Effects of Selective Estrogen Receptor Modulator Raloxifene Plus 17-beta Estradiol in Aorta and Mammary Gland of Female Experimental Atherosclerosis Rabbits and Possible Involvement of ERK Signal Transduction Pathway

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Abstract

The present study investigated the effects of raloxifene, a second generation selective estrogen receptor modulator (SERM), plus 17-βE$_2$ on aortic atherosclerosis and mammary gland hyperplasia in ovariectomized, cholesterol-fed rabbits. Following 10 weeks of raloxifene, 17-βE$_2$, or raloxifene plus 17-βE$_2$ administration, serum total cholesterol, triglyceride, low density lipoprotein were significantly decreased in the drug groups compared to the placebo group. Consistent with serum lipid results, the total lesion area for each aorta of the drug groups decreased significantly as compared to the placebo group. HE staining of aorta paraffin section showed that in the drug groups the endothelial monolayer was almost continuous. While in mammary gland, HE staining of paraffin sections indicated the hyperplasia of epithelial cells (in 17-βE$_2$ group) was obviously inhibited in raloxifene plus 17-βE$_2$ group. In cultured vascular smooth muscle cell (VSMC), the results of MTT and [3H]TdR incorporation showed that the drug groups could inhibit AngII-induced proliferation of VSMC. Western blotting proved that raloxifene plus 17-βE$_2$ inhibited the expression of phosphorylated ERK protein, similar to 17-βE$_2$ but different from raloxifene. This effect was inhibited by PD98059 (inhibitor of MAPK) or ICI182780 (ER antagonist). In conclusion, this study suggests that SERM raloxifene plus 17-βE$_2$ improves the lipid metabolism and relieves the aorta changes of female experimental atherosclerosis rabbits, which are partly implemented by the inhibition of VSMC growth through ERK cascade. The hyperplasia of mammary gland epithelial cells could be significantly inhibited by raloxifene plus 17-βE$_2$.

Key Words: SERM, raloxifene, 17-βE$_2$, atherosclerosis, mammary gland, VSMC, ERK

Introduction

Cardiovascular disease is a major health problem. It is the leading cause of death among postmenopausal women in industrialized societies (14). Efficacious preventive strategies and therapies are therefore strongly needed now and in the future. A multi-center heart disease prevention study, part of the Women’s Health Initiative (WHI), found that estrogen-alone hormone therapy had no effect on coronary heart disease risk (women in the hormone trial are now in a follow-up phase, and will last until 2007, during which their
Selective estrogen receptor modulator (SERM) raloxifene is a non-steroidal selective estrogen receptor modulator being developed by Eli Lilly & Company as a therapeutic agent for postmenopausal osteoporosis. It has been shown to have estrogen agonist effects on bone and cardiovascular system (15, 27, 30), while having estrogen antagonist effects on mammary gland and uterus (4). The effects of not only raloxifene but also 17-β estradiol (17-βE₂) are mediated by estrogen receptors (ERs), while their association indexes to ERs are different. When both of them are presented, the effects are unknown. We hypothesized that raloxifene plus 17-βE₂ therapy may reserve the beneficial effects of cardiovascular system, but reduce side effects of mammary gland. Therefore, firstly the study was to determine the anti-atherosclerotic effects of raloxifene plus 17-βE₂ in ovariectomized, cholesterol-fed rabbits. At the same time, we observed the effect on the mammary gland. and studied the action of raloxifene plus 17-βE₂ on the proliferation of vascular smooth muscle cell (VSMC) and the involvement of extracellular signal-regulated kinase (ERK) signal transduction pathway.

Materials and Methods

Animal and Study Protocol

A total of 36 healthy female adult New Zealand rabbits (Center for Experimental Animals, Sun Yat-Sen University, Guangzhou, Guangdong, China) each weighing about 2.5 kg were continuously housed at the animal care facilities in standard rabbit cages with a room temperature of 20 ± 2°C and a 12-hour-light cycle. Rabbits received either a cholesterol diet, composed of 1% cholesterol (Guangzhou Reagent Chemicals Ltd., Guangzhou, Guangdong, China), 5% lard and 94% standard rabbit chow (Center of Experimental Animal Sun Yat-Sen University, Guangzhou, Guangdong, China) or standard rabbit chow (control group, n = 6) for 10 weeks from 7 days after ovariectomy.

