Synergistic Effect of a Physiological Ratio of Estradiol and Testosterone in the Treatment of Early-stage Atherosclerosis

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Received for publication May 21, 2015; accepted November 19, 2015 (ARCMED-D-15-00362).

Background and Aims. Clinical trials and epidemiological data suggest that estrogen replacement therapy (ERT) fails to reduce cardiovascular events in postmenopausal women with coronary heart disease (CHD). The high concentration of estrogen supplementation may increase the risk of thrombosis and result in testosterone deficiency, which is considered the main reason for failure. Thus, we hypothesized that a physiologic dosage of estradiol combined with testosterone may become a new therapeutic strategy in postmenopausal women with CHD.

Methods and Results. We used human umbilical vein endothelial cells (HUVECs) and female C57BL/6 mice as the experimental subjects. With the HUVECs, we found an appropriate E2/T ratio of 5:1 (5×10^-8 mol/L estradiol and 10^-8 mol/L testosterone), which has a significant anti-apoptotic effect on HUVECs by inducing a C-reactive protein. In the in vivo study, we verified the beneficial effects of the defined appropriate E2/T ratio in mice with early stage atherosclerosis. We found that replacement therapy with the defined appropriate E2/T ratio had beneficial effects of reducing the lipid lesions, reducing the formation of foam cells, reducing endothelial injury, modulating the coagulation system function and inhibiting inflammation and was significantly more effective than either estradiol or testosterone supplementation alone.

Conclusion. The present study demonstrated that estradiol and testosterone have a synergistic effect on early stage atherosclerosis, and replacement therapy with the defined appropriate E2/T ratio can significantly suppress the development of atherosclerosis through reducing the lipid lesions, reducing the formation of foam cells, reducing endothelial injury, modulating the coagulation system function and inhibiting inflammation. © 2015 IMSS. Published by Elsevier Inc.

Key Words: Estradiol, Testosterone, Synergistic effect, Apoptosis, Atherosclerosis.

Introduction

Human and animal studies have established that atherosclerosis is driven by a chronic inflammatory process within the arterial wall initiated mainly in response to endogenously modified structures (1). Dysfunction and apoptosis of endothelial cells is the initial step of atherosclerosis, which indicates that endothelial cells play an important role in atherosclerosis (2,3).

Basic studies have verified that estradiol is influential on cardioprotection, including inhibited oxidation stress, inflammation, vascular smooth muscle cells proliferation and promoted the release of nitric oxide (4,5). Owing to the positive effect of estradiol, estrogen replacement therapy has been in practice for a long time (6). However, an increasing number of clinical trials using estrogen replacement therapy for coronary heart disease (CHD) in postmenopausal women failed to demonstrate a reduced rate of CHD events (7). Meanwhile, experimental evidence suggests that androgen deficiency contributes to the onset and progression of CHD in men (8). Androgen deficiency is associated with endothelial dysfunction, high glucose...
and adverse lipid profiles, inflammatory responses, altered smooth muscle and hypertension (9–12), which are also associated with CHD. There are many studies reporting the role of estradiol (E2) or testosterone (T) in CHD (13–16). However, there are few studies that investigate the role of E2 combined with T in women with CHD.

Our previous study demonstrated that the E2 and T balance was disrupted in postmenopausal women with CHD (17). In this study, we aimed to determine the appropriate E2/T ratio in vitro and then verify the appropriate E2/T ratio in mice with early stage atherosclerosis to investigate whether the appropriate E2/T ratio has beneficial effects on the prevention of the development of atherosclerosis and to hint at a new strategy for hormone replacement treatment in CHD.

Materials and Methods

Cell Culture

HUVECs were maintained in endothelial basal media containing 5% fetal bovine serum and supplemented with an endothelial cell growth supplement (scienCell, catalog number: 1001). Cells were maintained at 37°C with 5% CO₂.

Reactive Oxygen Species (ROS) Assay

ROS was detected by ROS assay kit (Beyotime Institute of Biotechnology, S0033, China). HUVECs (10⁶) were isolated and treated with 10 μmol DCFH-DA at 37°C for 20 min. The fluorescence intensity was detected by flow cytometer.

