Chronic Estrogen Supplementation Following Ovariectomy Improves the Emotional Stress-Induced Cardiovascular Responses by Indirect Action on the Nervous System and by Direct Action on the Heart

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Background  Takotsubo cardiomyopathy is triggered by emotional or physical stress especially in post-menopausal women. A reduction in estrogen levels following menopause might underlie the high incidence of takotsubo cardiomyopathy.

Methods and Results  The left ventricular contraction between ovariectomized rats (OVX) and OVX with estrogen supplementation (OVX+E) while subjected to immobilization stress (IMO) was compared. The IMO in combination with general anesthesia impaired the left ventricular contraction in both OVX and OVX+E. Estrogen supplementation tended to improve the IMO-induced cardiac dysfunction and significantly attenuated the increase of blood pressure and heart rate. To understand the protective mechanism of estrogen, the expression of c-fos mRNA, a marker of cellular activation was compared. The mRNA expression of cardioprotective substances in the heart was also investigated. In the OVX+E, the levels of c-fos mRNA were significantly decreased in the paraventricular hypothalamic nucleus, adrenal gland and left ventricle, suggesting that an increase of estrogen attenuates the emotional stress-induced hypothalamo-sympatho-adrenal outflow from the central nervous system to the target organs. An expression of heat shock protein 70 and atrial natriuretic peptide was significantly augmented in the OVX+E.

Conclusions  These data suggest that estrogen supplementation partially prevents emotional stress-induced cardiovascular responses both by indirect action on the nervous system and by direct action on the heart. (Circ J 2007; 71: 565 – 573)

Key Words:  Emotional stress; Estrogen; Paraventricular hypothalamic nucleus; Sympathetic nervous system

The incidence of cardiovascular diseases is low in pre-menopausal women, whereas it is increased in post-menopausal women. Estrogen supplementation therapy prevented the increase of cardiovascular diseases in post-menopausal women1 while recent meta-analysis of randomized trials showed no significant merits.2 Effects of estrogen on cardiovascular diseases were attributed principally to the modification of serum lipid concentration and coagulation pathways, while direct actions of estrogen on the cardiovascular system contributed substantially to the cardiovascular protective effects of estrogen because estrogen receptors (ERα and ERβ) are expressed in the blood vessels and in the heart3,4 Also both ERα and ERβ are also widely expressed in the central nervous system5 Estrogen plays crucial roles in sexual behavior, learning and memory processes, protection against ischemic insults and modulation of autonomic nervous function6 In fact, a reduction of estrogen levels following menopause might increase the vulnerability of women to stress while estrogen supplementation attenuates the exaggerated response to stress or to increased sympathoadrenal activity7 A unique form of acute cardiac attack called “takotsubo cardiomyopathy”, or “transient left ventricular apical ballooning” similarly occurs predominantly in post-menopausal women in association with emotional or physical stress8–14 Although the etiology of this syndrome is yet to be clarified, an increase of serum norepinephrine, epinephrine and neuropeptide Y levels at the onset of takotsubo cardiomyopathy compared with acute myocardial infarction suggests that the exaggerated sympathoadrenal activation triggered by stress is the primary cause of this cardiomyopathy9,10 Immobilization stress (IMO) in the rat provides an excellent animal model of emotional stress, which activates the hypothalamic-pituitary-adrenocortical system and the sympathoadrenal system15 We have succeeded in developing a model of this clinical condition in rats16–20 The characteristic changes such as elevation of
the ST segment in the electrocardiography (ECG)\textsuperscript{17} left ventricular dysfunction including the reversible left ventricular apical ballooning in left ventriculography (LVG)\textsuperscript{19} and the induction of immediate early genes in the heart\textsuperscript{16} were prevented by pretreatment with a combined blockade of $\alpha$- and $\beta$-adrenoceptors, suggesting that enhanced sympathoadrenal outflow is involved in these cardiac changes. We also found that the increase of serum $\beta$-estradiol ($E_2$) improved the IMO-induced left ventricular dysfunction and tachycardia.\textsuperscript{19,20} Recently, we reported that c-Fos immunoreactivity, a marker of enhanced neuronal activity, was attenuated in the multi-synaptically sympathoadrenal-projecting central regions such as the lateral septum, paraventricular hypothalamic nucleus (PVH), dorsomedial hypothalamic nucleus, medial amygdaloid nucleus and lateral periaqueductal gray in $E_2$-supplemented rats.\textsuperscript{20} These data suggested that estrogen reduced the sympathoadrenal outflow from the brain that was induced by stress, however, the mechanism of IMO-induced cardiac dysfunction and the protective effects of estrogen have yet to be clarified.