Cholesterol-fed rabbits were divided into five groups. Four groups were ovariectomized and the fifth group received sham operation. After one-week recovery period, the first group of the ovariectomized rabbits received a subcutaneous injection of 17-βE₂ (20 µg/kg/d, subcutaneous injection, in 50 µl/kg/d sesame oil) for 10 weeks (17-βE₂ group, n = 6). Raloxifene (3 mg/kg/d) was administered orally by gavage daily to the second group (raloxifene group, n = 6). The third group was given both 17-βE₂ (20 µg/kg/d, subcutaneous injection, in 50 µl/kg/d sesame oil) and raloxifene (3 mg/kg/d) (raloxifene plus 17-βE₂ group, n = 6). The fourth group of overiectomized rabbits (placebo group, n = 6) and the fifth sham-operated rabbits ( sham, n = 6) received the same amount of solvent (50 µl/kg/d sesame oil).

Plasma Lipid Analyses

Blood samples were obtained before the animals were sacrificed. Total serum cholesterol (TC), high density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), apolipoproteinA1 (apoA1) and apolipoproteinB (apoB) were measured respectively (Hitachi 7170A Biochemical Test Equipment, Tokyo, Japan).

Areas of Aorta Atheromatous Plaque Assay

The aortas were separated carefully from aortic arch to iliac artery. A longitudinal cut was made to each of the aorta, then photos were taken and the aorta was scanned (JVC Co, Kanagawa, Japan). The images were analyzed and the areas of aorta atheromatous plaque were calculated (IBAS image analysis system, Kontron Elektronik CO, Eching, German).

Histological Preparation of Aortas and Mammary Glands

Tissue samples of aortas from 1.5 cm left to the aortic arch and the forth pair of mammary glands were collected for histological study. Aortas of 0.4-0.8 cm² and the mammary glands were prepared, soaked in 10% formalin saline solution, and were then processed for normal histological section. The tissue samples were ultra-sectioned (5-6 µm thickness), stained with hematoxylin and eosin (HE) and examined under Axiotron light microscope (Zeiss CO, Oberkochen, Germany) for observation of structural abnormality.

Cell Culture

VSMC was obtained from aortas of Sprague-Dawley rats (Center for Experimental Animals, Sun Yat-Sen University, Guangzhou, Guangdong, China).
Cells were isolated, cultured, and characterized as previously described (5). Briefly, the abdominal segments of the aortas were removed and the fascia was cleaned away. The aortas were cut longitudinally and small pieces of the media were carefully stripped from the vessel wall. The strips were placed in culture flask. Within 1 to 2 weeks, VSMC migrated from the explants; they were capable of being passaged 1 week after the first appearance of cells. They were identified as VSMC by their characteristic hill-and-valley growth pattern and immunohistochemistry for VSMC-specific \( \alpha \)-actin. All cultures were maintained in dulbecco’s minimum essential medium (DMEM) a medium containing 10% FCS at 37°C in a humidified, 5% CO\(_2\)/95% air atmosphere.

**Cell Proliferation Assays**

Cells were plated at a density of 10,000 cells/cm\(^2\) in 96-well plates. After 24 hours, the cells were washed, followed by an addition of 200 \( \mu \)l DMEM without phenol-sulfonphthalein or serum. After 24 hours, the cells were treated with angiotensinII (AngII) for 24 hours before co-cultured with 17-\( \beta \)E\(_2\) or raloxifene for 24 hours. Cells were then added 20 \( \mu \)l (5mg/ml) 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenylterazolium bromide (MTT). After incubation at 37°C for 4 hours, culture media were removed and 0.2 ml dimethyl sulfoxide (DMSO) were added. MTT assay was performed as described (11).

For the thymidine incorporation study, subconfluent cells were deprived of serum for 24 hours to make them quiescent. The cells were then fed with methyl-[\(^3\)H]thymidine at 1 \( \mu Ci/ml\) for 16 hours, after which thymidine incorporation was measured by trichloracetic acid (TCA) counting.