Western Blot

HUVECs were lysed in RIPA buffer (Thermo Scientific) with 1 mM PMSF. Equal amounts of proteins were separated by SDS-PAGE and then probed overnight at 4°C with the following primary antibodies: PARP (1:1000), Bax (1:1000), Bcl-2 (1:500), caspase 3 (1:1000), (Millipore, Temecula, CA), ABCG1(1:1000), VCAM (1:1000), Akt (1:1000), p-Akt (1:1000) (Cell Signaling Technology, Danvers, MA). After washing, the membrane was incubated with goat anti-mouse or goat anti-rabbit secondary antibodies (1:3000; Millipore, Temecula, CA) at 37°C for 1 h. Immunoreactive bands were detected with a chemiluminescence detection system (Thermo Scientific).

Mice

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and were approved by the local animal care and use committee.

Female C57BL/6 mice were housed in the Renmin Hospital of Wuhan University experimental animal center. They were all kept in standard cages (ten mice per cage) in a temperature-controlled (23 ± 2°C) specific pathogen-free room with a 12 h dark/12 h light cycle. Eight-week old mice were randomly assigned to two groups: sham and experimental. The sham group served as the control and was operated on and received daily injections of the vehicle. The experimental group underwent bilateral ovariectomy (Ovx) and was further divided into four subgroups. Two weeks after Ovx, the four subgroups of the Ovx mice received daily gavage administration of: a) the vehicle (Ovx group), b) 17β-estradiol (1 μg/day, Sigma) (Ovx+E group), c) testosterone (7 μg/day, Sigma) (Ovx+T group), or d) 17β-estradiol (1 μg/day, Sigma) + testosterone (7 μg/day, Sigma) (Ovx+E/T group) for 120 days. The sham group was fed a regular chow diet, and the experimental group was fed a high fat diet (SLACCAS Co., Ltd., China) containing 21% fat (18% added cocoa butter and 3% fat within the basic diet), 0.15% cholesterol, 7% casein, 7% sucrose, and 3% maltodextrin. All mice had free access to water and food except during a 14-h fast period prior to blood sample collection.

Blood Analysis

Two weeks after ovariectomy and before the mice were sacrificed, we collected blood through the orbital venous. Plasma total cholesterol, triglyceride, HDL, LDL, and Hs-CRP were determined by an automatic biochemical analyzer (AU5400 Olympus, Japan). Plasma PT and APTT were analyzed by an automatic blood coagulation analyzer (CA7000 Sysmex, Japan). TNF-α, IL-6, estradiol and testosterone concentrations were measured with an ELISA kit (BOGOO, China).

Immunohistochemistry

The animals were perfusion-fixed with 4% paraformaldehyde (PFA). The tissues were excised and fixed with 4% PFA (4 h) and transferred to 20% sucrose (overnight) before embedding in Tissue-Tek medium at −40°C according to routine procedures. Aortic root sections were fixed in ice cold acetone or ethanol for 15 min at 4°C. A primary antibody against α-smooth muscle actin (α-SMA, 1:50, Santa Cruz) was used. α-SMA was detected by a sheep-anti-rabbit secondary antibody (1:200, Sigma). Macrophages were detected by an antibody against mac2 (1:400, Santa Cruz). Lipid deposition was determined by oil red O staining of the aortic valve.

Transmission Electron Microscopy

Heart tissue blocks were fixed in 2.5% glutaraldehyde in 0.1 mol PBS (pH 7.4) overnight. Fixed heart tissue blocks were washed in 0.1 mol PBS (3 × 15 min) and immersed in 1% osmium tetroxide in 0.1 mol PBS for 1 h. The heart tissue blocks were then dehydrated using different concentrations of ethanol. To embed the tissue, heart blocks were
immersed in a 1:1 EMBed 812 and propylene oxide mixture for 1–2 h. After embedding in the EMBed solution for 1 h, tissues were incubated at 37°C for 24 h and then baked at 60°C for 48 h. Thick tissue sections were cut and observed under the microscope where the precise location to cut for ultrathin sections was determined. Sections were stained with uranyl acetate for 15 min and lead citrate for 5 min. A transmission electron microscope (H-600 Hitachi, Japan) was used to observe the stained sections.

