New Phenomenon

Raloxifene hydrochloride treatment leads to better outcomes than medroxyprogesterone acetate when paired with estrogen in ovariectomized cholesterol-fed rabbits

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The benefits of estrogen in cardiovascular system include a reduction in low-density lipoprotein cholesterol (LDL-C), decrease in LDL oxidation, and enhancement of vascular function [1]. Estrogen replacement therapy, however, has been linked to an increased risk of tissue-specific side effects including breast cancer and uterine cancer [2]. These issues have led to the development of hormone replacement therapy (HRT) which combines estrogen and progestin. Progestin can reverse endometrial hyperplasia induced by estrogen. The most commonly used progestin in HRT is medroxyprogesterone acetate (MPA), a synthetic progestin, although there is some evidence that the administration of MPA is not as beneficial as natural progesterone [3]. Findings from randomized placebo-controlled trials have demonstrated that the combination of estrogen and MPA does not confer cardiac protection and may increase the risk of coronary heart disease among healthy postmenopausal women, especially in the first year after initiation of hormone therapy. Furthermore, an increase in the risk of breast cancer was also found with this therapy [4]. Although the role of progestin remains poorly defined, it is possible that the coadministration of progestin could counteract the cardioprotective effects of estrogen [5].

In an effort to obtain the beneficial effects and reduce the risks associated with HRT, new replacement therapy formulations including selective estrogen receptor modulators (SERMs) have been developed. However, in a preclinical trial, treatment with bazedoxifene acetate (BZA), a new third-generation SERM, and conjugated equine estrogen (CEE) in combination did not show significant effects on plasma lipid profiles, and BZA treatment had no adverse effects on atherosclerosis but attenuated the atheroprotective effects of CEE in both the coronary and iliac arteries [6]. Raloxifene hydrochloride (RLX), a benzothiophene SERM, confers estrogen-like effects on lipids but anti-estrogenic effects on breast tissue and uterine endometrium. Our previous study had shown that the combination of RLX, aspirin, and estradiol valerate (E2) exhibits positive lipid, MCP-1 and atherosclerotic responses with minimal stimulation of breast and uterine tissue or aggregation of platelet in ovariectomized cholesterol-fed rabbits [7]. In this study, whether RLX could demonstrate an improved pharmacological profile compared with MPA was determined in the same rabbit model. All the treatments on experimental rabbits were approved by the Animal Care Committee of Shandong University and performed according to the Guidelines for the Use of Experimental Animals by the Ministry of Health, China.

Sixty healthy and sexually mature (age, 3 months; weight, 2.25 ± 0.20 kg) female New Zealand white rabbits (Agricultural Sciences Institute Products, Jinan, China) were used in this study. The rabbits were housed individually in standard cages at a room temperature of 20 ± 2°C and 12 h light/12 h dark cycle for 2 weeks. For the control group, 10 rabbits were sham-operated and received a 1.5% cholesterol diet for 12 weeks, but received no hormone treatment. Other 50 rabbits were bilaterally ovariectomized under general anesthesia (30 mg/kg sodium pentobarbital, intravenously), received a 1.5% cholesterol diet for 12 weeks, and then randomized into five groups (10 rabbits in each group): Veh group (vehicle: saline, 2% Tween 80 and 0.5% methylcellulose); E2 group (0.1 mg/kg/day E2); RLX group (10 mg/kg/day RLX); E2/RLX group (0.1 mg/kg/day E2 and 10 mg/kg/day RLX); and E2/MPA group (0.1 mg/kg/day E2 and 0.4 mg/kg/day MPA). Compounds were administered orally in a saline vehicle, and the doses of E2 [8], MPA [9], and RLX [10,11] were determined according to the previous reports. Then rabbits were fasted for 12 h and sacrificed.
Serum was harvested immediately for the measurement of lipids. The biochemical evaluation of serum lipids was carried out following the criteria established for the World Health Organization Lipid Reference Laboratories.

The aorta was freed from the adventitia, opened longitudinally and stained with Oil Red O solution. The percentage of aorta stained positively with Oil Red O was determined. Quantification was performed by capturing images of the aortas with a digital camera and analyzed using the computer-based quantitative color image analysis system IPP6.0 (Media Cybernetics, Bethesda, USA). The acquisition of images and analysis were performed in a blind fashion.

A 5 mm section of aorta was fixed in 10% buffered formaldehyde, embedded in paraffin, then cut into serial 5 μm thick sections, and stained immunohistochemically with RAM-11 antibody (Lab Vision and Neomarkers, Fremont, USA). The areas of macrophages were acquired by IPP6.0. The areas containing macrophages were digitally ‘painted’ and detected by the density of staining. The quantity of digitally painted areas was then calculated electronically as a percentage of the overall selected area of interest.

Breast tissue specimens were fixed in 10% neutral buffered formalin, routinely processed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. The percentage of the extent of gland branches and ducts in the mammary gland was determined using IPP6.0.