**Western Blot Analysis**

Protein samples were prepared from subconfluent proliferating cells. The cells were lysed in hot Laemmeli lysis buffer. Protein concentrations were determined by Bradford colourimetry and each sample was diluted by 2X sodium dodecyl sulphate (SDS) sample buffer (0.125 M Tris-Cl/0.1% SDS, pH 6.8, 20% glycerol, 4% SDS, 0.2% 2-mercaptoethanol, 0.001% bromphenol blue), and the samples were boiled for 5 min. Samples of 30 \( \mu \)g protein per lane were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated for 2 hours with anti-phosphorylated ERK antibodies. The membrane was then incubated in phosphate buffered solution (PBS) containing goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase for 1 hour. The membrane was washed, and positive signals were developed with the use of enhanced chemiluminescence (ECL) plus substrate-chromogen system.

**Drugs**

Raloxifene was obtained from Eli Lilly and Company (Indianapolis, IN, USA); 17-\( \beta \)E\(_2\), AngII, phenylmethylsulfonyl fluoride (PMSF), leupeptin, trypsin, MTT, sesame oil were purchased from Sigma Chemical Co. (Saint Louis, MO, USA); ICI182780 was provided by Prof. Ji-qiu Chen (Harvard Medical School). Cell medium DMEM was purchased from Gibco Ltd. (Paisley, Strathclyde, UK); \([\text{H}]\)Thymidine was obtained from Chinese Atomic Energy Research Institute (Beijing, China). The rabbit polyclonal anti-phosphorylated ERK antibody was purchased from New England Biolabs (Ipswich, England). IgG conjugated with horseradish peroxidase was obtained from Boster Biological Technology Ltd. (Wuhan, Hubei, China). Cholesterol was purchased from Guangzhou Reagent Chemicals Ltd. (Guangzhou, Guangdong, China).

**Statistics**

Values are expressed as (\( \bar{x} \pm SD \)). One-way ANOVA was used to analyze the data. \( P < 0.05 \) was considered statistically significant.

**Results**

**Plasma Lipid Level**

Lipid metabolism treated with raloxifene, 17-\( \beta \)E\(_2\) or raloxifene plus 17-\( \beta \)E\(_2\) resulted in lower TC, TG, and LDL concentrations relative to those in the placebo group (Table 1), but HDL, apoA1 or apoB concentrations were not affected relative to those in the placebo group.

**Aorta Morphologic Changes**

The analysis of plaque area was shown in Table 2. The ratio (area of lipid plaque to total area of aortic intima) was the highest in the placebo group. The rates of raloxifene group, 17-\( \beta \)E\(_2\) group, and raloxifene plus 17-\( \beta \)E\(_2\) group were all lower than that of the placebo group (\( P < 0.05 \)).

The aortas of control rabbits showed normal histology and that the endothelial monolayers were continuous. However, aortas of the placebo group showed spaces within the intima tunica and media tunica. These spaces had originally contained fat droplets which were dissolved during the Hematoxylin-eosin (HE) staining procedure. There were plenty of
Table 1. Changes of serum TC, TG, HDL, LDL, apoA1 and apoB levels in each group (x ± SD) showed raloxifene, 17-βE2 and raloxifene plus 17-βE2 improved the lipid metabolism. *P < 0.01 vs. placebo, #P < 0.01 vs. control, n = 6

