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A comparative study of efficacy of tibolone and simvastatin on atherosclerosis in ovariectomized cholesterol-fed rabbits

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Abstract

Background: After menopause women are more susceptible to coronary heart disease due to increased risk of atherosclerosis. Tibolone (Livial) is an innovative synthetic steroid analogue for the treatment of postmenopausal climacteric symptoms including atherosclerosis, but the mechanisms of its effect are still unclear. The present study investigated the effect of tibolone and simvastatin on atherosclerosis and the expression of both estrogen receptor A (ERA) and LDL receptor (LDLR) mRNA in ovariectomized cholesterol-fed rabbits.
Methods: Fifty New Zealand white rabbits were included for the study. Of them, 40 underwent bilateral ovariectomy and the other 10 were sham-operated. The sham-operated group only received atherogenic diet (group SC) and the ovariectomized rabbits were divided into 4 groups of 10 each, with group N received normal diet, group C received atherogenic diet, group T received atherogenic diet and tibolone (2.5 mg/day) and group SI received atherogenic diet and simvastatin (20 mg/day). After 12 weeks of the treatments, the animals were euthanized and the extent of thoracic aortic atherosclerosis was measured morphologically and the level of ERA and LDLR mRNA in heart and liver was determined by real-time RT-PCR.
Results: The extent of atherosclerosis in the thoracic aorta was 0.75 ± 0.24 for group C, 0.56 ± 0.27 for group SC, almost 0 for group N, 0.10 ± 0.02 for group T and 0.09 ± 0.08 for group SI ($P < 0.01$; groups T versus C, T versus SC, SI versus C, SI versus SC). The relative copies of ERA at group C, SC, N, T and SI were 0.29, 0.53, 0.46, 0.85 and 0.30, respectively in heart and 0.32, 0.51, 0.49, 0.68 and 0.30, respectively in liver; the relative copies of LDLR at group C, SC, N, T and SI were 0.22, 0.24, 0.33, 0.27 and 0.23, respectively in heart and 0.68, 0.93, 1.52, 1.27 and 0.88, respectively in liver.
Conclusion: Both tibolone and simvastatin prevented the atherosclerosis in ovariectomized cholesterol-fed rabbits and this effect was associated with up-regulation of ERA and LDLR expression by tibolone but not by simvastatin.
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Keywords: Tibolone; Simvastatin; Atherosclerosis; Rabbit; Real-time RT-PCR; ERA; LDLR

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1. Introduction

Cardiovascular disease (CVD) is the most common cause of morbidity and mortality among postmenopausal women in the developed world [1,2]. The primary symptoms of CVD are heart attack and stroke, which are the clinical sequelae of a systemic vascular process known as atherosclerosis. Atherosclerosis affects large- and medium-sized arteries and the disease is characterized by deposition of fatty material in the inner lining of an artery and manifested in three stages known as early, developing and mature lesions. Early lesions are characterized by nodular areas of lipid deposition that have been termed "fatty streaks" morphologically. These represent lipid-filled macrophages and smooth muscle cells in focal areas of the vascular intima. Developing lesions are also called early plaques and represent the next stage beyond fatty streaks. Ultimately, lesions may progress to become complicated and advanced, and these are characterized by calcified fibrous areas of the artery with visible ulceration [3]. When fully advanced, these plaques restrict the flow of blood through the vessel and this often results in tissue ischaemia.

Because of the increased risk of atherosclerosis and CVD in women around menopause [4], it has been speculated that endogenous estrogen has an important cardiovascular-protective effect and many studies have demonstrated that estrogen replacement therapy in postmenopausal women can decrease the risk for CVD. Although many observational data suggest that estrogen has cardio-protective effect, its actual benefits and risks have not been fully evaluated. Recently, estradiol valerate and conjugated estrogen are commonly used in hormonal replacement therapy [5,6]. These agents increased the risk of endometrial hyperplasia and cancer as well as mammary cancer. The therapy of estrogen plus progesterone has higher vaginal bleeding rate. Furthermore, in nonhuman primates, continuous low-dose medroxyprogesterone acetate (MPA) substantially reduces estrogen's preventive effect on coronary atherosclerosis [7].

