İst Tıp Fak Derg 2008;71:39-45
J Ist Faculty Med 2008;71:39-45
www.itfdergisi.com

EFFECTS OF MENOPAUSE AND POSTMENOPAUSAL HORMONE REPLACEMENT THERAPY ON PLASMA NITRATE/NITRITE AND ENDOTHELIN-1 LEVELS

MENOPOZUN VE HORMON REPLASMAN TEDAVİSİNİN PLAZMA NİTRAT/NİTRİT VE ENDOTELİN-1 DÜZEYLERİNE ETKİSİ

Pervin VURAL*, Gülnar VATANDAŞ GÜLAÇTI*, Cemil AKGÜL **

ABSTRACT

Objective: The aim of this study was to investigate the changes of plasma nitrate/nitrite (NOx) and endothelin-1 (ET-1) in postmenopause in comparison with premenopausal state and assess the effects of different hormone replacement regimens on these vasoactive substances.

Materials and methods: The study involved 50 healthy postmenopausal (PMW), and 25 healthy premenopausal women. PMW were randomly divided into two subgroups: women receiving cyclic hormone replacement therapy (HRT) [0,625mg conjugated estrogen (CEE) from day 1 to day 28 + 5mg medroxyprogesterone acetate (MPA) from day 18 to day 28] (n=25); second subgroup consisted of women receiving continuous HRT [0.625mg CEE + 2,5mg MPA from day 1 to day 28] (n=25) for two months. Statistical significance was analysed by Kruskal-Wallis, Mann-Whitney U, Wilcoxon signed ranks, and Spearman correlation tests.

Results: Compared with premenopausal individuals, PMW have decreased NOx and increased ET-1. There is a positive correlation between NOx and ET-1 in premenopausal women, suggesting that there is a balance between NOx and ET-1. This balance is changed with menopause. Two month durated HRT significantly elevates NOx that is diminished with menopause and does not change ET-1, thereby improves the NOx/ET-1 balance.

Conclusion: Continuous HRT is more successful than cyclic HRT in improving the endothelial function and maintaining the critical balance between NOx and ET-1. Our data provide evidence for the existence of increased (3/4 of subjects) and reduced (1/4 of subjects) NOx levels in response to HRT, which suggest that a significant number (but not all) of the HRT receiving postmenopausal women profit from the beneficial effects of HRT.

Key words: Nitric oxide, endothelin-1, hormone replacement therapy (HRT), estrogen, progestin, menopause

ÖZET

Amaç: Bu çalışmanın amacı menopoz sonrası (postmenopozda) plazma nitrat/nitrit (NOx) ve endotelin-1 (ET-1) düzeylerinde meydana gelen değişiklikleri, ve farklı hormon replasman tedavilerinin (HRT) bu vazoaktif maddeler üzerindeki etkisini incelemekti.

Gereç ve yöntem: Çalışmaya 50 sağlıklı postmenopozal (PMW) ve 25 sağlıklı premenopozal kadın dahil edildi. Postmenopozal kadınlar rastgele iki alt gruba ayrıldı: siklik HRT ve kesintisiz HRT alan alt gruplar. 1. grup (siklik HRT) 2 ay boyunca siklüsün 1-28 günleri arasında 0,625mg konjuge estojen (CEE) + 18-28 günleri arasında 5mg medroksiprogesteron asetat (MPA) alan kadınlardan oluşturuldu. 2. grup (kesintisiz HRT) ise 2 ay boyunca siklüsün 1-28 günleri arasında 0,625mg CEE + 2,5mg MPA alan kadınlardan oluşturuldu. Sonuçların istatiksel değerlendirilmesinde Kruskal-Wallis, Mann-Whitney U, Wilcoxon signed ranks, ve Spearman korelasyon testleri kullanıldı.