In this study, we investigated the effects of IMO and estrogen on cardiac function by using non-invasive and highly reproducible 2-dimentional echocardiography. To know the protective mechanism of estrogen, we estimated the expression of c-fos mRNA, a functional marker of cellular activation in the brain, adrenal gland and heart. We also estimated the mRNA expression of some cardioprotective substances in the heart.

**Methods**

**Materials**

Female Sprague-Dawley rats (8 weeks old) were purchased from Japan SLIC Inc (Shizuoka, Japan) and housed in a temperature-controlled environment. Under anesthesia with sodium pentobarbitol (40 mg/kg, ip), bilateral ovariectomy (OVX) was performed ($n=36$). Following bilateral ovariectomy, half of the rats were subcutaneously implanted with a pellet containing $E_2$ (5 mg, release time 21 days) (Innovative Research of America, Sarasota, FL, USA) (OVX + E). Experiments were performed after allowing the rats free access to food and water for 2 weeks. The rats in each group were restrained for 30 min by securing them on their back to a board using adhesive tape (immobilization stress: IMO) (OVX + E/Stress, $n=18$; OVX/Stress, $n=18$). Anesthetic, analgesic and tranquilizing drugs were not given during IMO in order to reproduce the clinical manifestations of emotional stress.

**Estimation of Cardiac Function by 2-Dimentional (D) Echocardiography and Electrocardiography**

Immediately after IMO for 30 min, the rats were anesthetized with sodium pentobarbitol (40 mg/kg, ip). Fractional left ventricular area change (FAC) was measured at the papillary muscle levels as shown in A and B. Dotted lines indicate the endocardial margin. Fractional left ventricular area change (FAC) was defined as indicated in the formula (C).
natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are shown in Table 1. A computer-assisted homology search revealed no identical sequences in any genes in the database (GenBank). In situ hybridization histochemistry was performed by using a Bioimaging-analyzer BAS2500 Film autoradiography and estimation of radio-activities were performed by using powdered dry ice within 1 min after decapitation. The adrenal glands were fixed in 4% paraformaldehyde/0.1 mol/L phosphate buffer pH 7.4 overnight, then cryoprotected in phosphate-buffered saline (PBS) containing 30% sucrose for 3 days. The frozen tissues were stored at –80°C until sectioned. All animal manipulations were approved by the Osaka University Animal Use Committee.

**In Situ Hybridization Histochemistry**

Frozen sections of 60μm in thickness were cut in a cryostat and thaw-mounted onto silane-coated slides. The probe sequences for c-fos heat shock protein (HSP) 70, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are shown in Table 1. A computer-assisted homology search revealed no identical sequences in any genes in the database (GenBank). In situ hybridization histochemistry (ISH) was performed as we previously described. Film autoradiography and estimation of radio-activities were performed by using a Bioimaging-analyzer BAS2500 (Fuji Film, Tokyo, Japan). Next, the slides were coated with K-5 emulsion (Ilford, Knutsford, UK) diluted 1:2 with water for auto-radiography and then exposed for 6–10 weeks at 4°C. Slides were developed in D-19 (Kodak, Rochester, NY, USA) and the sections were counter-stained with hematoxylin–eosin for morphological examinations.

**Real-Time Reverse (RT) Transcription-Polymerase Chain Reaction (PCR)**

Following the preparation of frozen sections of the heart, an additional section of 50μm in thickness was cut in a cryostat. Total RNAs from these sections were extracted by using a RNeasy® Mini Kit (QIAGEN, Tokyo, Japan) and digested with RNase free-DNase (QUIAGEN). The expression of c-fos, HSP70, ANP and BNP mRNAs was determined by real-time RT-PCR. As an internal control, we also estimated the expression of rat glyceraldehydes-3 phosphate dehydrogenase mRNA. Primer sequences are shown in Table 1. PCR amplification using a LightCycler instrument was carried out in 20μl of reaction mixture consisting of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Penzberg, Germany), 4.0 mmol/L of each probe, and 2μl of template cDNA in a LightCycler capillary. Relative mRNA in each sample was then quantified automatically by reference to the standard curve constructed each time according to the LightCycler software. The levels of mRNA were calculated with reference to external standard curves constructed by plotting the log number of 10-fold serial diluted cDNA samples against the respective crossover point (Ct). The expression of mRNA level in each sample was normalized against its GAPDH mRNA level.