Tibolone is an innovative synthetic steroid analogue for the treatment of postmenopausal climacteric symptoms. It has a combination of estrogenic, progestogenic and mild androgenic activities by reacting with estrogen, gestagen and androgen receptors following oral administration. Several studies have shown that tibolone is effective in eliminating or significantly alleviating climacteric symptoms with a low incidence of side effects [8–10], and animal tests have shown that tibolone treatment decreased the extent of atherosclerosis in ovariectomized cholesterol-fed rabbits [16,17]. Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, is a cholesterol-lowering drug widely used in the treatment of atherosclerosis [11,12]. However, the mechanisms of effects of tibolone and simvastatin on atherosclerosis are still unclear. In mammals, low-density lipoprotein (LDL), a major cholesterol carrier, is taken into cells via low-density lipoprotein receptor (LDLR) on peripheral tissues to supply cholesterols to cells. Studies have shown that tibolone has positive effects on several lipid parameters such as serum lipoprotein (a) (Lp (a)) and triglycerides [13,14].

The present study replicated the atherosclerosis model with ovariectomized rabbits on atherosclerotic diet and investigated the influence of tibolone, in comparison with simvastatin, on the development of atherosclerosis by examining the extent of atherosclerotic plaque formation and by determining the level of expression of estrogen receptor (ER) and LDLR transcripts.

2. Materials and methods

2.1. Animals

Fifty healthy, 7–9 months old, female New Zealand white rabbits weighing around 4 kg were included in this study. The rabbits were housed at approved animal facilities in standard rabbit cages at room temperature and fed with standard commercial rabbit chow (LKK20, Keyu Experimental Animal Institute, Beijing, China) at 100 g per day with free access to water.

2.2. Experimental design

After 3 weeks of acclimatization, 40 rabbits underwent bilateral oophorectomy through the abdominal route under general anaesthesia and 10 rabbits underwent sham-operation. The rabbits were allowed 14 days to recover from the surgery and the ovariectomized rabbits were then randomly divided into 4 groups (C, N, T

and SI) of 10 rabbits each. The group N was administered with the standard commercial rabbit chow, group C received high cholesterol chow made of the standard rabbit chow and additives of cholesterol at 0.4%, coconut oil at 3.75% and earthnut oil at 3.75%, group T received high cholesterol chow plus tibolone (Oujianong Inc., Nangjing, China) which was mixed into the chow at 2.5 mg per day and group SI received high cholesterol chow plus simvastatin (Meke Inc., Beijing, China) at 20 mg per day. The groups SC, which underwent sham-operation, were given high cholesterol chow. The dosage of tibolone and simvastatin applied in the present study was calculated according to those in estrogen replacement used in human [9]. After 12 weeks of treatment, all rabbits were euthanized and tissues were harvested for the following analysis.

2.3. Aortic atherosclerosis

The thoracic aortas (including aortic arch) were resected, cut open longitudinally and any remaining blood was rinsed away with phosphate buffered saline (PBS). The aortas fixed in 10% formalin for 24 h. The aortas were then washed briefly in 70% alcohol, stained for 20 min in Herxheimer solution (500 ml of 70% ethanol, 500 ml of acetone and 5 g of Sudan IV) and destained in 80% ethanol for 20 min and tap water for 1 h [15]. The inner surface of the aorta segment was flattened and photographed and the images were processed with an image analysis program (Verily Color Pathologic Image Analysis System TD2000, Tiandibainian Inc., Beijing, China). The severity of aortic atherosclerosis was evaluated as the lesion area relative to the inner surface.

