed using isolated mitochondria from the left ventricle. With pyruvate and malate as substrates, maximum ATP production (plateau) is significantly reduced in the preparation from OVX rats (150 ± 13 nmol/mg protein) versus the sham control (256 ± 35 nmol/mg protein), while the half-time is not altered (Fig. 3A–C). Estrogen supplementation can prevent the suppression of ATP production in OVX, whereas progesterone offers no protective effect. However, ATP production by complex II activity, using succinate as a substrate, is not significantly different among the four groups (data not shown).

Both estrogen and progesterone are found to have a significant role in regulating mitochondrial ROS production. At basal levels, the amount of ROS emission detected by DCFH-DA is not different among the four groups (Fig. 4A). However, when challenged with 10 μM Ca^{2+}, the level of ROS emission in the mitochondrial preparation from the OVX rat heart (533 ± 47) is significantly higher compared to the sham control (413 ± 34). A similar change is detected in the OVX mitochondrial sample under activation of antimycin A (Fig. 4B and C). The increase in ROS production by either Ca^{2+} overload or antimycin A induction in the OVX group is prevented by either estrogen or progesterone supplementation. Progesterone appears to be more effective in preventing antimycin A induced ROS production compared to estrogen.

To identify the potential mechanisms underlying changes in mitochondrial ATP and ROS production, the relative expression levels of five oxidative phosphorylation complexes in mitochondrial preparations were determined using immunoblot analysis (Fig. 5). When normalized with the level of porin, the amount of each complex is not significantly changed among the four experimental groups. Fig. 6 demonstrates the representative protein band of mitochondrial SOD2 and mitofusin 2 versus porin. The level of mitochondrial SOD2, which might affect the emission of ROS does not change after the deprivation of female sex hormones. Thus, enhanced stress-induced ROS emission in mitochondria from the OVX rat heart is likely due to an increase in ROS production rather than a decrease in ROS elimination. Similarly, the amount of mitofusin 2 expression, which could affect the level of mitochondrial fusion, is also not changed, which suggests that the enlargement of mitochondria in the cardiomyocyte of the OVX rat potentially contributes to membrane swelling.

4. Discussion

Previously, we demonstrated that many cellular and molecular changes in cardiac contraction and relaxation after a 10-week deprivation of ovarian sex hormones were similar to those observed in the heart disease condition. Supplementation with estrogen or progesterone after an ovariectomy yielded different protective results [25]. In this study, we further demonstrate that both estrogen and progesterone have regulatory roles in modulating mitochondrial structure and function, but exhibit some differences. Although estrogen or progesterone supplementation can prevent mitochondrial swelling in the OVX rat heart, progesterone supplementation unexpectedly decreases the amount of mitochondrial volume fraction. Estrogen supplementation in the OVX rat can prevent both the decrease in mitochondrial ATP synthesis and increase in stress-induced ROS production. In contrast, progesterone normalizes the ROS production, but does not restore ATP synthesis.

Chronic deprivation of female sex hormones induces mitochondrial swelling by potentially causing an imbalance of intracellular Ca^{2+} mobilization. Our previous studies demonstrated a decrease in the amplitude of intracellular Ca^{2+} transients with a prolonged Ca^{2+} decay in cardiomyocytes isolated from 10-week OVX rat [23], with the latter being primarily a result of suppressed SERCA activity [31]. We also demonstrated that the lack of female sex hormones induced an increase in Na^{+}–H^{+} exchange activity in cardiomyocytes and potentially defective intracellular Ca^{2+} homeostasis [23]. These previous findings suggested a potential increase in the cytosolic Ca^{2+} level as being responsible for mitochondrial swelling. In addition, the increase in myofilament Ca^{2+} sensitivity found in 10-week OVX rat [32] might also disrupt the Ca^{2+} buffering system in cardiac myocytes. Because mitochondrial function is related to the cellular Ca^{2+} buffering system, changes in intracellular Ca^{2+} homeostasis in the OVX rat cardiomyocyte could have an effect on mitochondrial function. Under the resting condition, approximately 1% of cytosolic Ca^{2+} is taken up by mitochondria [33]. However, the supraphysiological Ca^{2+} concentration theoretically increases the mitochondrial Ca^{2+} load due to functional saturation of SERCA and NCX [34]. In addition, dysfunction of the sarcoplasmic reticulum (SR) Ca^{2+} handling in the heart of OVX rats could alter SR-mitochondrial Ca^{2+}