**Estimation of Serum E2 Levels**

Serum E2 levels were estimated with an E2 radioimmunoassay kit (Diagnostic Products Corp, Los Angeles, CA, USA). No cross-reactivities to other steroids were observed.

Examine the effects of anesthesia, per se.

**Estimation of Blood Pressure (BP)**

Immediately following IMO, the left femoral artery of the rat was cannulated with polyethylene tubing (PE50) under local anesthesia with lidocaine (1%; 0.5 ml, sc) for BP monitoring via a pressure transducer (UPS-801, Unique Medical Co, Ltd, Tokyo, Japan). The IMO was maintained for 30 min and was followed by general anesthesia with sodium pentobarbital (40 mg/kg, ip). The BP was measured for 30 min and was followed by general anesthesia with sodium pentobarbital (40 mg/kg, ip). The BP was measured at the end of IMO (stress), and at 20 min following the end of IMO (post-stress).

**Tissue Preparations**

Another group of rats with OVX (n=6) or OVX + E (n=7) were immediately decapitated at 30 min from the start of IMO. The hearts, the adrenal glands and the brains were rapidly removed. The heart and the brain were immediately frozen using powdered dry ice within 1 min after decapitation. The adrenal glands were fixed in 4% paraformaldehyde/0.1 mol/L phosphate buffer pH 7.4 overnight, then cryoprotected in phosphate-buffered saline (PBS) containing 30% sucrose for 3 days. The frozen tissues were stored at –80°C until sectioned. All animal manipulations were approved by the Osaka University Animal Use Committee.
Statistical Analysis

Data are shown as mean ± SEM. Statistical analysis was performed using 1-way, 2-repeated-measures ANOVA, or a Student’s unpaired t-test using StatView software (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at p<0.05. Refer to the figures for more detail.

Results

Stress Impaired Left Ventricular Contraction and Estrogen Tended to Improve the Stress-Induced Cardiac Dysfunction

Left ventricular contraction estimated at the papillary muscle level was not impaired during IMO stress for 30 min. Following induction of general anesthesia, the left ventricular contraction was significantly altered as demonstrated by changes in the FAC (OVX, p<0.001; OVX + E, p<0.05) (Fig 2A). However, the induction of general anesthesia without prior stress did not significantly influence the change in FAC (Fig 2B). A reduction in the FAC was most prominent at 20 min after the induction of anesthesia, and recovery of systolic function was observed at 60 min. When OVX + E was compared with OVX, the differences in reduction in the FAC between the 2 groups were not significant, although the decline in FAC seemed mild in OVX + E (p=0.192). An elevation of the ST segment on the ECG monitor was also observed in all cases (Figs 2C,D).

Estrogen Attenuated the Stress-Induced Increase of HRs and BP

Following the end of IMO stress and induction of general anesthesia, HR was gradually and significantly decreased in both groups (p<0.0001; Fig 3A). The HR was generally higher in OVX than in OVX + E, and 2-way repeated-measures ANOVA confirmed the effect of estrogen supplementation (p<0.05). These changes were also observed in pre-stress free conditions. There were no significant differences between OVX and OVX + E in terms of diastolic BP (DBP) at the end of the stress period, and in terms of SBP and DBP at post-stress.

Estrogen Attenuated the Stress-Induced Expression of c-fos mRNA in the Brain, the Adrenal Gland and the Heart

A clear expression of c-fos mRNA was observed in the brain, the adrenal gland and the heart. In the brain, signals were observed in the cerebral cortex, amygdaloid complex, midline thalamus and PVH (Fig 5). In the adrenal glands, signals were observed in both the cortex and medulla. In the heart, signals were observed in both ventricles, especially in the myocardium in the area surrounding the left ventricular cavity. Microscopic observation revealed that c-fos mRNA was expressed in the myocardium and coronary artery (Figs 7A,B). These signals were not observed in pre-stress control tissues (data not shown). Excess amounts of non-labeled probes completely eliminated the hybridization signals for c-fos mRNA, indicating that these signals were specific.