2.4. Real-time RT-PCR

To determine the expression of ERA and LDLR mRNA in response to oophorectomy and high cholesterol chow with or without tibolone or simvastatin, heart and liver tissue were collected, snap-frozen in liquid nitrogen and stored at −86 °C. The tissues were taken from 8–10 rabbits in each group. The hearts or livers of each group were mixed and homogenized. Total RNA was extracted from the tissues using RNA-Solv Reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNaseI (Invitrogen, Carlsbad, CA, USA). All the RNA samples were quantified by spectrophotometry and stored at −86 °C. Two micrograms of total RNA was reverse-transcribed into cDNA in a total volume of 40 μl using oligo (dT) primers and AMV reverse transcriptase (Takara, Kyoto, Japan). The expression level of the above genes was then measured by real-time quantitative PCR using DyNAmo SYBR Green qPCR kit (MJ Research Inc., Watertown, MA, USA) on a DNA Engine Opticon^{TM}2 fluorescence detection system (MJ Research Inc., Watertown, MA). The sequences of the PCR primers were 5′-CAGATCCAAGGGAACGAG-3′(forward) and 5′-TCTCCAGGTAGTAGGGCA-3′(reverse) for ERA with the amplification size of 362 bp, 5′-TCTACAGCGCACAGATGGAC-3′(forward) and 5′-CGGCAGGCACAGGTATTGG-3′(reverse) for LDLR with the amplification size of 698 bp and 5′-AGCGGGAAATCGTGCGTG-3′(forward) and 5′-CAGGGTACATGGTGGTGCC-3′(reverse) for PCR of a housekeeping gene B-actin with the amplification size of 328 bp. Each of the PCR reaction mixture (20 μl) contained 10 μl of DyNAmo SYBR Green qPCR mix, 5 μl of primers at 0.3 uM and 5 μl of cDNA template. The PCR program consisted of a pre-incubation with UNG enzyme at 50 °C for 2 min, then an initial denaturation at 95 °C for 10 min. This was followed by 36 cycles of 20 s at 94 °C for denaturation, 30 s at varying annealing temperatures depending on primers (48 °C for ERA, 52 °C for B-actin and 54 °C for LDLR), 30 s at 72 °C for extension for fluorescent data acquisition. A final extension of 10 min at 72 °C was performed to allow formation and agarose gel electrophoresis of fully duplexed DNA. Melt-curve analysis was carried out immediately after the amplification protocol by heating the reaction from 65 °C to 95 °C at 0.2 °C step and each step being held for 1 s to capture the fluoresce. All samples were measured in triplicate. The specificity of the PCR amplification of target genes was confirmed by sequencing. Briefly, PCR products were purified with E.Z.N.A Gel Extraction kit (Takara, Kyoto, Japan) and directly sequenced using DyeDeoxy terminators in an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA).

2.5. Quantification of mRNA expression

The quantification of expression was carried out by normalizing the target genes to an endogenous gene B-actin as controls using a relative standard curve method.

The amplified fragments of each gene were purified using E.Z.N.A Gel Extraction kit and cloned into pGEM-T Easy Vector for use as standards (Takara, Kyoto, Japan). A standard curve was obtained by plotting the cycle threshold (C_t) values over serial dilutions from 10^1 to 10^6 copies of the plasmid. For each experimental sample, the amount of mRNA of each target gene and B-actin was determined from the respective standard curves, and the quantity of each target gene was divided by that of B-actin to obtain a normalized value for each transcript.

2.6. Statistical analysis

All analyses were performed using Statistical Analysing System (SAS8.2). Values for the results are presented as mean ± S.E.M. Student's *t*-test was used for the group-by-group comparison. $P < 0.05$ were considered significantly in this study.

3. Results

During the experimental period, six rabbits in total died. Two rabbits in group C or SC died of coronary occlusion without treatment. After surgery, the other two rabbits in group T and group SI were not used. All the others passed the 12-week feeding period and their tissues were analyzed.

The extent of atherosclerosis in each experimental group was estimated by calculating the percentage of the plaque area over total aorta surface area measured. It was shown that the group N, which received standard rabbit chow, exhibited the least atherosclerosis among all groups, with fatty streaks almost being not detectable. In contrast, the group C, which received high cholesterol rabbit chow, showed the most severe atherosclerosis with the plaque area being 0.75 ± 0.24 and the group SC, which underwent sham ovariectomy and received high cholesterol rabbit chow, showed less atherosclerosis (0.56 ± 0.27) than that in group C, although the reduction was not statistically significant ($P > 0.05$) (Table 1). When tibolone or simvastatin was added to the high cholesterol rabbit chow, the degree of atherosclerosis was reduced significantly by 87% or 88%, respectively, compared to the controls (all $P < 0.01$).

