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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 (E_2) 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 $20 \pm 2^{\circ}$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); E_2 group (0.1 mg/kg/day E_2); RLX group (10 mg/kg/day RLX); E_2/RLX group (0.1 mg/kg/day E_2 and 10 mg/kg/day RLX); and E_2/MPA group (0.1 mg/kg/day E_2 and 0.4 mg/kg/day MPA). Compounds were administered orally in a saline vehicle, and the doses of E_2 [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 $<0.1\%$. The concentration of PRP was adjusted to 5×10^5 platelets/μl. Platelet aggregation to 10 μM adenosine diphosphate was performed in PRP by a turbidimetric method using a whole-blood aggregometer in optical mode (Model No. 560-CA; Chrono-log, Havertown, USA).

Our results showed that dyslipidaemia was improved in E_2, E_2/MPA, E_2/RLX, and RLX groups compared with the Veh group (Table 1). When RLX was coadministered with E_2, an ~20% reduction in LDL-C level relative to the E_2/MPA group was observed. As shown in **Fig. 1A**, Oil Red O staining demonstrated that the size of fatty streaks in the aorta was significantly decreased in the E_2 group compared with the Veh group, and a similar result was observed in the RLX group. Although the difference was not significant, the reduction was somewhat less in the E_2/MPA group than in the E_2 group, whereas the E_2/RLX group showed the maximal reduction, which was ~20% greater than E_2/MPA treatment. As shown in **Fig. 1B**, the relative proportion of macrophage-stained areas to the fatty streak areas was also assessed and the macrophage-positive area decreased significantly in all E_2-treated rabbits compared with the Veh group. And the macrophage positive area was somewhat lower in the RLX group than in the Veh group. The maximal reduction was observed in the E_2/RLX group, which showed an ~15% reduction in the proportion of macrophage-stained areas relative to the E_2/MPA group. In ovariectomized rabbits, an estrogenic stimulatory response was found in breast tissue following E_2 treatment. As shown in **Fig. 1C**, the alveoli and ducts were dilated, and the extent of gland branches and ducts in the mammary glands increased significantly in the E_2 group compared with the Veh group. A similar response was found in the E_2/MPA group. When RLX was coadministered with E_2, breast tissue stimulation induced by E_2 was reduced to similar level as the Veh group and the extent of gland branches and ducts in the mammary glands decreased significantly compared with the E_2/MPA group. As shown in **Fig. 1D**, 12 weeks of E_2 treatment at 0.1 mg/kg/day also resulted in a significant increase in uterine wet weight compared with the Veh group. Concomitant use of MPA with E_2 reduced uterine weight significantly compared with E_2 alone, but the weight was still much higher than the Veh group. When RLX was co-administered with E_2, the increase in uterine weight induced by E_2 was reduced to the level of the Veh group, and was much lower than the E_2/MPA group. As shown in **Fig. 1E**, E_2 group demonstrated a significant increase in whole-blood platelet aggregation compared with the Veh group. Similar results were found in the E_2/RLX group and E_2/MPA group, but not in the RLX group.

As expected, a significant reduction (~20%) of serum LDL-C level was found in E_2/RLX group compared with E_2/MPA group, demonstrating the positive estrogen agonistic activity of RLX on lipid metabolism. Consistent with the reduction in serum LDL-C level, E_2/RLX rabbits had ~20% fewer fatty streaks than E_2/MPA rabbits. The fatty streaks in E_2/RLX rabbits were less macrophage-rich than those in E_2/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

Table 1. Serum lipid levels in different groups

Group	TC (mM)	LDL-C (mM)	HDL-C (mM)
Control	22.0 ± 4.21	17.1 ± 5.30	3.67 ± 0.65
Veh	30.1 ± 3.30	26.4 ± 5.03	2.38 ± 1.00
E_2	23.5 ± 3.90**	16.7 ± 6.23**	3.24 ± 0.90*
RLX	24.7 ± 7.31**	20.1 ± 6.61*	3.02 ± 1.06*
E_2/RLX	19.3 ± 2.13**	13.4 ± 3.76**$^{\#}$	3.54 ± 0.70*
E_2/MPA	21.5 ± 2.74**	17.2 ± 3.45*	3.11 ± 1.19*

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 E_2/MPA group.