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Sham</th>
<th>High cholesterol</th>
<th>Raloxifene</th>
<th>17-βE2</th>
<th>Raloxifene plus 17-βE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mM)</td>
<td>1.24 ± 0.54</td>
<td>34.09 ± 1.82*</td>
<td>38.15 ± 3.06*</td>
<td>33.85 ± 1.93*</td>
<td>36.51 ± 2.11*</td>
<td>35.00 ± 1.82*</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.87 ± 0.03</td>
<td>1.08 ± 0.08*</td>
<td>5.78 ± 1.02*</td>
<td>1.89 ± 0.11*</td>
<td>1.41 ± 0.10*</td>
<td>0.96 ± 0.06*</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>0.15 ± 0.02</td>
<td>0.55 ± 0.07*</td>
<td>0.59 ± 0.09*</td>
<td>0.19 ± 0.02*</td>
<td>0.70 ± 0.15</td>
<td>0.42 ± 0.03*</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>0.41 ± 0.02</td>
<td>32.05 ± 2.01*</td>
<td>35.05 ± 1.84*</td>
<td>32.93 ± 2.47*</td>
<td>31.66 ± 3.11*</td>
<td>25.27 ± 2.12*</td>
</tr>
<tr>
<td>apoA1 (g/l)</td>
<td>0.11 ± 0.03</td>
<td>0.15 ± 0.02*</td>
<td>0.14 ± 0.02*</td>
<td>0.17 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>apoB (g/l)</td>
<td>0.11 ± 0.02</td>
<td>2.49 ± 0.26*</td>
<td>1.70 ± 0.23*</td>
<td>1.84 ± 0.12</td>
<td>2.62 ± 0.03</td>
<td>1.39 ± 0.09</td>
</tr>
</tbody>
</table>

MTT Assay

AngII was added to cultured cells for 24 hours. It induced the proliferation of VSMC in a concentration-dependent manner. The amount of cells increased 7.00%, 14.06%, and 21.37% at the concentration of 10^{-9} M, 10^{-8} M, and 10^{-7} M, respectively, as compared to control. Raloxifene attenuated AngII (10^{-7} M)-induced proliferation of VSMC by 15.60%, 14.47% and 26.02% at 10^{-7} M, 10^{-6} M and 10^{-5} M individually. While 17-βE_2 (10^{-10} M–10^{-6} M) inhibited the proliferation by 21.06%, 25.13%, 25.20%, 17.69% and 12.01%. Raloxifene (10^{-9} M–10^{-5} M) plus 17-βE_2 (10^{-8} M) inhibited it by 13.17%, 14.71%, 12.08%, 17.22% and 28.91%. (Fig. 3)

Thymidine Incorporation Measurement

AngII was added to cultured cells for 24 hours. AngII (10^{-7} M) increased the incorporation of [^{3}H] thymidine to 339%, as compared to the control group. Raloxifene attenuated AngII (10^{-7} M)-induced incorporation by 44.48%, 53.13% and 85.78% at the concentration of 10^{-7} M, 10^{-6} M and 10^{-5} M individually, as compared to the control group, while 17-βE_2 (10^{-10} M–10^{-6} M) inhibited the incorporation by 42.12%, 50.33%, 51.47%, 35.21% and 30.24%. Raloxifene (10^{-9} M–10^{-5} M) plus 17-βE_2 (10^{-8} M) inhibited it by 36.81%, 48.15%, 40.46%, 43.44% and 54.78%.

Western Blot

AngII was added to cultured cells. AngII (10^{-7} M) increased the expression of phosphorylated ERK, as compared to the control group. The effects of raloxifene (10^{-7} M), 17-βE_2 (10^{-8} M), and raloxifene (10^{-7} M) plus 17-βE_2 (10^{-8} M) were shown as the following figures. 17-βE_2, raloxifene plus 17-βE_2 inhibited the ERK phosphorylation but raloxifene did not. The inhibition of 17-βE_2 and raloxifene plus 17-

Mammary Gland Morphologic Changes

There was no obvious red swelling in mammary glands of the raloxifene group, neither of the control, sham or placebo group. While in the 17-βE_2 group, there was obvious red swelling in mammary glands. The red swelling was inhibited in raloxifene plus 17-βE_2 group. Normal breast parenchyma, with no evidence of any atypical features or carcinoma was noted in mammary glands of animals in the control group, sham group, placebo group and raloxifene group, while ductal epithelial hyperplasia were seen in the sections of the 17-βE_2 group. The ductules were lined by several layers of large epithelial cells and the lumen may be obliterated by a solid proliferation. The proliferation was inhibited in raloxifene plus 17-βE_2 group. (Fig. 2a-f)
have shown that ERT reduces the incidence and severity of cardiovascular disease such as atherosclerosis in postmenopausal women, while many studies have also shown that high dose estrogens have the potential to act as both initiators and promoters of malignancies. Although a few studies of estrogen plus progestin therapy showed improvement of the risk of endometrial hyperplasia, progestin weakened the beneficial effects on cardiovascular diseases, and it did not confer protection against breast cancer. We are searching

\[ \beta E_2 \] was reversed by PD98059 (inhibitor of MAPK) (Fig 4). The inhibition of 17-\( \beta E_2 \) and raloxifene plus 17-\( \beta E_2 \) was attenuated by ICI182780 (inhibitor of ER) (Fig 5).