The amplified ERA, LDLR and B-actin gene products by real-time RT-PCR were specific as confirmed by electrophoresis on 1.2% agarose gel (Fig. 1A, B and C) and direct sequencing (data not shown). Using relative standard curve method as described above, the relative copies of ERA at groups C, SC, N, T and SI were 0.29, 0.53, 0.46, 0.85 and 0.30, respectively in heart and 0.32, 0.51, 0.49, 0.68 and 0.30, respectively in liver; the relative copies of LDLR at groups C, SC, N, T and SI were 0.22, 0.24, 0.33, 0.27 and 0.23, respectively in heart and 0.68, 0.93, 1.52, 1.27 and 0.88, respectively in liver (Fig. 2A and B). Whereas the copies of ERA in both the heart and liver were not significantly different among groups N, C, SC and SI, the expression level of ERA in both heart and liver from group T were significantly higher than those from groups C and SI (a, b; c, d; $P < 0.05$). However, it was noted that the copies of LDLR in heart were similar to each other among all groups, but the copies of LDLR in liver were higher from groups N and T than those from groups C, SC and SI (a, b; $P < 0.05$). Simvastatin had no remarkable effect on the expression of both ERA and LDLR compared with group C.

4. Discussion

The present experiment examined the extent of formation of the atherosclerotic plaque in ovariectomized

Table 1
The atherosclerotic plaque area relative to the inner surface

	Treatment groups				
	N	SC	C	T	SI
Atherosclerotic plaque area (M ± S.E.M.)	0	0.56 ± 0.27	0.75 ± 0.24	0.10 ± 0.02	0.09 ± 0.08
n	10	8	8	9	9

N: ovariectomized and on standard chow; SC: sham-operated and on high cholesterol chow; C: ovariectomized and on high cholesterol; T: ovariectomized and on high cholesterol plus tibolone; SI: ovariectomized and on high cholesterol plus simvastatin; *n*: sample number.

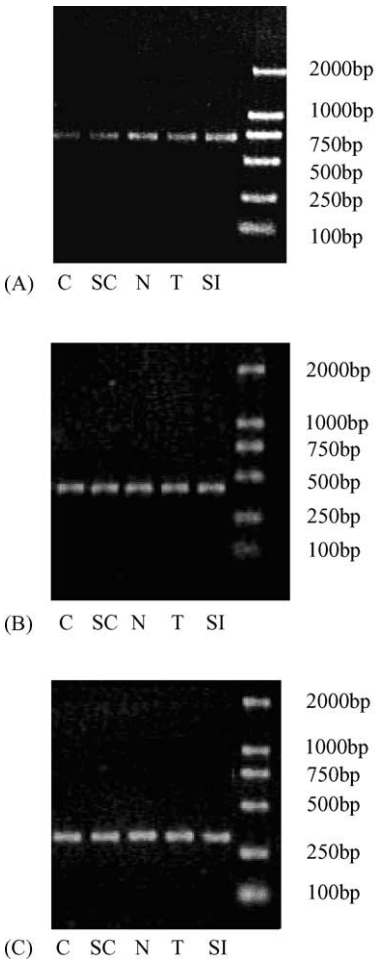

Fig. 1. RT-PCR products for liver of LDLR mRNA (A, product size 698 bp), ERA mRNA (B, product size 362 bp) and B-actin mRNA (C, product size 328 bp) shown on 1.2% agarose gel. (Marker: DL 2000).

rabbits on high cholesterol diet with or without the tibolone in order to evaluate the effect of tibolone on atherosclerosis. The high cholesterol diet induced apparent formation of atherosclerotic plaque in both ovariectomized and sham-operated rabbits in 12 weeks. Tibolone significantly decreased the thoracic aorta atherosclerotic plaque in ovariectomized rabbits fed with high cholesterol rabbit chow. Simvastatin showed similar effect to that of tibolone. These results agree with previous reports, suggesting that tibolone plays an important role in prevention of experimental primary CVD [16,17]. The extent of atherosclerotic plaque in