Uteri were excised and weighed after removal of associated fat and luminal fluids. Platelet-rich plasma (PRP) was separated from acid citrate dextrose-anticoagulated blood. Purity of PRP was validated by a Coulter counter (Qilu Hospital Hematology Laboratory, Jinan, China) with contamination of PRP was validated by a Coulter counter (Qilu Hospital rated from acid citrate dextrose-anticoagulated blood. Purity fat and luminal fluids. Platelet-rich plasma (PRP) was separated from acid citrate dextrose-anticoagulated blood. Purity of PRP was validated by a Coulter counter (Qilu Hospital Hematology Laboratory, Jinan, China) with contamination of acid citrate dextrose-anticoagulated blood.

Table 1. Serum lipid levels in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mM)</th>
<th>LDL-C (mM)</th>
<th>HDL-C (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.0 ± 4.21</td>
<td>17.1 ± 5.30</td>
<td>3.67 ± 0.65</td>
</tr>
<tr>
<td>Veh</td>
<td>30.1 ± 3.30</td>
<td>26.4 ± 5.03</td>
<td>2.38 ± 1.00</td>
</tr>
<tr>
<td>E2</td>
<td>23.5 ± 3.90**</td>
<td>16.7 ± 6.23**</td>
<td>3.24 ± 0.90*</td>
</tr>
<tr>
<td>RLX</td>
<td>24.7 ± 7.31**</td>
<td>20.1 ± 6.61*</td>
<td>3.02 ± 1.06*</td>
</tr>
<tr>
<td>E2/RLX</td>
<td>19.3 ± 2.13**</td>
<td>13.4 ± 3.76***</td>
<td>3.54 ± 0.70*</td>
</tr>
<tr>
<td>E2/MPA</td>
<td>21.5 ± 2.74**</td>
<td>17.2 ± 3.45*</td>
<td>3.11 ± 1.19*</td>
</tr>
</tbody>
</table>

TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

All values expressed as mean ± SEM, n = 10. *P < 0.05, **P < 0.01 vs Veh group. #P < 0.05 vs E2/MPA group.

As expected, a significant reduction (~20%) of serum LDL-C level was found in E2/RLX group compared with E2/MPA group, demonstrating the positive estrogen agonistic activity of RLX on lipid metabolism. Consistent with the reduction in serum LDL-C level, E2/RLX rabbits had ~20% fewer fatty streaks than E2/MPA rabbits. The fatty streaks in E2/RLX rabbits were less macrophage-rich than those in E2/MPA rabbits. Estrogen treatment significantly stimulated breast tissue compared with placebo, and concomitant use of MPA did not reverse the changes induced by estrogen. The stimulatory effects of estrogen, however, were significantly

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inhibited when RLX was added, suggesting that the pairing of RLX with estrogen is safer for breast tissue than estrogen and MPA. The stimulatory effects of unopposed estrogen on uterus have been well documented and RLX was found to have estrogen antagonist effect on the uterus. A previous study had demonstrated that RLX intervention can cause a decreased uterus weight compared with placebo controls in ovariectomized, cholesterol-fed rabbits [12].

In postmenopausal women receiving RLX (60 mg/day) in combination with placebo controls in ovariectomized, cholesterol-fed rabbits [12]. In postmenopausal women receiving RLX (60 mg/day) in combination with placebo controls in ovariectomized, cholesterol-fed rabbits [12]. In postmenopausal women receiving RLX (60 mg/day) in combination with placebo controls in ovariectomized, cholesterol-fed rabbits [12]. In postmenopausal women receiving RLX (60 mg/day) in combination with placebo controls in ovariectomized, cholesterol-fed rabbits [12]. In postmenopausal women receiving RLX (60 mg/day) in combination with placebo controls in ovariectomized, cholesterol-fed rabbits [12].

The increase in wet weight of the uterus in the ovariectomized rabbits was in the following order: E2 > E2/MPA > E2/RLX. Compared with placebo controls, estrogen treatment significantly increased the weight of uteri. The concomitant use of MPA reduced the increase, although not to the level of placebo controls. RLX in combination with estrogen reduced uteri weight to almost the same level as placebo controls. This result indicated that RLX might be more effective than MPA in rabbits in preventing the unwanted effects of estrogen on the uterus. Platelets contain both ERα and ERβ, but the effect of estrogen on the platelet aggregation is still controversial [14]. In the current study, significant stimulation in platelet aggregation was observed with E2 alone, E2 plus RLX, and E2 plus MPA, but not with RLX alone in the ovariectomized rabbits.

In summary, to the best of our knowledge, this is the first study to compare RLX with MPA when paired with estrogen. RLX was found to effectively abrogate the stimulatory effects of E2 on the breast and uterus, and positive effects on the lipid profile and aortic atherogenesis were observed in ovariectomized rabbits fed with a high cholesterol diet, which indicated that RLX might represent a promising therapeutic option for pairing with estrogen. However, in this research, we could not determine the mechanism involved, only the fact that RLX might be better than MPA was supported, and the histological analysis was not enough. Thus, additional research is needed to verify the results reported here. In this study, we found that RLX could not abrogate
the stimulatory effects of E₂ on platelet aggregation, thus continued efforts should be made to provide ideal HRT for postmenopausal women.

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References