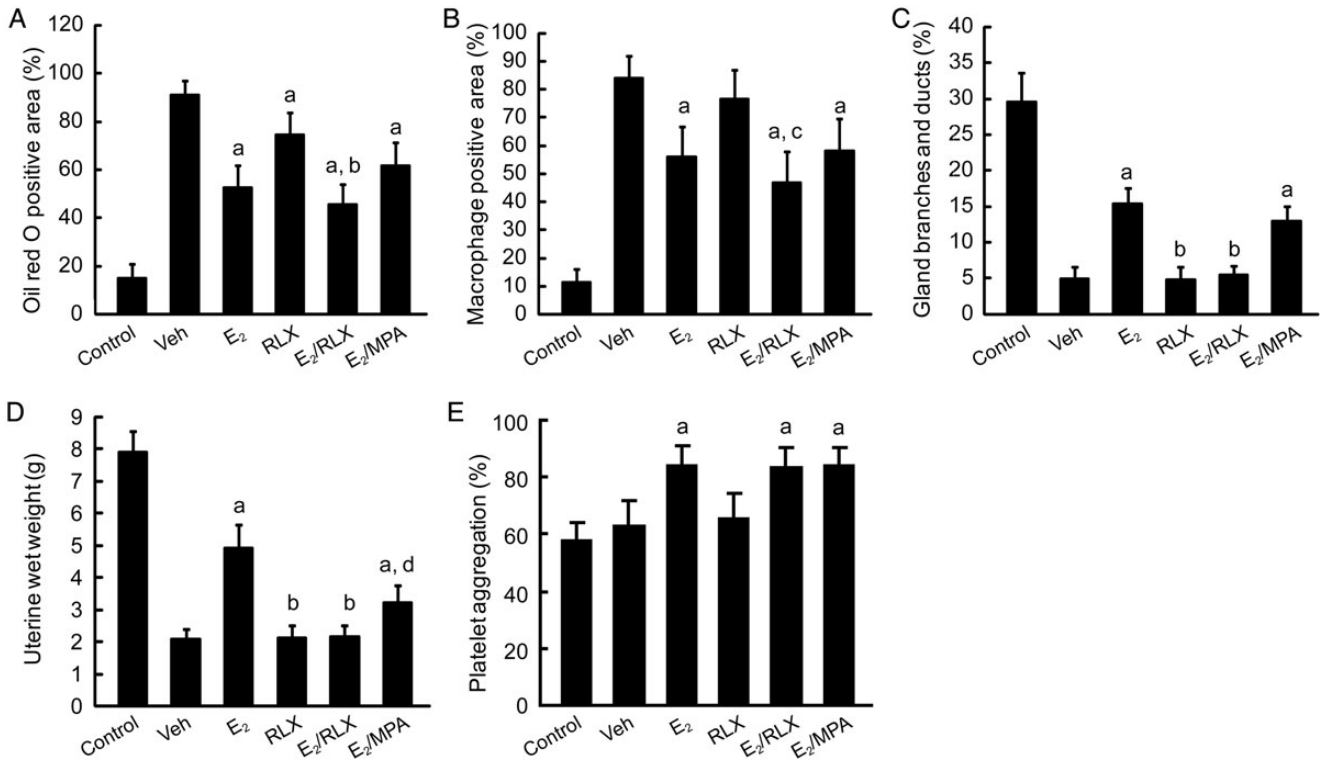

Figure 1. Effects of E_2/RLX on aortic fatty streaks, macrophage recruitment, breast, uterine, and platelet aggregation response (A) Effects of E_2/RLX on aortic fatty streaks. Following 12 weeks of treatment, lesions in the aorta (%) in response to E_2, E_2/MPA, and E_2/RLX were determined by Oil Red O staining. $^aP < 0.01$ vs. the Veh group; $^bP < 0.01$ vs. the E_2/MPA group. (B) Effects of E_2/RLX on aortic macrophage recruitment. Following 12 weeks of treatment, the macrophage positive area was determined by immunohistochemistry. $^aP < 0.01$ vs. the Veh group; $^cP < 0.05$ vs. the E_2/MPA group. (C) Breast response to E_2/RLX. The extent of ductal elongation and gland branches in the mammary glands was analyzed following treatment. $^aP < 0.01$ vs. the Veh group; $^bP < 0.01$ vs. the E_2/MPA group. (D) Uterine response to E_2/RLX. Uterine wet weight was determined following treatment. $^aP < 0.01$ vs. the Veh group; $^bP < 0.01$ vs. the E_2/MPA group; $^dP < 0.01$ vs. the E_2 group. (E) Platelet aggregation response to E_2/RLX. After 12 weeks of treatment, whole-blood platelet aggregation was determined. $^aP < 0.01$ vs. the Veh group.

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 17β-estradiol (1 mg/day), signs of endometrial stimulation were observed, indicating that further studies using different estrogen doses and preparations are needed before concomitant use of RLX with systemic estrogens can be recommended clinically [13].

The increase in wet weight of the uteri in the ovariectomized rabbits was in the following order: $E_2 > E_2$/MPA $> E_2$/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 E_2 alone, E_2 plus RLX, and E_2 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 E_2 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 E_2 on platelet aggregation, thus continued efforts should be made to provide ideal HRT for postmenopausal women.

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