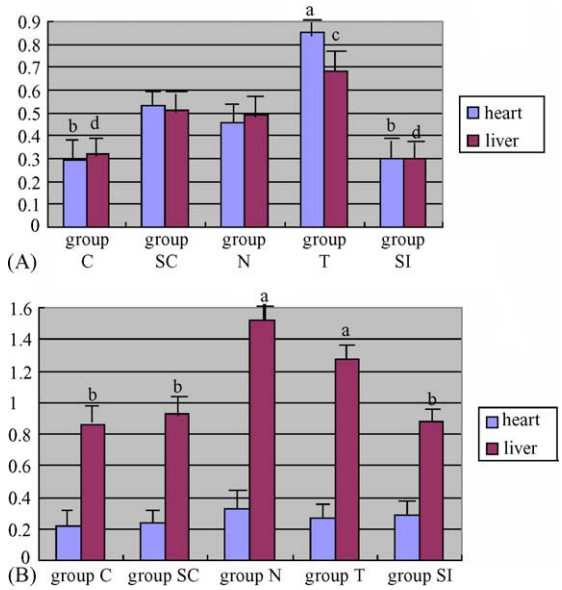

Fig. 2. The normalized copies of estrogen receptor A mRNA (A: a, b: $P < 0.05$; c, d: $P < 0.05$) and LDLR mRNA (B: a, b: $P < 0.05$) in heart and liver.

sham-operated rabbits on high cholesterol diet was less than that in ovariectomized rabbits on the same diet. This finding indicates that physiologically functioning ovary has protective effect against atherosclerosis.

Previous studies have shown that the preventive effect of estrogen against atherosclerosis occurs days after initiation of estrogen treatment [18,19]. This activity of estrogen mainly results from binding of the hormone to intra-cellular ER, but the mechanisms of estrogen-modulated cardiac pathophysiology are still poorly understood. It has been shown that the protective effect of estrogen on CVD is attributed mainly to the fact that estrogens reduce serum TC and LDL-C levels and increase HDL-C levels, thus improve the HDL-C:LDL-C ratio [20]. In the present study, blood was collected at 5, 9 and 12 weeks from ear artery and at 17 weeks by cardiac puncture after 12 h fast. The serum LDL-C and TC of administer group (groups T and SI) were significantly lower than that of groups SC and C; the results were similar to others [8]. Data of lipid and hormone assays were not shown in this paper.

The present study mainly investigated the expression of ERA and LDLR transcripts by real-time

RT-PCR to elucidate the mechanism of tibolone against atherosclerosis. It was found that the expression of ERA in heart and liver from ovariectomized rabbits on high-fat diet decreased by about 35% when compared with the group N. Tibolone increased the expression of ER in heart and liver from ovariectomized rabbits with high-fat diet by 100–200% compared with the group N. The inhibitory effect of tibolone on atherosclerosis was, therefore, associated with the enhanced expression of ER. The LDLR is a target of multiple signaling pathways, one of which is a sterol-sensitive pathway. In vitro and in vivo studies have shown that estradiol stimulates expression of the LDLR gene. Estradiol- and tamoxifen-induced activation of LDLR transcription is selectively mediated through ERA that can activate transcription of the LDLR promoter through Sp1 interaction [21]. The present results showed that the expression of LDLR in heart and liver from ovariectomized and atherogenic rabbits decreased by 30–40% compared to that from the control group and tibolone improved the LDLR expression by 30–50% in liver. Thus, the effect of tibolone on atherosclerosis involved up-regulation of both ERA and LDLR expression, a mechanism consistent with above discussion that estradiol-induced activation of LDLR transcription is mediated through ERA [21]. With the enhancement of LDLR, the clearance of plasma LDL-C was accelerated [18,20]. Statin, a lipid-lowering pharmaceutical, has anti-atherosclerosis effect by slightly increasing HDL (5–15%) and highly lowering LDL level (18–55%) and TC level (20–40%) of plasma [20]. However, simvastatin, although showing similar effect on formation of atherosclerotic plaque to tibolone, had no effect on the expression of ERA and LDLR, suggesting that the protective effect of simvastatin to the diet-induced atherosclerosis appeared to be unrelated to regulation of expression of ER and LDLR.

In conclusion, the present experiments demonstrated that the protective effect of tibolone on atherosclerosis in ovariectomized, cholesterol-fed rabbits is associated with its stimulation on expression of ER and LDLR.

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