Relative levels for c-fos mRNA were compared by real-time RT-PCR (heart) and by in situ hybridization (adrenal gland and brain) between OVX and OVX + E (Fig 5). In the brain, we focused on the expression in PVH, which regulates the autonomic nervous system and the hypothalamic-pituitary-adrenal axis. As shown in Fig 5, the signals for c-fos mRNA in PVH were significantly higher in OVX (OVX, n=5; 100.0±7.4% vs OVX + E, n=5; 66.8±4.9%, p<0.01). In the adrenal glands, signals for c-fos mRNA were significantly higher in OVX compared with OVX + E (OVX, n=4; 100.0±19.3% vs OVX + E, n=4; 38.2±2.9%, p<0.05). The levels of c-fos mRNA in the heart were significantly higher in OVX than those in OVX + E (OVX, n=5; 100.0±11.5% vs OVX + E, n=6; 64.3±9.5%, p<0.05).

Estrogen Upregulated the mRNA Levels of Cardio-Protective Substances in the Heart

Finally, we investigated the mRNA expression of some...
cardio-protective substances by ISH and real-time RT-PCR (Figs 6, 7). Signals for HSP70 mRNA were observed in both ventricles. Microscopic observation revealed that HSP70 mRNA was expressed in the myocardium and coronary artery (Figs 7C, D). Signals for ANP and BNP mRNA were observed in both ventricles, especially in the myocardium in the area surrounding the left ventricular cavity. Microscopic observation revealed that ANP and BNP mRNA were expressed in the myocardium (Figs 7E, F).

Relative levels for each mRNA were compared by real-time RT-PCR between OVX (n=6) and OVX+E (n=7) (Fig 6). The levels of HSP70 and ANP mRNAs were significantly lower in OVX than in OVX+E (HSP70, OVX: 100.0±21.2% vs OVX+E: 291.4±69.3%, p<0.05; ANP, OVX: 100.0±13.3% vs OVX+E: 177.8±25.6%, p<0.05). The levels of BNP mRNA were not significantly different between OVX and OVX+E (OVX: 100.0±10.9% vs OVX+E: 115.6±14.1%, p=NS).
Fig 6. Film autoradiography showing expression of heat shock protein (HSP)70 (A), atrial natriuretic peptide (ANP) (B) and B-type natriuretic peptide (BNP) (C) mRNA in the heart, and quantitative comparison of mRNA levels between ovariectomized rats (OVX) and OVX with estrogen supplementation for 2 weeks (OVX+E). Signals for HSP70 mRNA were observed in both ventricles. Signals for ANP and BNP mRNA were observed in both ventricles, especially in the myocardium in the area surrounding the left ventricular cavity. The data are expressed as the mean value ± SEM. *p<0.05. NS, no significant differences; GAPDH, glyceraldehydes-3 phosphate dehydrogenase.

Fig 7. Bright-field photographs showing signals for c-fos (A, B), heat shock protein (HSP)70 (C, D), atrial natriuretic peptide (ANP) (E) and B-type natriuretic peptide (BNP) (F) mRNA in the heart. Bar: 50 μm (A–D, F), 100 μm (E).
Estimation of Serum E2 Levels

Serum E2 levels were significantly increased in OVX + E (mean±SEM; OVX, n=6; 11.3±3.7 pg/ml vs OVX + E, n=7; 2005.0±389.7 pg/ml, p<0.001).

Discussion

The present study demonstrated that IMO stress in combination with general anesthesia temporally impaired left ventricular contraction. Chronic estrogen supplementation in ovariectomized rats attenuated the IMO-induced increase of HR and BP and tended to improve the systolic dysfunction. Cellular activation indicated by c-fos mRNA expression was also reduced in PVH, adrenal gland and the heart as a result of estrogen treatment, while the mRNA expression of some cardio-protective substances such as HSP70 and ANP was upregulated.