Bulgular: Premenopozal olanlara kıyasla postmenopozal kadınlarda NOx konsantrasyonları azalırken, ET-1 kon-

Date received/Dergiye geldiği tarih: 03.01.2008- Dergiye kabul edildiği tarih: 26.05.2008

* Istanbul University, Istanbul Faculty of Medicine, Department of Biochemistry, Çapa, Istanbul
(İletişim kurulacak yazar: pervinvural@yahoo.com)

** Istanbul University, Istanbul Faculty of Medicine, Department of Gynecology, Çapa, Istanbul

santrasyonları artmaktadır. Premenopozal kadınlarda NOx ve ET-1 arasında görülen pozitif korelasyon, bu iki parametre arasında bir denge olduğunu göstermektedir. Bu denge menopozda değişmektedir. İki aylık HRT menopozla azalan NOx'nın artmasına neden olurken, ET-1 üzerine bir etkisi yoktur, böylece HRT NOx ve ET-1 arasındaki dengeyi düzenlemektedir. Siklik HRT'ye kıyasla kesintisiz HRT endotel fonksiyonu ve NOx/ET-1 arasındaki kritik dengenin sürdürülmesinde daha başarılı olduğunu söyleyebiliriz.

Sonuç: Sonuçlarımızdan da görüldüğü gibi, HRT'ye cavaben postmenopozal kadınların bir kısmında (3/4) plazma NOx düzeyleri artmakta, bir kısmında ise (1/4) azalmaktadır; bu da, hepsi olmasa da postmenopozal kadınların çoğunun HRT'nin pozitif etkilerinden yararlandığını söyleyebiliriz.

Anahtar kelimeler: Nitrik oksit, endotelin-1, hormon replasman tedavisi (HRT), estrojen, progestin, menopoz

INTRODUCTION

At menopause, ovarian estradiol and progesterone declines and risk for osteoporosis and cardiovascular disease increases. Postmenopausal osteoporosis is a heterogenous disorder, characterized by a progressive loss of bone tissue. This progressive bone loss is related with altered equilibrium between bone formation and resorption, and is due to increased osteoclast and decreased osteoblast formation.

The incidence of cardiovascular disease is lower in premenopausal women than in men, while postmenopausal women have an incidence of coronary disease similar to that of men in same age. This is suggested to be dependent upon estrogen deficiency. Although it seems to be controversial, HRT and especially estrogen, might have some benefical effects on atherosclerosis (the underlying cause of cardiovascular disease) and bone loss (19), but its exact mechanism of action is still unknown. Estrogen positively affects plasma lipids and exerts a benefical effect upon carbohydrate metabolism and hemocoagulation profile (20, 28). Furthermore, by inhibiting the susceptibility of LDL (low density lipoprotein) to oxidative modification, estrogen diminishes plasma lipid peroxides, which have a significant role in the development of atherosclerosis (20, 28). Along with a decrease in plasma lipid peroxides, estrogen may positively influence all steps involved in the formation of the atherosclerotic plaque (accumulation of cholesterol in arterial walls, arterial smooth muscle proliferation, platelet aggregation and vasoconstriction) (3, 5). There are many endothelium-derived vasocostricting (e.g. thromboxane, endothelin-1, interleukin-1) and vasodilating substances (e.g. nitric oxide, prostacyclin) that regulate local blood pressure and maintain the fluidity of blood and the pathency of blood vessels (31). Recent studies have postulated the contribution of altered nitric oxide and ET-1 production by endothelium to the benefical effects of estrogen replacement (3, 4, 7, 17, 27, 29). Nitric oxide causes vasodilatation, inhibits platelet aggregation, suppresses smooth muscle proliferation (5) and acts as an antiatherogenic factor (5, 9). Continuous release of nitric oxide from the endothelium maintains basal vascular tone, and alterations in the nitric oxide release allow the autoregulation of blood flow (21).

ET-1 is a 21-aminoacid peptide produced by both endothelial and smooth muscle cells, which opposes the effects of nitric oxide. It causes potent vasoconstriction of the systemic and coronary vasculature, increases monocyte adhesion, activates macrophages, and promotes vascular smooth muscle cells proliferation and migration (24). Furthermore, it has been suggested that nitric oxide and ET-1 interact. Nitric oxide inhibits ET-1 synthesis and modulates the number and affinity of the ETA receptors. While impaired nitric oxide production promotes vasoconstricting properties of ET-1; activating locally, ET-1 may increase nitric oxide secretion through ETB receptors (8,24).

Studies on the role of HRT on nitric oxide and ET-1 production in postmenopause have yielded conflicting results. Nitric oxide production was variously shown to be reduced (7), unchanged (6,38), or elevated (3,4,17,27,29) after HRT, and that HRT results in decreased (4,7,36) or unchanged ET-1 levels (38) in postmenopausal women.

The controversial data about the effect of HRT on nitric oxide and endothelin-1 prompted us to investigate nitric oxide and ET-1 in postmenopausal women before and after different regimens of oral estrogen/progestestin administration and to compare them to the levels measured in young premenopausal healthy women.