Results

Investigation of the Appropriate E2/T Ratio in HUVECs

To explore the role of E2 and T in protection against CHD, we chose HUVECs as our in vitro study model (18). According to our preliminary experiments (data not shown), we chose a 5:1 (E2/T) ratio as the center ratio, and we chose another three ratios (1:1, 10:1, 15:1) ranging before and after the center ratio to test as well. First, we treated cells with 10 μg/mL of the C-reactive protein (CRP) (19) to induce apoptosis. We then added the four different E2/T ratios, 10^{-8} and 10^{-8} (mol/L, 1:1), 5 \times 10^{-8} and 10^{-8} (mol/L, 5:1), 10^{-7} and 10^{-8} (mol/L, 10:1) and 1.5 \times 10^{-7} and 10^{-8} (mol/L, 15:1) and cultured for 24 h. Results showed in tunel and apoptotic-related proteins. According to the tunel, we can directly observe that CRP can improve the apoptosis of HUVECs, and among the four different ratios, “E2/T = 5:1” group can significantly resistant the apoptosis of HUVECs (Figure 1). We then detected the apoptotic-related proteins. After CRP treatment, the cleaved caspase 3, the cleaved PARP, and Bax had significantly increased expression levels. Conversely, the prosurvival protein Bcl-2 (20) expression level decreased

Figure 1. Investigation of the appropriate E2/T ratio in HUVECs. (A) Cells were first treated with CRP for 24 h and then added with four different E2/T ratios, including E2 (10^{-8} mol/L) and T (10^{-8} mol/L) (1:1), E2 (5 \times 10^{-8} mol/L) and T (10^{-8} mol/L) (5:1), E2 (10^{-7} mol/L) for another 24 h. Western blotting detected the levels of cleaved caspase-3, cleaved PARP, Bax, Bcl-2, caspases-8 and β-actin. (B–E) Statistical analysis of the cleaved caspase-3, cleaved PARP, Bax and Bcl-2 protein levels. (F) Cells were first treated with CRP for 24 h and then added with the fixed E2/T ratios (5:1) but different concentrations including E2 (5 \times 10^{-10} mol/L) and T (10^{-10} mol/L), E2 (5 \times 10^{-9} mol/L) and T (10^{-9} mol/L), E2 (5 \times 10^{-8} mol/L) and T (10^{-8} mol/L) for another 24 h. Western blotting detected the levels of cleaved caspase-3, cleaved PARP, Bcl-2, and β-actin. (G–I) Statistical analysis of the cleaved caspase-3, cleaved PARP and Bcl-2 protein levels. *Compared with control, p < 0.05. **Compared with control, p < 0.01.
Together these results demonstrate that CRP treatment promoted apoptosis. Expression levels of all proteins returned to baseline levels when E2 \((5 \times 10^{-8})\) and T \((10^{-8})\) (mol/L) at a 5:1 ratio were added; however, the other ratios \((1:1, 10:1 \text{ or } 15:1)\) did not recapitulate this effect (Figure 1). Based on these results, we chose a 5:1 ratio as the best E2/T ratio for HUVEC culture.

However, the best concentrations of E2 and T to use within this ratio were still not clear. We fixed the E2/T ratio at 5:1 and varied the concentrations of E2 and T. The concentrations of E2 and T tested were \(5 \times 10^{-10}\) and \(10^{-10}\) mol/L, \(5 \times 10^{-9}\) and \(10^{-9}\) mol/L, and \(5 \times 10^{-8}\) and \(10^{-8}\) mol/L, E2/T, respectively. Western blot results showed that the expression levels of cleaved caspase 3 and PARP increased when treated with CRP but significantly decreased when treated with the concentration of \(5 \times 10^{-8}\) and \(10^{-8}\) mol/L E2/T (Figure 1). The expression level of Bcl-2 decreased when treated with CRP but increased in a concentration-dependent manner compared with the control (Figure 1). In summary, we defined the HUVEC E2/T balance point as an E2/T ratio of 5:1 with a concentration of \(5 \times 10^{-8}\) (E2) and \(10^{-8}\) (T).