Suppression of the stress-induced increase in HR and blood pressure in OVX + E compared with OVX was previously observed.19,20,28 Regardless of higher estrogen levels than those used in previous studies, therapeutic effects of estrogen on IMO-induced cardiac dysfunction were not complete. In OVX + E, the HR and BP at the end of IMO were still higher than those at post-stress. An elevation of the ST-segment was also observed in both OVX and OVX + E.

Left ventricular contraction, as evaluated by 2-D echocardiography was not impaired during IMO, but it was significantly impaired following the end of IMO and induction of general anesthesia (Fig 2A). General anesthesia itself did not influence the left ventricular contraction as shown in Fig 2B. During IMO, a pronounced increase in plasma adrenaline and noradrenaline was noted.29 Increased levels of catecholamines augment ventricular contraction, whereas an abrupt cessation of positive inotropic drive might reduce contractility. In addition, high levels of catecholamine can damage cardiac tissue and diminish left ventricular function because of Ca2+ overload, coronary occlusion, free radical formation, myocardial ischemia, increase of ventricular afterload and heart rate.27,28 When OVX + E was compared with OVX, the decline of FAC seemed to be mild in OVX + E but not significant. In the present study, we estimated the left ventricular contraction only at the papillary muscle level. This could not be excluded as a possible under-estimation of cardiac function and an oversight of regional ventricular akinesis at the apex (apical ballooning). Further studies using apical 4-chamber imaging or 3-D echocardiography, both of which were technically very difficult to measure, will be required.

We analyzed the mechanisms of estrogen in stress-induced cardiovascular responses based on the gene expression in the brain, the adrenal gland and the heart. Expression of c-fos mRNA, a marker of cellular activation, at the pre-stress control tissues is very low, while it prominently and maximally increased at 30 min in the brain, the adrenal gland and the heart.16 Therefore, we compared the levels of c-fos mRNA at 30 min from the onset of stress in the heart, adrenal gland and the brain between OVX and OVX + E to know the effect of estrogen on stress responses. The levels of c-fos mRNA in these areas were lower in OVX + E than in OVX, suggesting that cellular activation was attenuated by estrogen treatment. Although we did not estimate plasma catecholamine levels, a lower expression of c-fos mRNA in the adrenal gland in OVX + E suggests the hypo-excitation of chromaffin cells and diminished secretion of catecholamines. We recently demonstrated that IMO-induced c-Fos immunoreactivities were lower in the multi-synaptically sympato-adrenal-projecting central regions such as the lateral septum, PVH, dorsomedial hypothalamic nucleus, medial amygdaloid nucleus and lateral periaqueductal gray in E2-supplemented rats.20 We reconfirmed that the levels of c-fos mRNA in PVH were also significantly lower in OVX + E. Both ERα and ERβ are expressed in these brain areas, especially in the lateral septum, PVH, medial amygdaloid nucleus and lateral periaqueductal gray. These results suggest that estrogen treatment could modify the reactivity in these ER-positive and sympathoadrenal-projecting central neurons, thereby decreasing the sympatho-adrenal outflow from the central nervous system to the target organs. Further analysis to elucidate the precise mechanisms by which chronic estrogen treatment modifies neuronal activity in response to stress is needed.

Because ERβ and ERα are expressed in the cardiac cells,3,4 estrogen can directly act to reduce the reactivity to catecholamines in the heart. Administration of estrogen reduced isoproterenol-induced tachycardia and the incidence of ischemia/reperfusion-induced arrhythmia and cAMP production in rat hearts.40 OVX increased the Ca2+ sensitivity of cardiac myofilaments and estrogen replacement abolished this change.31 The density and protein content of the β-adrenergic receptor were upregulated in OVX, and estrogen/progesterone supplementation reversed these changes.32 These previous observations are in accordance with the diminished HR, BP response, reduced expression of c-fos mRNA in the heart and improvement of cardiac function results seen in the present study. The protective effects of estrogen in the heart have been demonstrated in several pathological conditions such as ischemia-reperfusion injury33,34 and pressure-overload hypertrophy.35 The ERβ-selective agonist but not the ERα-selective agonist reduced the infarct size of an ovariec- tomized rabbit heart.36 Systemic deletion of ERα increased the mortality of myocardial infarction.37