MATERIALS and METHODS

Fifty healthy postmenopausal women weighing within 15% of their ideal body weight were selected according to following inclusion criteria: (1) natural menopause occurred at least 12 months before the onset of the study; (2) body mass index lower than 30kg.m^{-2}; (3) sustained FSH rise (higher than 40IU.mL^{-1}).

Twenty-five premenopausal healthy women in productive era constituted the control group. They were multiparas, were in the follicular phase of the menstrual cyclic at the sampling, and had no estrogen deficiency symptoms. All women (controls and postmenopausal ones) were not on medication, and were free from hepatic, renal, vascular or endocrinological disease, or genital tract and breast malignancy. Women reporting alcohol and tobacco use were excluded.

The age of postmenopausal women was 48.6 ± 3.5 years; the age at menopause was 47 ± 2.5 years; and time since menopause was 1.6 ± 1 year. The age of premenopausal women (control group) was 30 ± 2.5 years. Postmenopausal women were randomly divided into two subgroups. While 25 postmenopausal women were treated for two months with cyclic HRT schedule (0.625mg conjugated estrogen from day 1 to day 28, and 5mg medroxyprogesterone acetate from day 18 to

Table 1. Clinical data (mean ± SD) of postmenopausal women receiving cyclic and continuous HRT

Clinical Data	Cyclic HRT receiving women	Continuous HRT receiving women
Age (years)	47.2 ± 3.1	51.2 ± 3.5
Age at menopause (years)	45.8 ± 4.1	48.1± 2.5
Time since menopause (years)	1.9 ± 1.5	1.5 ± 1
Systolic blood pressure (mmHg) - At baseline	142.2 ± 7.8	140.5 ± 10.1
- At month 2	140.1 ± 6.0	139.7 ± 4
Diastolic blood pressure (mmHg) - At baseline	84.5 ± 3	81.5 ± 4.4
- At month 2	85.7 ± 4.2	80.7 ± 3.2

Table 2. Plasma NOx (µmol/L) and ET-1 (pmol/L) levels in premenopausal and postmenopausal women (both cyclic and continuous HRT receiving subgroups) before and after 2 month HRT (mean ± SD)

		Postmenopausal Women (n=50)	
	Premenopausal Women (n= 25)	Before HRT	After HRT
NOx	57.92 ± 7.28	47.24 ± 5.18 $p^a < 0.0001$	54.90 ± 8.80 $p^b < 0.05$ $p^c < 0.0001$
ET-1	11.39 ± 2.1	20,8 ± 8,81 $p^a < 0,0001$	19.62 ± 8.20 $p^b < 0.0001$ NS^c

a) When postmenopausal women before HRT were compared with premenopausal ones
b) When postmenopausal women after HRT were compared with premenopausal ones
c) When posttreatment values in postmenopausal women receiving HRT were compared with baseline (before treatment) values

day 28), the remaining 25 women were treated for two months with continuous HRT schedule (0.625mg conjugated estrogen and 2.5mg medroxyprogesterone acetate from day 1 to day 28). Overnight fasting venous blood samples were collected in EDTA.K3 tubes from the antecubital vein at 10:00, and centrifuged immediately at 1000g (10min) to obtain the plasma. It is known that dietary nitrates are excreted in the urine within 18h of ingestion (15), so after overnight fasting (approximately 14h after the last meal) most of dietary nitrates would have been eliminated at the time of blood sampling. The plasma samples were kept at -70^{0}C pending analysis. Blood drawing and blood pressure measurements were made after 30 min of rest at supine position.

Nitric oxide, a free radical, rapidly and spontaneously reacts in oxygenated solutions with molecular oxygen (O_2) to yield a variety of nitrogen oxides. The only stable products, formed by spontaneous decomposition of nitric oxide in oxigenated solutions are nitrite (NO_{-2}) and nitrate (NO_{-3}). For measurement of plasma nitrite/nitrate (NOx) concentrations, the Grisham's method was used (12). The intraassay and interassay coefficients of variation (CV%) were 5,2 % and 10.3 %, respectively.