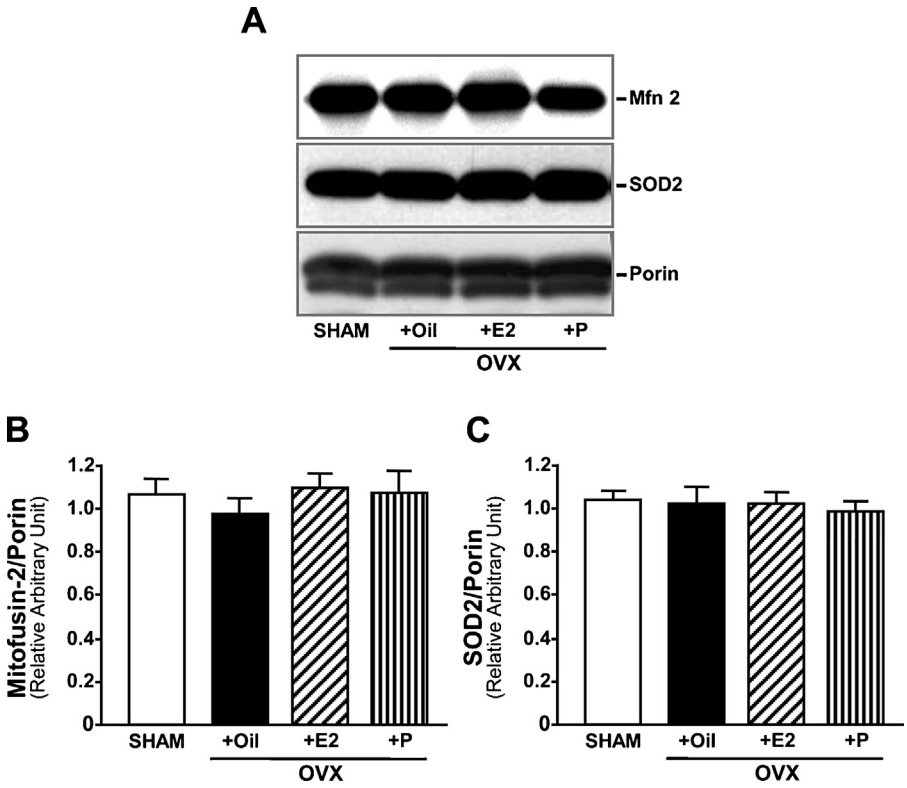

Fig. 6. Effect of ovarian sex hormone deprivation on expressions of mitochondrial mitofusin 2 and superoxide dismutase 2. Representative immunoblot analyses of cardiac mitofusin 2 (Mfn 2), superoxide dismutase 2 (SOD2), and porin of isolated mitochondria from SHAM and OVX rats, with/without E_2 or P. Bar graphs demonstrate densitometric analysis of Mfn2/porin expression ratio (B) and SOD2/porin ratio (C). No significant difference in the ratio of each protein to porin was detected using one-way ANOVA.

transmission [35]. In particular, an increase in mitochondrial Ca^{2+} uptake may subsequently activate ATP production to match the energy requirements. In addition, excessive mitochondrial Ca^{2+} uptake activates the opening of the mitochondrial permeability transition pore (mPTP), which results in an influx of water and solutes into the matrix. Thus, mitochondrial swelling in the OVX rat heart could be due to an impairment of intracellular Ca^{2+} homeostasis. This possibility is supported by the fact that either estrogen or progesterone supplementation could improve heart function by normalizing the reduced SR Ca^{2+} uptake in this OVX model [31]. Nevertheless, a decrease in the mitochondrial volume fraction heart after OVX progesterone supplementation was unexpected, particularly because a previous investigation showed that progesterone administration induced increases in mitochondria size and volume fraction in the glandular cells of the OVX uterus [36]. Differences in these finding could not be explained, but it was possible that the actions of progesterone exhibited tissue specificity.

Unlike progesterone, the effect of estrogen in stabilizing mitochondrial function in cardiac myocytes is well-established. Our present data further strengthened the significance of estrogen in reducing cardiac injury after pathological stress. A previous study demonstrated that estrogen supplement prevented ischemia-reperfusion injury in the heart of OVX rats by attenuating mitochondrial disruption [16]. The protective mechanism partly involved inhibition of the mPTP opening and subsequent cytochrome *c* release [20]. Thus, swelling mitochondria in the heart of the OVX rat might be more susceptible to apoptosis under stress. In addition, the lower level of stress-activated mitochondrial ROS production in the OVX rat with estrogen supplementation is consistent with the benefit of estrogen on preventing myocardial damage. In contrast, information regarding the protective effect of progesterone on the heart is presently lacking. It is well-known that progesterone exerts a neuroprotective effect against traumatic and ischemic brain injury. Similar to estrogen, progesterone administration enhanced mitochondrial respiratory activity, but reduced the rate of mitochondrial peroxide formation in brain tissue [37]. However, a single administration of progesterone had no protective effect on the myocardium against ischemia-reperfusion injury [38]. Moreover, progesterone acutely activated H_2O_2 emission in permeabilized skeletal myofibers [39]. These reports, however, did not support the use of progesterone for cardioprotection. Interestingly, the results obtained from the present study revealed a positive effect of long-term progesterone supplement action on the heart, particularly on mitochondrial ROS production under stress.

No change in mitochondrial respiratory complex protein expression after ovariectomy or hormone supplementation indicated that estrogen exerts an effect on mitochondrial ATP production by altering complex activity. This implication was supported by previous studies in other tissues [21,40,41]. Mitochondrial complex I and IV activities were reduced in the brain in OVX rats, and estrogen supplementation could reverse this suppression [41]. While estrogen administration had no effect on mitochondrial ATP production under normal conditions, estrogen could restore mitochondrial ATP production in the heart under hemorrhagic stress by upregulating complex IV [21]. Because suppressed ATP production in mitochondria from the OVX heart was detected only when complex I, and not complex II was activated, the target of estrogen in heart mitochondria is potentially complex I. In contrast, estrogen was reported to suppress the respiratory activity of liver mitochondria by interacting with complex I at the flavin mononucleotide site [40]. We could not provide an explanation for this controversial finding, and future studies are focused on the interaction of estrogen and mitochondrial complex I activity specifically in

cardiac cells. A previous study also demonstrated that progesterone supplement could prevent the reduction of complex I activity in the brains of transgenic neurodegenerative mice [42], but this benefit was not reflected in the mitochondrial ATP production in the heart. Thus, the action of progesterone on mitochondrial complex proteins in heart muscle also needs to be clarified.

Although ATP production from complex I activity was decreased in the mitochondria of the OVX rat heart, normal ROS production indicated no disruption of the electron transport chain. However, interruption of the electron transport chain (such as by antimycin A) slows the rate of electron transport from complex I to complex III, thereby increasing the electron leak from complex I, with electrons reducing O_2 to oxygen radicals. It has been demonstrated that the main sites of oxygen radical formation are the flavin mononucleotide (FMN) site of complex I and the Q cycle of complex III [43]. Thus, a potential impairment of complex I activity in the mitochondria of OVX rat heart may result in high susceptibility of mitochondria to produce oxygen radicals. It is not currently known whether the protective effects of estrogen and progesterone on mitochondrial ROS production share a similar underlying mechanism. One mechanism of the protective action of female sex hormones is antioxidant regulation. While many studies have provided evidence supporting the antioxidative role of estrogen, very few studies have examined the role of progesterone. However, the present finding of no change in the expression of SOD2 in the experimental groups suggest that oxidative stress in the OVX rat heart was not due to a decrease in this antioxidative enzyme, but was more likely due to an increase in ROS production. Moreover, the non-genomic effect of female sex hormones on antioxidant activity in cardiac myocytes also needs to be clarified.