Figure 2. The defined E2/T ratio could better protect the cells from apoptosis induced by CRP in HUVECs through PI3K/Akt signaling pathway. (A) Cells of HUVECs were first treated with CRP for 24 h and then single estradiol \((5 \times 10^{-8}\) mol/L), single testosterone \((10^{-8}\) mol/L) and E2/T balance point \((5 \times 10^{-8}\) mol/L estradiol and \(10^{-8}\) mol/L testosterone) were added for another 24 h. Western blotting detected the levels of cleaved PARP, p-Akt and Akt. (B–C) Statistical analysis of the cleaved PARP and p-Akt protein levels. (D) Cells were first treated with PI3K inhibitor \((20 \mu g/mL)\) for 1 h and then CRP was added for 24 h and finally treated with E2, T or E2/T for 24 h. Western blotting detected the levels of PARP and p-Akt. (E–F) Statistical analysis of the cleaved PARP and p-Akt protein levels.*Compared with control, \(p < 0.05\). **Compared with control, \(p < 0.01\).
Comparison of the Anti-apoptotic Effect Between the Defined E2/T Ratio and Estradiol or Testosterone Treatment Alone

After we determined the E2/T balance point, we were interested to see whether treatment with the defined E2/T ratio could better protect the cells from apoptosis compared with estradiol or testosterone treatment alone. HUVECs were treated with CRP for 24 h and then with estradiol ($5 \times 10^{-8}$ mol/L) alone, testosterone ($10^{-8}$ mol/L) alone, or the E2/T balance point ($5 \times 10^{-8}$ E2 and $10^{-8}$ T, mol/L).

As shown in Figure 3, cleaved PARP expression levels were restored to levels similar to those of the control group when treated with the defined E2/T ratio. At the same time, we also checked the related signaling pathways and found a decrease in the expression level of p-Akt when the cells

![Figure 3](image-url)

*Figure 3.* Protective effect of the defined E2/T ratio on endothelial cell function in HUVECs. (A) ROS formation assay was performed according to the manufacturer. HUVECs were first treated with 10 μg/mL CRP for 24 h and then single estradiol ($5 \times 10^{-8}$ mol/L), single testosterone ($10^{-8}$ mol/L) and E2/T balance point ($5 \times 10^{-8}$ mol/L estradiol and $10^{-8}$ mol/L testosterone) were added for another 24 h. Fluorescence intensity of ROS was detected by flow cytometer. (B) Expression of ABCG1 and VCAM were detected by Western blot. *Compared with control, $p < 0.05$. 
were treated with CRP. Similarly, p-Akt expression levels were restored to levels similar to baseline levels only when treated with the E2/T balance point (Figure 2A and C).

To explore whether E2 and T treatment regulated cell apoptosis through the Akt pathway, we used the PI3K/Akt pathway inhibitor (LY294002) (21). We found that after treatment with the inhibitor, the cleaved PARP expression levels increased when treated with E2 or T alone, and expression levels did not return to baseline when treated with the E2/T balance point (Figure 2D and E). Furthermore, we found that the p-Akt expression levels also significantly decreased with all treatments (E2 or T alone as well as the E2/T balance point) (Figure 2D and F). These results demonstrated that blocking the Akt signaling pathway inhibited the E2/T balance point treatment completely, which suggests that the E2/T balance point treatment works through the PI3K/Akt pathway.

### Protective Effect of the Defined E2/T Ratio on Endothelial Cell Function in HUVECs

As shown in Figure 3, we detected ROS formation and expression of ABCG1 and VCAM to illustrate the protective effect of estradiol combined testosterone on endothelial cell function. Figure 3A shows that the intensity of ROS significantly increased after inducing by CRP. Treating with estradiol combined with testosterone could reduce the formation of ROS.

Compared with control, we observed the decreased level of ABCG1 and increased level of VCAM after inducing by CRP. When treated with estradiol alone, it showed the increasing expressions of ABCG1 and VCAM. Testosterone showed the opposite expression. However, when we treated with the E2/T balance point, it upregulated the level of ABCG1 and downregulated the level of VCAM, which showed the better protective effect on endothelial cell function than estradiol or testosterone alone (Figure 3B).

### General Features of Experimental Animals

Two weeks after OVX, female mice exhibited a significant decrease in serum estradiol ($p < 0.001$) and total testosterone ($p < 0.001$) levels and a significant increase in body weight ($p < 0.001$) compared with control mice. Mice were treated with estradiol and/or testosterone for 120 days. Estradiol replacement alone increased the serum estradiol level but further decreased the total serum testosterone level and had no significant effect on body weight. Testosterone replacement alone markedly increased the total serum testosterone level and body weight. However, when we treated with the defined appropriate E2/T ratio, the estradiol and testosterone serum levels both increased to levels similar to the control serum levels (Table 1).