ET-1 was extracted from the plasma by using column chromatography (Sep-pak C-18 cartridges) and its concentration was measured by radioimmunoassay (Euro-Diagnostica, Sweeden). The analytical sensitivity was 2 pmol/L. The intraassay and interassay CV were 9.5% and 11,4 respectively (manufacturer's data). Data were given as means ± SD. Because the data were not normally disturbed, we used the Kruskal-Wallis test for nonprametric data plus post hoc Mann-Whitney U tests to compare differences between groups. Wilcoxon signed ranks test for paired data was used for comparison of baseline and posttreatment values, and Spearman correlation test was used to test the correlation between variables.

Informed consent was given by each woman, and the study was approved by the institutional review board of Istanbul Faculty of Medicine.

RESULTS

Both cyclic and continuous HRT treated subgroups were shown to be homogenous for age, age at menopause, time since menopause and blood pressure (Table 1). Blood pressure in both subgroups showed no significant variation at the second evaluation. Table 2 shows mean plasma NOx and ET-1 levels

Table 3. Plasma NOx (µmol/L) and ET-1 (pmol/L) levels in postmenopausal women before and after 2 month HRT (mean ± SD)

	Cyclic HRT Receiving (n=25)		Continuous HRT Receiving (n=25)	
	Before HRT	After HRT	Before HRT	After HRT
NOx	47.18 ± 6,03	51.92 ± 7.8 NS	47.3 ± 4.3	57.9 ± 8.87 p< 0.0001
ET-1	19.42 ± 9.62	18.82 ± 8.27 NS	20.74 ± 8.1	20.42 ± 8.23 NS

Table 4. Plasma NOx (µmol/L) levels (mean ± SD) in increasing and reducing subjects after HRT vs baseline

Increasing Subjects (3/4 , n=37)		Reducing Subjects (1/4 , n=13)	
Before HRT	After HRT	Before HRT	After HRT
46.20 ± 4.83	59.03 ± 7.98 p< 0.0001	50.16 ± 5.23	46.02 ± 3.08 p < 0.05

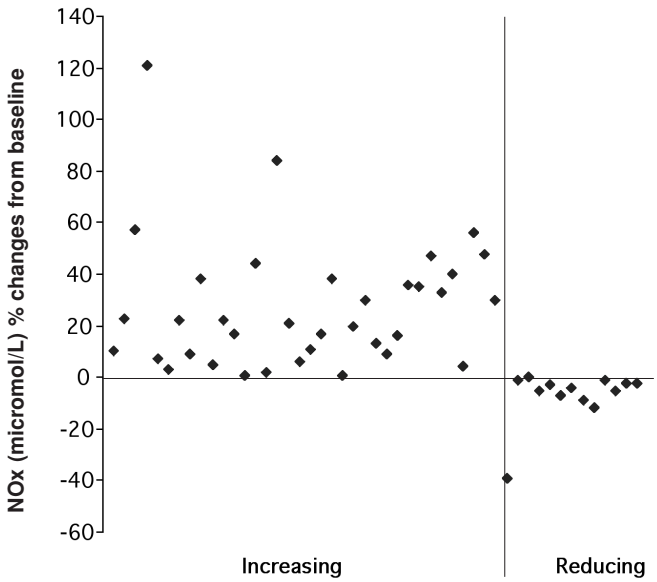

Figure 1. Plasma NOx levels in postmenopausal women after HRT. There are two distinct populations: increasing (3/4 , n= 37) and reducing (1/4 , n= 13) NOx vs. baseline.

in premenopausal and postmenopausal women before and after HRT. The results revealed that, compared with premenopausal individuals, postmenopausal women have decreased plasma NOx and increased ET-1 levels (p< 0.0001). HRT leads to the significant increase (p< 0.0001) of diminished with menopause NOx levels, and has no effect on increased with menopause plasma ET-1 levels. It is seen from Table 3 that mean baseline concentrations of NOx and ET-1 are similar in both cyclic and continuous HRT received subgroups. While cyclic HRT schedule has no effect on NOx and ET-1 concentrations, continuons HRT schedule significantly increases NOx to the premenopausal level (p< 0.0001) and does not change ET-1.

A detailed analysis demonstrates that out of 50 treated postmenopausal women 74% (3/4 ; n=37) have a significant increase (p<0.0001) in NOx levels (59.03 ± 7.98 µmol/L) when compared with baseline (46.2 ± 4.83 µmol/L), and classified as increasing subjects, whereas 26% (1/4 ; n= 13) have a significant decrease (p< 0.05) in NOx levels (46.02 ± 3.08 µmol/L) when compared with baseline (50.16 ± 5.23 µmol/L), and were classified as reducing subjects (Figure 1) (Table 4).