In summary, the present study demonstrated the importance of female sex hormones on mitochondrial energy synthesis and ROS production in the heart. Although estrogen has a prominent role in the regulation of ATP production compared to progesterone, both hormones can prevent stress-induced ROS production. Thus, alterations of both mitochondrial ATP synthesis and ROS production in the heart after female sex hormone deprivation may serve as another mechanistic process to induce cardiac disease in postmenopausal females. Furthermore, our finding that estrogen sufficiency preserves both cardiac energy metabolism and antioxidant activation provides additional insight in the use of only estrogen for postmenopausal replacement therapy. However, additional clinical studies on cardiac mitochondrial functions using future relevant techniques are needed to provide strong support for this implication.

Acknowledgements

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ORIGINAL PAPER

Regular exercise modulates cardiac mast cell activation in ovariectomized rats

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Abstract It is well accepted that regular exercise is a significant factor in the prevention of cardiac dysfunction; however, the cardioprotective mechanism is as yet not well defined. We have examined whether regular exercise can modulate the activity of cardiac mast cells (CMC) after deprivation of female sex hormones, as well as the density and percentage degranulation of mast cells, in ventricular tissue of ovariectomized (OVX) rats after an 11-week running program. A significant increase in CMC density with a greater percentage degranulation was induced after ovarian sex hormone deprivation. Increased CMC density was prevented by estrogen supplements, but not by regular training. To the contrary, increased CMC degranulation in the OVX rat heart was attenuated by exercise training, but not by estrogen supplement. These findings indicate a significant correlation between the degree of CMC degranulation and myocyte cross-section area. However, no change in the expression of inflammatory mediators, including chymase, interleukin-6, and interleukin-10, was detected. Taken together, these results clearly indicate one of the cardioprotective mechanisms of regular aerobic exercise is the modulation of CMC activation.

Keywords Estrogen · Chymase · Interleukin-6 · Interleukin-10

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Introduction

Regular exercise is highly recommended in post-menopausal women for its beneficial effects on cardiac function, whether or not they eventually receive hormone replacement therapy. It is well known that regular exercise reduces the risk of heart disease and hypertension, but basic information regarding the mechanism of action of cellular activation in the heart remains incompletely understood [8, 25, 32]. The activation of mast cells (MCs) has recently been shown to be a key event in the induction of pathologic hypertrophy by volume overload or pressure overload [19, 23]. Patients with dilated cardiomyopathy have been found to have an increased density of cardiac mast cells (CMCs) [31, 34], as well as a twofold higher percentage degranulation of CMCs compared to normal hearts [31]. MC density has been demonstrated to be enhanced by two- to threefold in animal models of pressure overload or volume overload, coupled with an increase in chymase activity [19, 23]. The impact of MCs on cardiac remodeling is supported by evidence that MC stabilization effectively prevents volume overload-induced dilated cardiac hypertrophy [7]. Moreover, MC stabilizers and chymase inhibitors can attenuate cardiac hypertrophy and decrease the cardiac contraction induced after aortic constriction [19]. A rather large body of evidence suggests that MC activation is a critical step in pathologic cardiac remodeling. Several studies have demonstrated that regular exercise can attenuate such pathologic remodeling [10, 40] and that regular exercise might be able to modify MC activation.

Previously reported studies from our research team have shown that regular exercise can prevent all of the cardiac contractile changes that resemble those seen upon estrogen supplementation in ovariectomized (OVX) rats [8, 9]. Based on these results, we proposed that regular exercise

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and estrogen share the same mode of activation of cardioprotective signaling. Recent reports on estrogen-based regulation of CMC activation [19, 23, 37] has led us to hypothesize that regular exercise might also modulate CMC activity. In two of these studies, the presence of female sex hormones was able to prevent the left ventricular hypertrophy and increased CMC density induced after volume overload [23], and estrogen supplementation attenuated increases in MC density and chymase release in the hearts of OVX rats after transverse aortic constriction [19]. In the third study, increases in MC density and chymase expression in the hearts of mRen2.Lewis rats after OVX were abolished using a G protein-coupled estrogen receptor agonist [37]. It would therefore appear that cardiac changes in the absence of female sex hormones might be due (at least in part) to increased CMC activation. This in turn suggests the possibility that the mechanism underlying the cardioprotective effects of regular exercise in ovarian sex hormone-deprived conditions could result from CMC stabilization.

The aim of our study was to test whether regular exercise inhibited CMC activation after deprivation of female sex hormones in a rat model. OVX rats were subjected to a 10-week running program, during which time CMC density, percentage degranulation of CMCs, and levels of various inflammatory chemokines were evaluated and correlated with myocardial changes.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical (St. Louis, MO), and electrophoretic reagents were purchased from Bio-Rad (Hercules, CA), Amersham Pharmacia Biotech (Buckinghamshire, UK), Omnipur (EMD Millipore, Billerica, MA) or Thermo Scientific (Waltham, MA).

Animals and treatment

The animal protocol was approved by the Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with the guidelines of the National Laboratory Animal Center, Thailand based on "The guide for the care and use of laboratory animals, 8th edition" (NIH). Female Sprague-Dawley rats (8–9 weeks old) were ovariectomized or sham operated. One week after surgery, OVX rats were randomly divided into three treatment groups for vehicle injection, estrogen supplementation and regular exercise, respectively. The lack of female sex hormones was confirmed by the reduced uterine weight at the end of study. All animals were fed ad libitum (Betagro Public Co., Ltd., Bangyor, Thailand) and had access to tap water during the entire experiment. Each rat was housed individually under a 12:12 light/dark cycle with temperature and humidity control. Estrogen supplementation was started by subcutaneous injection of 0.1 mL of estrogen (5 μg/rat) 3 days per week as previously described [38]. In the sham and OVX controls, 0.1 mL of corn oil was injected as placebo.

The regular exercise program including a 1-week acclimatization period followed by 10 weeks of moderate intensity running (65–75 % maximum oxygen consumption) as previously described [9]. Each rat in the regular exercise group was made to run for two 30-min periods per day, 5 days per week, with a 10- to 15-min rest period between each exercise period. Adequacy of the running program was verified by echocardiography at the end of study.