**Discussion**

Although the results of WHI indicated that estrogen had no effect on coronary heart disease risk (1), many previous clinical and laboratory studies have shown that ERT reduces the incidence and severity of cardiovascular disease such as atherosclerosis in postmenopausal women, while many studies have also shown that high dose estrogens have the potential to act as both initiators and promoters of malignancies. Although a few studies of estrogen plus progestin therapy showed improvement of the risk of endometrial hyperplasia, progestin weakened the beneficial effects on cardiovascular diseases, and it did not confer protection against breast cancer. We are searching

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Fig. 1. The aortas of control group (a) showed normal histology and the endothelial monolayers were continuous. Aortas of placebo group (c) showed spaces within the intima tunica and media tunica. These spaces had originally contained fat droplets which were dissolved during the HE staining procedure. There were plenty of foam cells, cholesterol deposits, calcium, and blood clots or thrombus. In raloxifene group (d), 17-\( \beta E_2 \) group (e), raloxifene plus 17-\( \beta E_2 \) group (f) or sham group (b), the endothelial monolayers were almost continuous. Only a few foam cells can be detected. (n = 6)
for a therapeutic agent that would produce estrogen-like effects on cardiovascular system without proliferative effects on breast or uterus. Since the appropriate selectivity profile of raloxifene, raloxifene plus 17-βE₂ offer the opportunity to dissociate favorable cardiovascular effects of estrogen from unfavorable stimulatory effects on the breast and uterus. In the present study, we investigated the anti-atherosclerotic effects of raloxifene, 17-βE₂, raloxifene plus 17-βE₂ in the in vivo atherosclerosis animal model or in vitro proliferation cell model and observed their side effects on mammary gland.

We proved that raloxifene plus 17-βE₂ improved the lipid metabolism and relieved the aorta changes of female experimental atherosclerosis rabbits. Several studies have demonstrated that raloxifene has tissue specificity, with estrogen agonistic effects on cardiovascular system (25) and bone, but with estrogen antagonistic effects on breast and uterus (6). Delmas et al. have reported that daily therapy with raloxifene...
increases bone mineral density, decreases serum concentrations of total and low-density lipoprotein cholesterol, and does not stimulate the endometrium (8). The protective properties of estrogen on the cardiovascular system are also well-known (18), although some recent clinical studies have failed to show a favorable effect (13). Our studies showed that SERM raloxifene played anti-atherosclerotic roles. SERM raloxifene plus 17-βE2 kept their protective effects in cardiovascular system. On the other hand, many studies have shown that estrogen may promote the malignancies such as mammary gland cancer (3).

Therefore, in this study, the proliferation of mammary gland epithelial cells was observed. HE staining of mammary gland paraffin sections showed that mammary gland epithelial cells were common in the raloxifene, sham and control groups. The obvious hyperplasia in 17-βE2 group was partially inhibited in raloxifene plus 17-βE2 group. Not only raloxifene but E2 can bind to estrogen receptors. When both of them are presented, raloxifene plugs part of the estrogen-binding sites on the ERs, thus competing estrogen with gaining access. The number of the sites plugged by raloxifene is mainly decided by the concentration. Since both raloxifene and 17-βE2 play the protective actions in cardiovascular system, the global effect is protection. While on mammary gland, 17-βE2 stimulated the proliferation of epithelial cells, but raloxifene did not. Since raloxifene occupied some of the estrogen-binding sites, in raloxifene plus 17-βE2 group, it inhibited the proliferation effect of 17-βE2. We are also interested in the effect of raloxifene in endometrium and will focus on it in our further studies.