There is a weak but significant positive correlation (r= 0,460; p< 0,05) between NOx and ET-1 in premenopausal women, and significant negative correlation in postmenopausal women before treatment (r= -0.405; p< 0.05). Baseline ET-1 values of continuous HRT received women correlate significantly with post treatment values (r= 0.825; p< 0.0001). There is not any correlation between blood pressure and NOx , ET-1 concentrations.

DISCUSSION

The results of the present study demonstrate the following: 1) Postmenopausal women have decreased plasma NOx and increased ET-1 levels in comparison with premenopausal healthy women; 2) HRT, in general, elevates significantly reduced with menopause NOx levels, and has no effect on increased with menopause ET-1 levels; 3) Cyclic HRT schedule (CEE from day 1 to day 28 + MPA from day 18 to day 28) has no effect on NOx and ET-1 concentrations; 4) Continuous HRT schedule (CEE + MPA from day 1 to day 28) leads to the significant elevation of NOx to the premenopausal levels, and does not change ET-1 levels; 5) HRT increases endogenous NOx levels in approximately 3/4 of postmenopausal women (classified as increasing subjects), whereas in 1/4 of postmenopausal women (classified as reducing subjects) HRT decreases NOx concentrations, 6) There is a weak, but significant negative correlation between NOx and ET-1 in postmenopausal women before treatment.

Decreased plasma NOx and increased ET-1 in postmenopausal women (compared with premenopausal ones) are suspected results. As women undergo menopause, circulating concentrations of estrogen decrease. It is well known that menopause is related with impaired lipid peroxidation/antioxidant defence balance (33), resulting in accentuated oxidative stres. Oxidative stress and accentuated lipid peroxidation may increase the nitric oxide scavengers, such as superoxide anions, which are known to accelerate the inactivation/breakdown of intracellular nitric oxide (26). Furthermore, oxidative stress may suppress nitric oxide synthase (NOS) activity or nitric oxide release to the circulation; also may stimulate the production of preproendothelin-1 (a precursor of ET-1) (10, 32, 36). Therefore oxidative stress due to estrogen deficiency and ageing may alter the balance between NOx and ET-1 in menopause, probably resulting in increased cardiovascular disease and bone loss. On the other hand accentuated oxidative stres in postmenopause is related with increased pro-inflammatory TNFα and IL-1β cytokines (34), which probably leads to increased osteoclast and decreased osteoblast formation, and thus disturb the equilibrium between bone formation and resorption resulting in bone loss (16). Pro-osteoblastic prostaglandin E_2 (PGE_2) is one of the indices of oxidative stres (35). Indeed, we have found that there is increased urinary PGE_2 in postmenopausal women compared with premenopausal ones (2).

What happens with plasma NOx and ET-1 levels after HRT? While cyclic HRT has no effect on these vasoactive substances, continuous HRT significanly increases diminished with menopause NOx to the premenopausal levels, and does not change ET-1. Previous studies by Ylikorkala et al. (38)(used cyclic HRT: oral estrogen + noretisterone acetate for 12 months) and by Imthurn et al. (15)(used cyclic HRT: oral 17β-estradiol + MPA for 12 months) are showing that these ways for HRT cause no change in NOx levels. Twelve month duration of cyclic HRT (38) also does not change plasma ET-1 levels, as in our study.

Our results (continuous HRT) agree with these of Silvestry et al. (29)(used continuous CEE + MPA for 1 month) and Kesim et al. (17) (used continuous CEE + MPA for 12 months) showing that continuous HRT leads to the significant increase of plasma NOx levels. Our results showing that HRT has no effect on ET-1 are in contrast with some studies (4, 29, 36) showing decrease of ET-1 levels after HRT. This contrast may be due to different estrogen type used (4, 36); not using any progestin for opposing estrogen (4); or different study population (while postmenopausal women in our study were healthy subjects, postmenopausal women in Wilcox's and Silvestry's studies were at increased cardiovascular risk). It is seen from the results that continuous HRT is more successful than cyclic HRT in improving the endothelial function and maintaining the critical balance between NOx and ET-1.