Cardiac structure and function by assessed by echocardiography

The echocardiographic study was performed as previously described [20]. Briefly, rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body weight). Using an echocardiography machine (Samsung Medison, Seoul, Korea) equipped with a 10-MHz echocardiographic probe (10 MHz), we scanned the chest wall using two-dimensional short-axis view at the mid-papillary muscle in M-mode. Used the modified recommendations off the American Society for Echocardiography, we measured the interventricular septum (IVS), left ventricular posterior wall (LVPW), diameter of the internal left ventricle (LVID), LV mass, relative wall thickness, and percentage of fractional shortening for least three consecutive cardiac cycles on the M-mode tracings. LV mass was calculated using the following formula [20]:

$$\text{LV mass} = 1.04 \times [(\text{LVID}_\text{d} + \text{LVPW}_\text{d} + \text{IVS}_\text{d})^3 - \text{LVID}_\text{d}^3]$$

To confirm the hypertrophy of the heart, heart weight per body weight was also determined. In addition, the cardiomyocyte cross-section area was measured on myocardial histological sections stained with hematoxylin and eosin stain.

MC density and morphological changes

After echocardiography examination, the rat was sacrificed and the heart was excised and perfused with Ca^{2+}-free Krebs–Henseleit solution before being fixed in 10 % formalin and embedded in Paraffin. The sample was then sectioned on a microtome (thickness 5 μm) and the sections placed on microscope slides. To stain the MCs, rehydrated

tissue sections were first stained with 0.001 g/mL of toluidine blue for 3 and then rinsed twice in distilled water to wash out the dye before dehydration. Counting was performed on a whole section at a magnification of 100×. The density of MCs was expressed as the mean number of mast cells per square millimeter of tissue section. The MCs were distinguished from other inflammatory cells as previously described [43]. Degranulated MCs were identified by extruded granules and the appearance of ruptured cell membranes or irregular border as granules are released from the cytoplasm. The result was expressed as the percentage of degranulated MCs per total number of MCs.

Immunoblot analyses

Frozen ventricular muscle tissue was mixed and homogenized with extraction sample buffer (50 mM Tris pH 6.8, 2.5 % SDS, 10 % glycerol, 1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and then subjected to sodium dodecyl

Table 1 General characteristics of sham-operated rats and ovariectomized rats receiving sham injection, estrogen supplementation or exercise training[a]

Parameters	Sham-operated group (n = 6 rats)	Ovariectomized rats		
		Oil injection (n = 8 rats)	Estrogen supplementation (n = 7 rats)	Exercise regimen (n = 7 rats)
Body weight (BW) (g)	268 ± 5	363 ± 9*	256 ± 7$^{\#}$	342 ± 9*
Heart weight (HW) (g)	1.31 ± 0.05	1.45 ± 0.03*	1.33 ± 0.04$^{\#}$	1.55 ± 0.03*
HW/BW (100×)	0.490 ± 0.017	0.400 ± 0.013*	0.518 ± 0.011$^{\#}$	0.456 ± 0.013$^{\#}$
Tibial length (TL) (cm)	4.15 ± 0.02	4.49 ± 0.03*	4.19 ± 0.03$^{\#}$	4.39 ± 0.03$^{*,\#}$
HW/TL (g/cm)	0.316 ± 0.012	0.322 ± 0.007	0.317 ± 0.011	0.354 ± 0.008
Soleus weight (g)	0.112 ± 0.002	0.146 ± 0.004*	0.098 ± 0.007	0.139 ± 0.005*
Uterine weight (g)	0.602 ± 0.054	0.154 ± 0.015*	0.618 ± 0.036	0.173 ± 0.025*

$^{*,\#}$ $P < 0.05$, significantly different from Sham-operated rats and ovariectomized (OVX) rats, respectively, using the Student–Newman–Keuls test after analysis of variance (ANOVA)

Data are presented as the mean ± standard error of the mean (SEM)

[a] There were 3 treatment groups for OVX rats: vehicle (0.1 mL corn oil) injection (OVX control group), estrogen supplementation and regular exercise, respectively. In the sham-operated group, 0.1 mL corn oil was also injected as placebo

Table 2 Echocardiographic parameters of sham-operated rats and ovariectomized rats and receiving sham injection, estrogen supplementation or exercise training

Parameters	Sham-operated	Ovariectomized rats		
		Oil injection	Estrogen supplementation	Exercise regimen
IVS_S (cm)	0.233 ± 0.007	0.240 ± 0.011	0.258 ± 0.014	0.261 ± 0.006
IVS_D (cm)	0.175 ± 0.007	0.190 ± 0.014	0.193 ± 0.017	0.208 ± 0.007
$LVPW_S$ (cm)	0.232 ± 0.009	0.282 ± 0.024	0.280 ± 0.026	0.302 ± 0.026
$LVPW_D$ (cm)	0.143 ± 0.014	0.201 ± 0.018	0.186 ± 0.016	0.183 ± 0.013
$LVID_S$ (cm)	0.373 ± 0.012	0.414 ± 0.022	0.314 ± 0.025$^{\#}$	0.367 ± 0.015
$LVID_D$ (cm)	0.588 ± 0.020	0.602 ± 0.037	0.523 ± 0.030	0.615 ± 0.015
LV FS (%)	36.6 ± 0.3	30.9 ± 0.6*	39.9 ± 1.0$^{*,\#}$	40.5 ± 1.2$^{*,\#}$
LV EF (%)	72.5 ± 0.4	64.8 ± 0.8*	76.6 ± 1.2$^{*,\#}$	76.7 ± 1.3$^{*,\#}$
RWT	0.463 ± 0.052	0.680 ± 0.092	0.728 ± 0.097	0.603 ± 0.058
Estimated LV mass (g)	0.541 ± 0.023	0.789 ± 0.063*	0.614 ± 0.045$^{\#}$	0.817 ± 0.016*
LV mass index	2.08 ± 0.11	2.04 ± 0.12	2.25 ± 0.08	2.51 ± 0.11$^{*,\#}$

$^{*,\#}$ $P < 0.05$, significantly different from Sham-operated rats and OVX rats, respectively, using Student–Newman–Keuls test after ANOVA

Data are presented as the mean ± SEM

IVS_S, *IVS_D* Systolic and diastolic parameters, respectively, of the interventricular septum (*IVS*), *$LVPW_S$*, *$LVPW_D$* systolic and diastolic parameters, respectively, of the left ventricular posterior wall (*LVPW*), *$LVID_S$*, *$LVID_D$* systolic and diastolic parameters, respectively, of the left ventricular internal diameter (*LVID*), *FS* fractional shortening, *EF* ejection fraction, *RWT* relative wall thickness, *LV* left ventricular

sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto PVDF membranes. Rabbit anti-rat interleukin-6 (IL-6; 1:1,000), rabbit anti-rat IL-10 (1:1,000), and anti-MCl chymase antibody-C-terminal (1:2,500) were obtained from Abcam (Cambridge, UK). Anti-β-actin antibody (1:5,000) was purchased from AVIVA Systems Biology (San Diego, CA). The secondary antibody used was goat anti-rabbit immunoglobulin G (1:10,000) from Zymed (San Francisco, CA). Band density was analyzed using Labscan version 5.0 (Amersham Biosciences) and ImageQuant TL version 7.0 (GE Healthcare Life Sciences, Cleveland, OH).