#### Atherosclerotic Lesions in the Aortic Sinus

To investigate the progression of the atherosclerotic lesions, mice were fed high-fat diets for 120 days, and the aortic sinus was stained with oil red O to detect vascular lipids. As shown in Figure 4A, no lipid-loaded lesions were observed in the control mice, whereas in the HF-fed mice, localized lipid-loaded lesions were observed. Estradiol replacement alone and testosterone replacement alone groups did not significantly reduce the lipid-loaded lesion area. However, mice treated with the defined appropriate E2/T ratio had no lipid-loaded lesions, similar to the control mice.

As shown in Figure 4B, histological examination disclosed a progressive increase in lesion complexity in the aortic sinus. VSMCs and macrophages can serve as markers of lesions. As indicated in Figure 4, the HF-diet group had an increase in VSMC and macrophage marker expression, α-actin and mac-2, respectively. Estradiol replacement alone and testosterone replacement alone groups had a slight decrease in α-actin and mac-2 expression. The group treated with the defined appropriate E2/T ratio had a significant inhibition of α-actin and mac-2 protein expression.

#### Endothelial Injury in the Aortic Sinus

Endothelial injury is the initial step of the atherosclerosis process. It is a marker of early stage atherosclerosis. Thus, we used a transmission electron microscope to detect the degree of endothelial cell injury. In every group, a total of ten endothelial cells were counted. The vascular endothelial cells in the HF-diet group (9/10) were shrinking,
dissociating from the vessel, and dying. When treated with estradiol alone or testosterone alone, the number of injured endothelial cells decreased slightly (5/10 and 7/10, respectively) compared with the HF-diet group. Notably, of the endothelial cells in the defined appropriate E2/T ratio treated group, only one endothelial cell was undergoing apoptosis (1/10), the other endothelial cells were similar to control endothelial cells (in good shape and close to the vessel) (Figure 5).

Coagulation System Function in Mice

Because the coagulation system function plays an important role in atherosclerosis and estrogen and androgen treatment has a controversial effect on it, we used PT and APTT to assess the coagulation system function in mice. Results shown in Figure 6A and B suggest that after receiving a HF-diet for 120 days, mice had a shorter PT and APTT ($p < 0.05$ vs. control mice). Mice in the estradiol replacement alone group and mice in the testosterone replacement alone group had a significantly longer PT and shorter APTT ($p < 0.05$, vs. control mice). However, mice in the defined appropriate E2/T ratio group had no significant differences in their PT and APTT compared with control mice.

Plasma Lipids, TNF-α and IL-6 Levels

As shown in Table 2, TC, TG and LDL levels of mice on the HF-diet increased dramatically, and the level of HDL significantly decreased. Although all three treatments had no effect on TC, HDL and LDL, the estradiol alone treated group and the defined appropriate E2/T ratio treated group significantly decreased the level of TG.

Because inflammation also affects the development of atherosclerosis, we measured the levels of plasma TNF-α and IL-6, which serve as two main markers of inflammation. As shown in Figure 6C and D, HF-diet induced a significant increase in plasma TNF-α and IL-6 levels. Mice fed with estradiol alone displayed an increased level of TNF-α

![Figure 4. Atherosclerotic lesions in the aortic sinus. (A) Representative sections stained with Oil Red-O and hematoxylin, original magnification: X40 or X100, as indicated. (B, C) Representative examples of VSMC-specific actin (α-actin) and macrophage antibody (Mac-2)-stained aortic sinus sections are provided. Brown particle represents positively for VSMC and macrophage, original magnification: X100 or X400.](image-url)
and a slight decrease in the level of IL-6. On the contrary, mice fed with testosterone alone showed an increased level of IL-6 and a decreased level of TNF-α. Most importantly, mice fed with the defined appropriate E2/T ratio had a decreased level of both inflammatory factors, and the levels were similar to control levels.

Discussion

In a previous study we measured the serum E2 or T concentrations in postmenopausal women without and with CHD (17). We found that the serum E2/T ratio is adversely altered in postmenopausal women with CHD. An imbalanced E2/T ratio also had a strong association with cardiovascular risk factors in postmenopausal women with CHD. This clinical study hinted at a new strategy of hormone replacement treatment: estradiol and testosterone combined in an appropriate ratio.