Our studies showed that serum TC, TG and LDL cholesterol decreased in the treatment groups, as
SERM RALOXIFENE PLUS 17-BETA ESTRADIOL IN AORTA AND GLAND AND ERK

compared to the placebo group. Several studies have demonstrated that intensive lowering of lipid metabolism may retard progression of atherosclerosis. Considerable data are available that document an decrease in plasma lipoprotein(a) levels (26) and a reduction of LDL cholesterol following estrogen therapy (22). Studies have clearly established that estrogen decreases total plasma cholesterol and increases or maintains plasma triglyceride levels (10, 16, 19). The effects of raloxifene on serum lipids in the present study could explain the decrease in cardiovascular events observed in a subset of women with increased risk for cardiovascular events in the Multiple Outcomes of Raloxifene Evaluation (MORE) study (2).

Although part of the beneficial effects of SERM and estrogen is mediated by improving serum lipid, evidence that the major part of the actions of SERM and estrogen is exerted directly on the vascular wall has been growing steadily in recent years. Ross reported that the lesions of atherosclerosis result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall (23). In the present experiment, raloxifene, 17-βE2 or raloxifene plus 17-βE2 inhibited the formation of atheromatous plaque and the histological changes of aortic tunica intima. These effects played important roles in the anti-atherosclerotic actions.

Proliferation of VSMC is a key event of atherogenesis. Growth of VSMC is induced by various agonists such as growth factors, cytokines, and hormones. AngII causes cell growth. This experiment demonstrated that pretreatment with raloxifene (10⁻⁷ M–10⁻⁵ M) or 17-βE2 (10⁻¹⁰ M–10⁻⁶ M) for 24 hours inhibited AngII-induced proliferation in cultured rat VSMC. Consistent with these results, our previous studies proved that 17-βE2 inhibited the proliferation of VSMC stimulated by endothelin (29). The inhibition effect reached a peak at the concentration of 10⁻⁷ M (raloxifene) or 10⁻¹⁰ M (17-βE2) individually. How about raloxifene plus 17-βE2? Our experiments showed that raloxifene plus 17-βE2 inhibited the proliferation, consistent with our results in the in vivo atherosclerosis animal model.

Recently important new developments are occurring about the mechanisms of cardiovascular protection by SERM. Takahashi et al. have reported that raloxifene exerts an antiproliferative effect in VSMC treated with platelet-derived growth factor (PDGF), at least in part through inhibition of phosphorylation of retinoblastoma protein (28). Raloxifene-induced inhibition of VSMC growth is due to induction of apoptosis through a p38 cascade (21). MAPK (mitogen-activated protein kinase), including the ERK, c-Jun-NH2-terminal kinase (JNK) and p38MAPK, is a family of protein-serine/threonine kinases that are believed to function as integrators for mitogenic signals originating from several distinct classes of cell surface receptors (9). In VSMC, Ang II induces a rapid increase in expression of the growth-associated nuclear proto-oncogenes, c-fos, c-jun, and c-myc and stimulates tyrosine phosphorylation of multiple substrates, including MAPKs. The results of our present study indicated that 17-βE2, raloxifene plus 17-βE2 inhibited the ERK phosphorylation, but raloxifene did not. The effects of 17-βE2 and raloxifene plus 17-βE2 were attenuated by PD98059 (inhibitor of MAPK) or ICI182780 (inhibitor of ER). The result of 17-βE2 confirmed the previously reported effects that ERK were involved in the proliferation of VSMC (31). Our results showed that phosphorylation of ERK was not changed by raloxifene individually, which suggested that raloxifene might exert its effect through other signal transduction pathways. We demonstrated that raloxifene plus 17-βE2 inhibited the ERK phosphorylation. Since 17-βE2 inhibited the ERK phosphorylation while raloxifene might exert its effect through other signal transduction pathway, ERK cascade was involved in the globle effect of raloxifene plus 17-βE2.

In conclusion, this study suggested that SERM raloxifene plus 17-βE2 improved the lipid metabolism and relieved the aorta changes of female experimental atherosclerosis rabbits, which were partly by the inhibition of VSMC growth through ERK cascade. The hyperplasia of mammary gland epithelial cells could be significantly inhibited by raloxifene plus 17-βE2.

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