Data and statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). For comparisons of four groups, one-way analysis of variance was used, and if statistically significant differences were detected, the Student–Newman–Keuls test was applied to further identify groups with different means. Differences were considered statistically significant at $P \leq 0.05$.

Results

General characteristics which could be attributed to the lack of ovarian sex hormones and regular exercise, respectively, are presented in Table 1. Deprivation of female sex hormones was confirmed by a marked decrease in uterine weight in OVX rats and OVX rats that were subjected to the regular exercise regimen. The 10-week deficiency of ovarian sex hormones resulted in an increase in body weight and heart weight, but the hypertrophic index by heart per body weight was decreased significantly (0.400 ± 0.013) as compared with sham controls (0.490 ± 0.017). Estrogen supplementation prevented changes in heart weight and body weight, but regular exercise could improve the heart per body weight ratio compared with sedentary OVX rats without any significant change in body weight.

Cardiac hypertrophy due to regular aerobic exercise was confirmed by the echocardiography (Table 2) and histochemistry studies (Fig. 1). Deprivation of ovarian sex hormones had no effect on hypertrophy of the LV, but significant decreases in the left ventricular ejection fraction and fractional shortening were observed in OVX rats. Reduction in cardiac contractility could be prevented by estrogen supplementation or regular exercise. Female sex hormones had no effect on the cross-section areas of cardiomyocytes, whereas the increased cross-section area of cardiomyocytes in exercise groups confirmed the hypertrophic effect of exercise.

Specific histochemical staining techniques revealed that chronic deprivation of female sex hormones increased CMC density compared with the sham control (3.13 ± 0.18 vs. 1.89 ± 0.12 cells/mm^2; Fig. 2). The lack of female sex

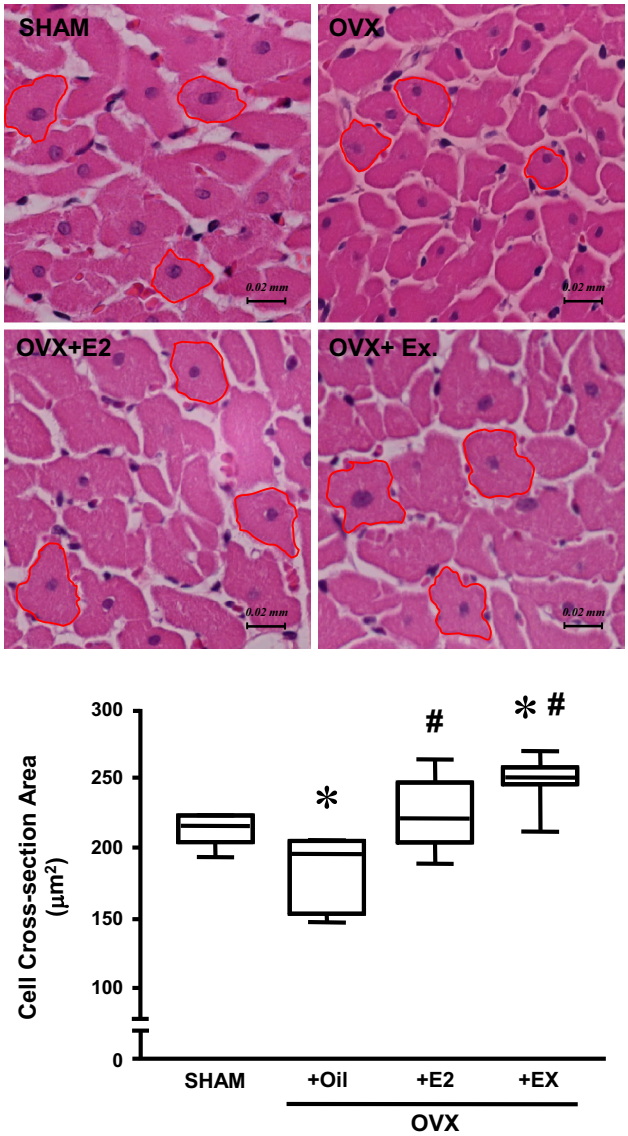

Fig. 1 Effect of regular exercise on cardiomyocyte cross-sectional area in ovariectomized (*OXV*) rats. *Stained images* are transverse sections of cardiomyocytes at ×400 magnification from the hearts of sham-operated controls (*SHAM*) and OXV rats with no further treatment (*OXV*), with estrogen supplementation (*OVX + E2*), or with exercise training (*OVX + Ex*). Only myocytes with a nucleus, a clear cell boundary, and a round or rectangular shape (length:width <1.5) were collected. *Box plot* of cross-sectional area of cells from the Sham-operated (control) group (*SHAM*) and from the three treatment groups of OXV rats (*+oil*, *+E2*, *+EX*). *Top* and *bottom* of *box* Minimum and maximum levels of cell cross-sectional area, *horizontal line within the box* median. Data are from 6–7 hearts in each group (150 cells per heart). *Asterisk* and *hash symbols* Significant difference ($P < 0.05$) from SHAM and OVX rats, respectively, using the Student–Newman–Keuls test after analysis of variance (ANOVA)