Mechanism by which estrogen effects nitric oxide and ET-1 metabolism is still under debate. Estrogen stimulates NOS activity in myometrium (18) and cultured endothelial cells (13); the estrogen induced increase in blood flow blocked by L-nitroarginine-methylester (known as inhibitor of NOS) (30). An additional evidence suggests that estrogen can directly increase the release of nitric oxide (11) or suppress the release or activity of ET-1 (4). Through its antioxidant effects, estrogen may also decrease nitric oxide degradation. This potent inhibitor of lipid peroxidation may hinder the accelerated breakdown of nitric oxide, that results from the increased production of oxide free radicals associated with hypercholesterolemia. Thus, estrogen may alter systemic nitric oxide and ET-1 levels through multiple mechanisms, improving the endothelial function and maintaining the critical balance between nitric oxide and ET-1.

What is the relationship between nitric oxide and ET-1? The results of this and our previous study (32) revealed that there is a weak, but significant positive correlation between plasma NOx and ET-1 values in healthy premenopausal women. This suggests that there is a balance between these two vasomotor tone regulating factors and this balance is changed with menopause (we have been found that there is a negative correlation between study parameters in postmenopausal women). It is likely that along with their opposing effects on vascular tone and smooth muscle proliferation, regulatory mechanisms of these factors interact. Nitric oxide inhibits the production of ET-1 and modulates both the number and affinity of ET_A receptors. Nitric oxide synthase is functionally coupled to the ET_B receptor (8, 24). Increased nitric oxide production is probably a compensatory mechanism against vasoconstriction and endothelial damage related with postmenopausal estrogen deprivation and increased lipid peroxidation.

An important observation of this study is that 3/4 of investigated subjects show an increase and 1/4 show a decrease in plasma NOx after 2 months HRT. This finding agree with an

our previous study observations investigating the effect of tibolone on NOx (1) and with those of Imthurn et al. (15). The fact that postmenopausal women response to HRT via different ways with respect to endogenouıs NOx production suggests that there are two distinct groups – increasing and reducing NOx. One reason for the different response of these groups on HRT may be different status of estrogen receptors, which are "normally" lost in postmenopause, and also different ratios of ERα and β receptors (22). Estrogen binding to ERβ leads to decreased production of c-Jun and JunD, two members of the AP-1 family of transcription factors in osteoblasts and monocytes. Decreased AP-1 production results in decreased AP-1 induced TNF gene expression and lower TNFα production (25) and probably stimulated nitric oxide synthesis or decreased degradation (34). Interestingly, binding of estrogen to ERα results in stimulation of TNF gene expression (25). It seems possible that in increasing NOx women estrogen binds more to ERβ, and in reducing NOx ones estrogen binds more to ERα. On the other hand, only in 3/4 of postmenopausal women (increasing subjects) CEE reacts with relatively preserved estrogen receptors, leading to a nitric oxide increase, and that in remaining 1/4 (reducing subjects) CEE can not react with estrogen receptors (because of their lost), leading to a nitric oxide decrease. So, it seems to be more advantageous to use direct nitric oxide supplementation together with HRT in respect for prevention and treatment of postmenopausal osteoporosis and cardiovascular problems. Indeed, using nitroglycerine in the prevention of oophorectomy-induced bone loss in women demonstrated an equivalent efficacy to estrogen in the prevention of bone loss (37).

Although, in general, studies adressing the action of HRT on the cardiovascular system of postmenopausal women have yielded promising results, the long term vascular effects of HRT are still unclear. While according to the most studies HRT have benefical effects on heart and blood vessels and decreases the risk of cardiovascular disease (4, 27, 36, 38) HERS and WHI studies indicate (14, 23) a negative effect of HRT during the first 2 years of use in postmenopausal women with a history of ischemic heart disease. A probable explanation of this discrepancy is the age of study population (postmenopausal women in our study are younger than these in HERS and WHI studies), and presence of coronary heart problem (HERS study).

In conclusion, the results of the present study demonstrate that plasma NOx decrease and ET-1 increase with menopause; 2 month duration of HRT (particularly continue schedule) elevates diminished with menopause NOx, and not change ET-1 levels, thereby improve the balance between NOx and ET-1. Continuous HRT (CEE + MPA) is more effective than cyclic HRT in improving the impaired balance between NOx and ET-1. Our data provide evidence for the existence of both increasing and reducing plasma NOx levels subgroups in postmenopausal women in response to the HRT as effect of endogenous nitric oxide production, suggesting that a significant number (but not all) of the HRT receiving women profit from the beneficial effects HRT.

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