The endothelium plays a pivotal role in maintaining vascular homeostasis, mainly by the production of the relaxing factor nitric oxide, which protects the vessel wall from the development of atherosclerosis (2). However, the relationship between sex hormones and the cardiovascular system in both men and women remain controversial. Premenopausal women are protected against the deleterious effect of aging on endothelial function by endogenous estrogen (22), but high local estrogen could lead to serious adverse effects (23–25). However, more and more evidence supports the finding that androgen displays beneficial effects on cardiovascular functions; however, the mechanism of androgen action remains to be elucidated (26,27). These findings suggest that treatment with estradiol combined with androgen might generate better effects on the cardiovascular system with CHD. To confirm our hypothesis, we first investigated the appropriate E2/T ratio in endothelial cells.

Our study shows that at a certain concentration and ratio (5×10⁻⁸ mol/L E2 and 10⁻⁸ mol/L T) E2 and T have an optimal effect on HUVECs such as downregulation of the expression levels of cleaved caspase 3, cleaved PARP, Bax and upregulation of the expression level of Bcl-2. These results show that there exists an appropriate E2/T ratio, which can protect endothelial cells through anti-apoptotic mechanisms.

After defining the appropriate E2/T ratio, we compared the anti-apoptotic effect of estradiol alone, testosterone alone and the defined appropriate E2/T ratio on HUVECs. Our results show that the defined appropriate E2/T ratio has a stronger anti-apoptotic effect than either sex hormone (estradiol or testosterone) alone. We also investigated the related signaling pathways and found that only the defined appropriate E2/T ratio can restore the p-Akt levels to baseline. Furthermore, to explore whether E2 and T treatment regulated cell apoptosis through the Akt pathway, we used the PI3K/Akt pathway inhibitor (LY294002) (21). We found that after culture with the inhibitor, the cleaved PARP expression level increased in the E2 or T alone treated...
groups, but the expression level did not return to baseline levels even when treated with the E2/T balance point. Furthermore, we found that the p-Akt expression level significantly decreased not only in E2 or T alone groups but also in the E2/T balance point group. These showed that blocking the Akt signaling pathway inhibited the E2/T balance point treatment completely, which suggests that the E2/T balance point works through the PI3K/Akt pathway. The results of this HUVEC study determined that the appropriate E2/T ratio actually had a great anti-apoptotic effect.

The dysfunction of endothelial cell is the first step of atherosclerosis. We found that treating with estradiol combined testosterone could inhibit the ROS formation, the

Table 2. Plasma lipid levels in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>HDL (mmol/L)</th>
<th>LDL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 9)</td>
<td>2.43 ± 0.08</td>
<td>1.18 ± 0.17</td>
<td>1.26 ± 0.06</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>OVX + HD (n = 9)</td>
<td>3.59 ± 0.23*</td>
<td>2.10 ± 0.29*</td>
<td>0.98 ± 0.06*</td>
<td>0.52 ± 0.05*</td>
</tr>
<tr>
<td>OVX + HD + E2 (n = 9)</td>
<td>3.41 ± 0.25*</td>
<td>0.74 ± 0.16*</td>
<td>1.17 ± 0.01</td>
<td>0.34 ± 0.03*</td>
</tr>
<tr>
<td>OVX + HD + T (n = 9)</td>
<td>4.42 ± 0.11*</td>
<td>2.15 ± 0.37*</td>
<td>1.88 ± 0.20*</td>
<td>0.46 ± 0.03*</td>
</tr>
<tr>
<td>OVX + HD + E2+T (n = 9)</td>
<td>4.30 ± 0.14*</td>
<td>1.14 ± 0.24*</td>
<td>2.17 ± 0.12*</td>
<td>0.27 ± 0.06*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.
\*Compared with control, p < 0.05.
\#Compared with OVX + HD, p < 0.05.
expression of VCAM and improve the expression of 
ABCG1 which help to regulate cellular lipid homeostasis. 
These evidences showed a protective effect of estradiol 
combined testosterone on endothelial cell function and gave 
us a theoretical basis for further animal studies.