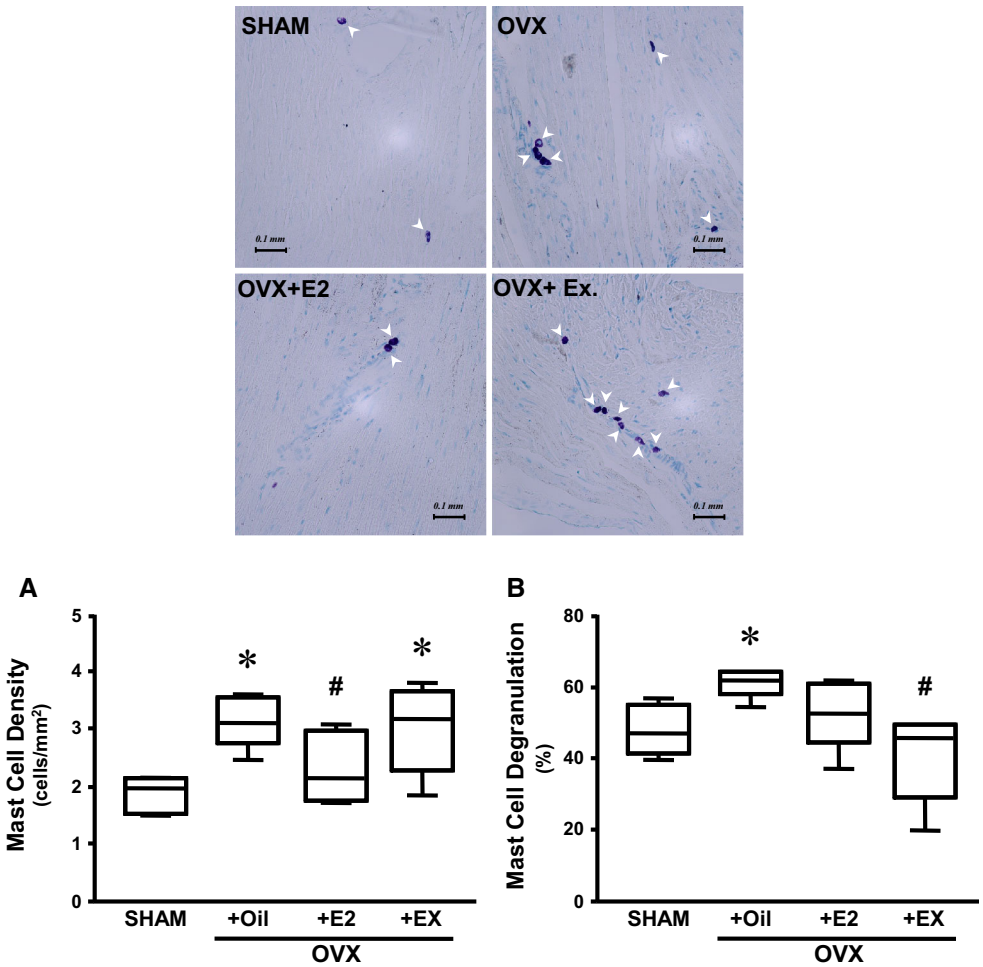

Fig. 2 Effect of regular exercise on the density (**a**) and percentage of degranulation (**b**) of cardiac mast cells (MCs) in ovariectomized (*OVX*) rats. *Images* are transverse sections of the ventricle at ×40 magnification from sham-operated controls (*SHAM*) and from OXV rats with no further treatment (*OXV*), with estrogen supplementation (*OVX* + *E2*), or with exercise training (*OVX* + *Ex*). Cardiac MCs were identifiable by their dark-purple coloration (*arrowheads*) due to toluidine staining. *Box plot* of number of cardiac MCs per tissue area (**a**) and percentage MC degranulation (**b**) from the Sham-operated (control) group (*SHAM*) and from the three treatment groups of OXV rats (+*oil*, +*E2*, +*EX*). *Top* and *bottom* of *box* Minimum and maximum values, *horizontal line within the box* median. Data are from 6–7 hearts in each group. *Asterisk* and *hash symbols* Significant difference ($P < 0.05$) from SHAM and OVX groups, respectively, using the Student–Newman–Keuls test after ANOVA

hormones also increased the percentage degranulation of MCs (61.7 ± 1.5 vs. 48.3 ± 2.9 % of sham controls). Estrogen supplementation was able to completely prevent the increases in MC density (2.32 ± 0.24 cells/mm^2), but only partially attenuated percentage degranulation. Conversely, regular aerobic exercise could not prevent increases in MC number due to deprivation of female sex hormones, but it did significantly decrease the percentage degranulation of MCs significantly (40.8 ± 4.9 %). Thus, the number of degranulated CMCs in exercised OVX rats was similar to that in sham controls [1.18 ± 0.15 (exercise group) vs. 0.91 ± 0.07 cell/mm^2 (sham group)]. These findings suggest a protective regulatory effect of estrogen and regular exercise on CMC function.

We then evaluated whether changes in MC activation could be the cause of cardiac hypertrophy, as well as the relationship between MC density and cross-sectional area of cardiomyocytes (Fig. 3). We found no correlation between cross-sectional area of cardiomyocytes and total density of MCs or percentage MC degranulation ($r^2 = 0.0893$, $P = 0.1560$; $r^2 = 0.07847$, $P = 0.1849$, respectively), but the number of degranulated MCs was correlated significantly with cardiomyocyte size ($r^2 = 0.237$, $P = 0.0157$).

An immunoblotting technique was used to estimate the levels of chymase, a major protease released from MCs, in the heart as a measure of CMC activity (Fig. 4a). We found that the number of MCs after deprivation of sex hormones had increased, but chymase expression was not altered. An effect of regular exercise on chymase levels in cardiac tissue was not observed.

Interleukin (IL)-6 levels were measured in the search for a possible signaling mechanism underlying the role of MCs in the regulation of cardiac remodeling (Fig. 4b). Deprivation of ovarian sex hormones had no effect on the myocardial expression of IL-6. Surprisingly, however, IL-6 levels in the hearts of OVX rats that underwent estrogen supplementation fell significantly by 40 %. To the contrary, regular exercise had no effects on cardiac levels of IL-6. Based on previous evidence that increased IL-10 level may underlie the protective action of regular exercise [15, 28], we then evaluated IL-10 expression; however, we detected no change in IL-10 levels among the four experimental groups (Fig. 4c).

