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

Q4 Wen Dai,[a] Wang Ming,[a] Yan Li,[a] Hong-yun Zheng,[a] Chuan-dong Wei,[a,b] Zhao Rui,[a] and Cui Yan[a]

[a]*Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan, China*
[b]*Department of Clinical Laboratory, Affiliated Hospital of You Jiang Medical University for Nationalities, Baise, Guangxi, China*

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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.

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

Address reprint requests to: Dr. Yan Li, Jiefang Road 238, Wuhan, 430060 China; Phone/Fax: +86 027-88071553; E-mail: yanlitf1120@163.com

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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% CO_2.

Reactive Oxygen Species (ROS) Assay

ROS was detected by ROS assay kit (Beyotime Insititude of Biotechnology, S0033, China). HUVECs (10^6) 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