In the experimental animal study, we combined the re-
ported physiological concentrations of estradiol and testos-
erone in the mouse (28) and the appropriate E2/T ratio we 
defined, and decided the dosage of estradiol and testos-
erone (1 μg/day estradiol and 7 μg/day testosterone at a 
1:5 testosterone:estradiol ratio) to be used. To mimic the or-
al administration of sex hormone replacement in humans, 
we administered the estradiol or testosterone treatment 
through gavage.

To our knowledge, this is the first study to address the 
administration of a physiologic dosage of testosterone-to-
estradiol in an appropriate ratio to enhance the cardioprotec-
tion of estradiol in OVX mice subjected to early stage 
atherosclerosis. Our results showed that either estradiol or 
testosterone replacement alone or replacement with the 
defined appropriate E2/T ratio reduced endothelial injury, 
inhibited the development of atherosclerosis, modulated 
the blood coagulation, and modulated the plasma levels 
of lipids and inflammatory factors. However, treatment with 
the combination of E2 and T in the appropriate ratio ap-
peared to be more effective than either estradiol or testos-
erone replacement alone.

A number of clinical and experimental studies confirmed 
that a high concentration of estradiol has beneficial effects 
on atherosclerosis (5). However, the effects of a physiologic 
concentration of estradiol are still controversial. Patten 
et al. found that treatment with a physiologic concentration 
of estrogen reduced cardiomyocyte apoptosis after myocar-
dial infarction in OVX mice (29). However, a study by 
Freudenberger et al. found that treatment with a physiologic 
concentration of estrogen accelerated atherosclerosis in 
OVX mice (28). In the current study, estradiol replacement 
alone downregulated the expression of α-actin and mac-2, 
reduced endothelial injury and slightly decreased IL-6 
expression compared with the HF-diet group, which 
together appear to suggest an anti-atherosclerotic effect. 
However, estradiol is known to have a detrimental 
effect on the coagulation system function. Estradiol may increase 
the blood viscosity and the risk of thrombosis, which is 
considered the main reason for the failure of estrogen hor-
monal replacement (30). Recently, a role for testosterone in 
reducing thrombogenesis has been discovered (31). This ef-
cfect can remedy the disadvantages of estradiol.

A large number of studies have demonstrated that testos-
erone replacement protects against atherosclerosis in male 
mice. However, few studies have focused on its effects in 
OVX female mice. In the present study, treatment with a 
physiologic dosage of testosterone downregulated the 
expression of α-actin and mac-2 and reduced endothelial 
injury to a lesser degree than replacement with estradiol 
alone. Testosterone alone also had a less detrimental effect 
than estradiol alone on the coagulation system function. 
Interestingly, testosterone had the opposite expression 
pattern of inflammatory factors compared with estradiol. 
These results may indicate that testosterone and estradiol 
may induce two different inflammatory reactions. Together 
our results indicated that a physiologic dosage of testos-
erone replacement also had an anti-atherosclerotic effect.

Although estradiol has a beneficial role in the cardiovas-
cular system, it can also cause an increase in cardiovascular 
events. Estradiol replacement alone results in a testosterone 
deficiency and causes an imbalance in the physiologic E2/T 
ratio. Testosterone replacement with estradiol replacement 
can also have a complementary effect. Vitale et al. demon-
strated that the estradiol/testosterone ratio, rather than the 
absolute levels of testosterone, was crucial in modulating 
the effect of testosterone on atherosclerosis in females (32).

In the present study, replacement therapy with the 
defined appropriate E2/T ratio had the beneficial effects 
of reducing the lipid lesions, reducing the formation of 
foam cells, reducing endothelial injury, modulating the 
coagulation system function and inhibiting inflammation 
and was significantly more effective than either estradiol 
or testosterone supplementation alone. Therefore, the com-
bination of estradiol and testosterone at the appropriate ra-
tio is a reasonable replacement therapy for prevention and 
treatment of atherosclerosis among postmenopausal 
women.

In conclusion, the present study demonstrated that estra-
diol and testosterone have a synergistic effect on early stage 
atherosclerosis, and replacement therapy with the appro-
priate ratio of E2/T can significantly suppress the develop-
ment of atherosclerosis. Further studies are warranted to 
delineate the potential mechanisms and to explore the 
possible therapeutic application of a treatment with a com-
bination of E2 and T in an appropriate ratio in postmeno-
pausal women with CHD.

Acknowledgments
This research was supported by National Natural Science 
Foundation of China (Grant Numbers: 81572069, 81501815).

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