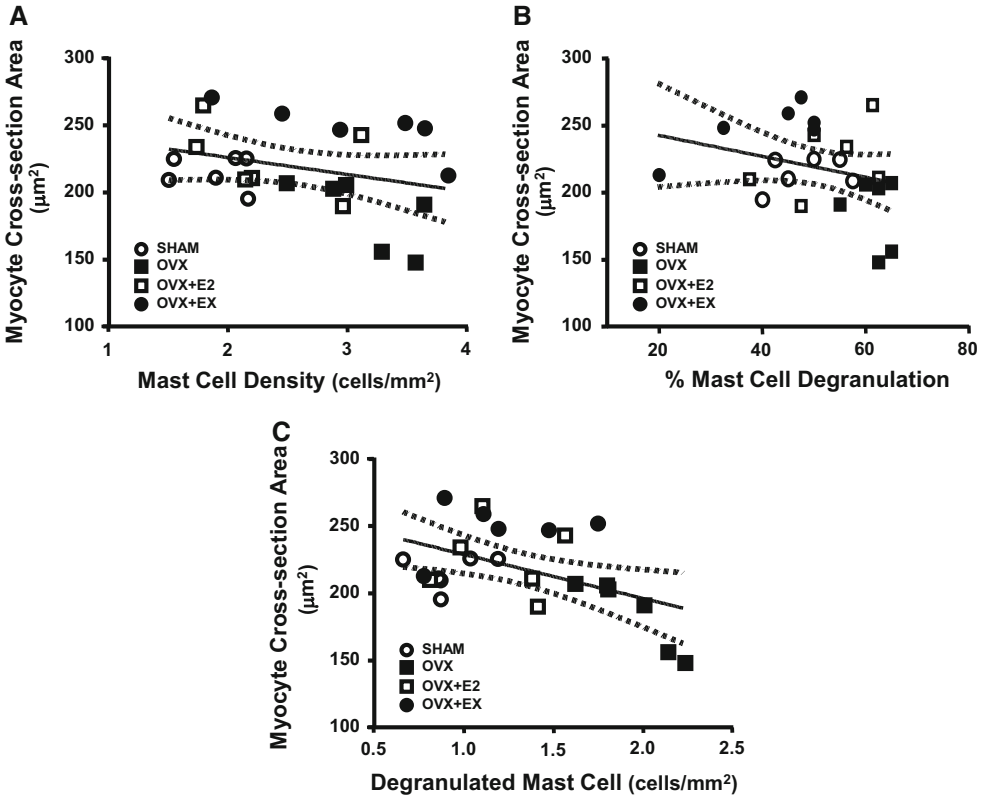

Fig. 3 Relationship between total cardiac MC density (**a**), percentage of MC degranulation (**b**), and degranulated MC density (**c**) to the cardiomyocyte cross-sectional area from all four experimental group combinations (see Fig. 1 caption for groups). Linear regression analysis indicated no relationship between cardiomyocyte cross-sectional area and total cardiac mast cell density ($r^2 = 0.08933$) or percentage of MC degranulation ($r^2 = 0.07847$), but a significant relation was found between degranulated MC density and cardiomyocyte size ($r^2 = 0.2376$)

Discussion

To our knowledge our study is the first to provide experimental evidence that chronic deprivation of ovarian sex hormones causes increases in the density and percentage degranulation of CMCs. In our rat model, estrogen supplementation had a major effect on the regulation of MC density, but regular exercise exerted a protective effect by inhibiting the increased degranulation of MCs. However, neither female sex hormones nor regular exercise appeared to have a regulatory impact on chymase expression in cardiac tissue. In addition, regular exercise had no effect on the expression of IL-6 and IL-10 proteins in the heart, but estrogen supplementation significantly suppressed IL-6 expression.

Female sex hormones (especially estrogen) are widely believed to be cardioprotective, but the mechanism of action and side effects of such protection are controversial [1, 29]. Female rats have been found to exhibit no adverse ventricular remodeling and to have a lower prevalence of mortality in response to volume overload than male rats [11]. This benefit of female sex hormones has been demonstrated to be due (at least in part) to an inhibition of MC activation by estrogen [23]. Estrogen and progesterone receptors are expressed in MCs [45]. Hence, whether MCs contribute to the cardiac changes after deprivation of sex hormones is an important question.

Information on the role of female sex hormones, especially estrogen, on cardiac hypertrophy is still largely inconclusive. Recent studies have found that estrogen regulates cardiomyocyte growth by inhibiting autophagy [13, 36] and that autophagy is associated with cardiomyocyte atrophy and therefore decreased contractility of the heart [21]. While it remains unclear at present whether cardiomyocyte autophagy is enhanced in the female sex hormone-deprived condition, an increase in osteocyte autophagy has been shown in OVX rats [41]. Exercise training-inhibited myocardial autophagy has also been demonstrated [39]. Importantly, the effect of MC activation, a key factor in our study, on cardiomyocyte autophagy has not yet been studied and further investigations in this area are needed.

Another potential protective mechanism of estrogen is the reduction of IL-6 activation. IL-6 is a pro-inflammatory cytokine that has been demonstrated to strongly regulate the proliferation and degranulation of MCs [16, 17]. Our observation of reduced IL-6 expression in OVX rats supplemented with estrogen strengthens the possibility that this hypothesis is correct. Several studies have confirmed the suppressive effect of estrogen on IL-6 levels in various tissues [18, 27]. However, the unexpected similarity of IL-6 levels in the heart between sham controls and OVX rats merits further discussion regarding the function of sex hormones. In a previous study we demonstrated that the