In the present study, we investigated the expression of cardio-protective substances such as ANP, BNP and HSP70 at the end of IMO stress for 30 min, and found that there was an upregulation of ANP and HSP70 mRNA but not BNP mRNA in OVX + E compared with OVX. In fact, the expression of ANP, BNP and HSP70 mRNA was also increased in response to IMO stress. However, we considered that these differences between OVX and OVX + E might reflect the pre-stress levels because the upregulation and translation of these substances need more time. In fact, the upregulation of ANP and HSP by chronic estrogen treatment was previously reported. Estrogen treatment reduced the aortic constriction-induced cardiac hypertrophy via the inhibition of phosphorylation of p38–mitogen-activated protein kinase and upregulation of ANP.16 Upregulation of ANP in the heart is mediated via the ERα-mediated pathway.37 Cardiac HSP70 levels in females were 2-fold higher than that in males.38 In addition, an ovariec- tomy reduced the level of HSP70 in female hearts, and this was prevented by estrogen supplementation.39 Both ANP and BNP exert the cardioprotective actions not only as circulating hormones but as local autocrine and/or paracrine factors.39 And both ANP and BNP can decrease the stress-induced increase of an afterload by natriuresis, inhibition of renin-angiotensin-aldosterone system and vasodilation. ANP also counteracts the norepinephrine-
stimulated Ca$^{2+}$ influx into cardiomyocytes by a cGMP-mediated mechanism.\textsuperscript{22} Involvement of HSP families in many pathological conditions has been also extensively studied.\textsuperscript{23} The levels of HSP following hypoxia were associated with the recovery of contractile function in isolated heart preparations probably via a energy metabolism.\textsuperscript{22} Hyperthermia-induced HSP70 production was negatively correlated with an increase of the left ventricular end-diastolic pressures in a chronic heart failure model.\textsuperscript{22} Taken together, increased levels of ANP and HSP70 levels in estrogen-treated rats can ameliorate the stress and catecholamine-induced left ventricular dysfunction.

**Clinical Implication and Study Limitation**

Pathogenesis of takotsubo cardiomyopathy is yet to be determined, while clinical observations strongly suggest that the trigger of the attack is emotional or physical stress and it occurs predominantly in post-menopausal women. In the present study, we confirmed by using an animal model that emotional stress induced left ventricular dysfunction and that estrogen supplementation partially improved the contractility. To date, no clinical provocation to induce takotsubo cardiomyopathy has been reported. Therefore, our experimental approach appears to mimic the clinical manifestations of takotsubo cardiomyopathy and potentially provides new insights into its pathogenesis. Because we could not demonstrate the “apical ballooning” in the present study, other approaches such as apical 4-chamber imaging or 3-D echocardiography, if possible, would permit more detailed analysis. We also demonstrated that chronic estrogen supplementation attenuated the stress-induced sympatho-adrenal outflow from the brain to the heart (indirect action on the nervous system) and upregulated cardioprotective substances such as ANP and HSP70 in the heart (direct action on the heart). Recently, we investigated the effect of estrogen on stress in male rats. In male rats, chronic estrogen treatment also improved the IMO-induced left ventricular dysfunction (unpublished observation). The upregulation of ANP mRNA in the heart was also observed in the estrogen-treated male rats compared with control male rats, while the levels of stress-induced \textit{c-fos} mRNA expression were not significantly altered (unpublished observation). From this preliminary data, the direct action of estrogen on the heart might be predominant compared with the indirect action on the nervous system.

A reduction of estrogen levels following menopause may augment the reactivity to stress via the modulation of autonomic functions and the downregulation of the cardioprotective substances in the heart, resulting in the high incidence of takotsubo cardiomyopathy in post-menopausal women. These data also suggest a potential merit of estrogen supplementation therapy for the possible prevention of stress-induced takotsubo cardiomyopathy in post-menopausal women. In premenopausal non-pregnant female rats, the basal \textit{E}2 levels are 7 pg/ml on estrus and reach peak levels of 50 pg/ml on proestrus.\textsuperscript{24} In the present study, serum \textit{E}2 levels in OVX + \textit{E} exceeded this normal range, suggesting that caution must be used in extrapolating these data to the clinical condition of takotsubo cardiomyopathy as observed in post-menopausal women, or to a possible role for estrogen in its treatment.


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