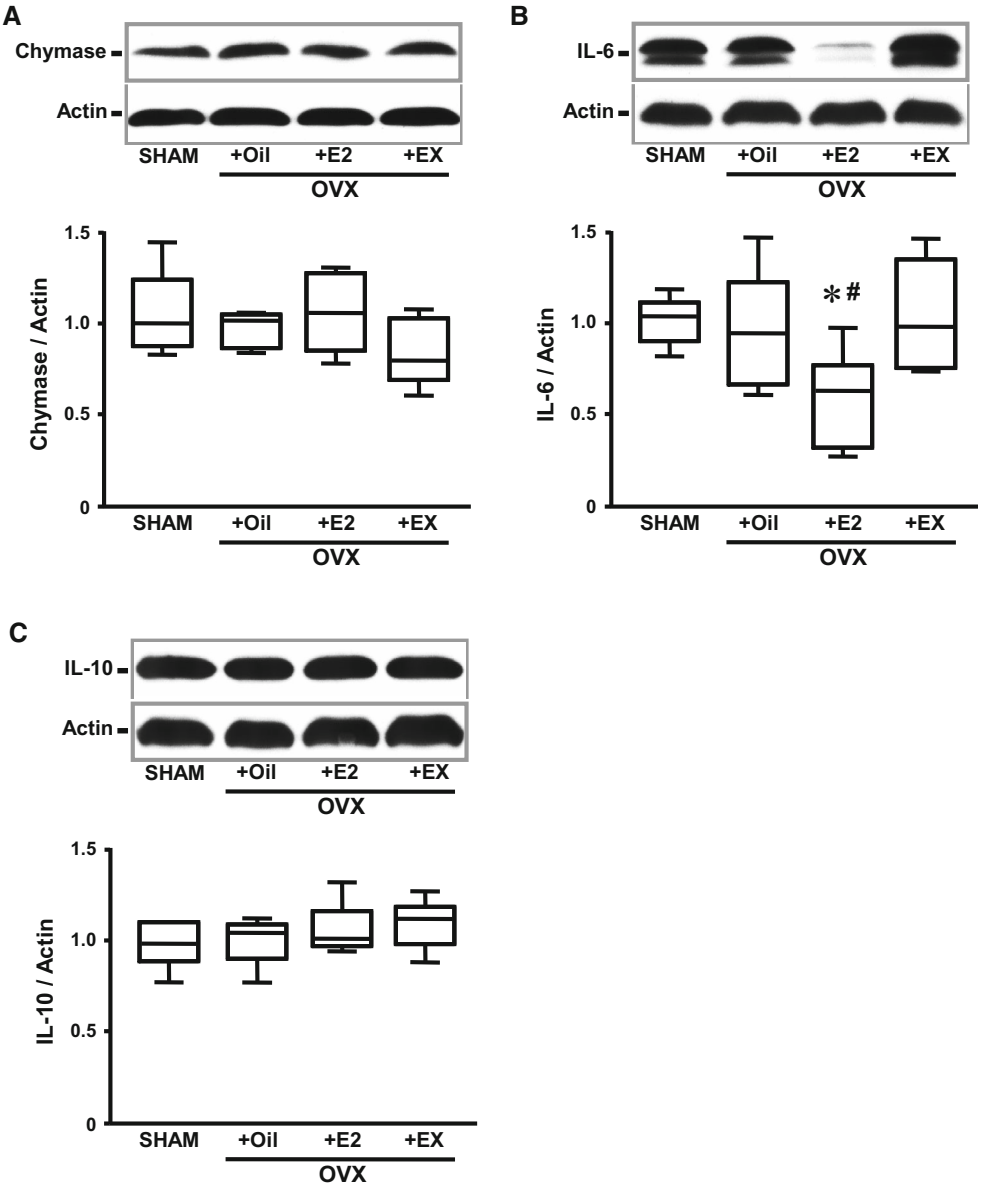

Fig. 4 Effect of regular exercise on the expression of inflammatory cytokines in the heart of OVX rats. Representative immunoblots of chymase (**a**), interleukin-6 (*IL-6*; **b**), and interleukin-10 (*IL-10*; (**c**) in comparison to β-actin expression are shown *above* the respective *box plots* (see Fig. 1 caption for groups). Data are the ratio of the density of each protein band to the density of the actin band from 6 hearts in each group. *Asterisk* and *hash symbols* Significant difference ($P < 0.05$) from SHAM and OVX groups, respectively, using the Student–Newman–Keuls test after ANOVA

dose of estrogen supplementation in OVX rats provided the same serum level of estrogen as that seen in sham-operated controls, leading us to conclude that a major difference is progesterone levels must be present [33]. Progesterone can reduce MC activity in various tissues [5, 35], but it increases IL-6 expression in mesenchymal stem cells [44]. Moreover, estrogen suppresses the levels of IL-6 and tumor necrosis factor (TNF)-α in monocytes, while progesterone increases TNF-α expression and has less of an effect on IL-6 expression [14]. Whether progesterone counteracts estrogen in regulating IL-6 expression in heart tissue is not yet known.

We previously proposed that regular exercise might regulate MC activation by inducing IL-10 production. IL-10 is an anti-inflammatory cytokine that can also inhibit MC activity [24]. Several studies have demonstrated that regular exercise can increase IL-10 levels in plasma and cardiac tissue [15, 28], possibly suggesting that IL-10 is an endogenous MC stabilizer activated by regular exercise. However, as the IL-10 levels were unchanged in our OVX rats subjected to the exercise regimen, the MC-suppressive effect of regular exercise observed in our study might be due to other pathways. One possible effect of exercise in preventing MC activation is through sympathetic activation. Epinephrine can inhibit the histamine release induced by antigens in passively sensitized human lungs [2]. Beta-2 adrenoceptor agonists also suppress the immunoglobulin E receptor-dependent release of histamine and inhibit the stem cell factor-induced proliferation and migration of MCs [12]. In addition, preconditioning with norepinephrine can

attenuate CMC degranulation induced by ischemia–reperfusion injury [30]. Therefore, regular sympathetic stimulation during exercise might be the underlying mechanism that inhibits MC degranulation. However, this hypothesis is counterbalanced by the increase in MC degranulation observed after a single dose of isoproterenol [22].

The results of our study also suggest that MCs are not involved in the induction of physiologic cardiac hypertrophy. Our hypothesis is supported by a study demonstrating that MC stabilizers cannot prevent the cardiac hypertrophy induced by norepinephrine infusion [6]. However, the inverse relationship between degranulated MCs and cross-sectional areas of cardiomyocytes suggests that MC activation might be associated with myocardial adaptation. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are potent stimulators of histamine release from peritoneal MCs [42]. Decreased levels of BNP upon endurance exercise training have been documented [4], so it is possible that MC degranulation is relatively reduced with exercise. However, the impact of ANP and BNP on CMCs is controversial [3, 26] and merits further investigation.

Conclusions

The results of this study demonstrate that estrogen and regular exercise have a differential effect on regulation of MC activation. An increase in MC activation after deprivation of ovarian sex hormones could be involved in the cardiac changes observed in postmenopausal women. These results also support implementation of regular aerobic exercise as an alternative to hormone replacement therapy in this population. Moreover, suppression of MC degranulation by regular exercise suggests that MC activation could be another indicator to differentiate between physiologic and pathologic cardiac hypertrophy.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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