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Effect of isoflavone on balloon catheter-induced neointimal hyperplasia in ovariectomized rabbit carotid artery

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Running title: isoflavone and carotid artery

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Abstract

Background: This study was designed to investigate the effects of phytoestrogen isoflavone on balloon catheter-induced hyperplasia of carotid artery.

Methods: Forty-eight female New Zealand rabbits were randomly divided into 4 groups: control (balloon-induced carotid artery injury only); ovariectomy control (ovariectomy and carotid artery injury), estrogen (ovariectomy, carotid artery injury and nilestriol, 5 mg/kg daily for 28 days), and isoflavone (ovariectomy, carotid artery injury and isoflavone 120 mg/kg daily for 28 days). The arterial wall thickness was assessed by colored ultrasonography, and the estrogen-α and estrogen-β receptors in the abdominal aorta were measured by western blotting.

Results: The medial layer thickness in the isoflavone group was less than in the ovariectomy control group (0.28±0.03 vs 0.35±0.04 mm, $p < 0.01$), and the intimal/medial layer (I/M) ratio is the isoflavone group was also less than in the ovariectomy control group (16.85±3.79 vs 48.94±8.92, $p < 0.01$). There was no statistically significant difference in the medial layer thickness or I/M ratio between the isoflavone and the estrogen groups. The optical density of the estrogen-α receptors in the isoflavone group (0.317±0.002) was less than in the estrogen (0.633±0.002) or ovariectomy control group (0.590±0.001, $p < 0.01$). The optical density of the estrogen-β receptors in the isoflavone group (1.350±0.002) and the ovariectomy control group (1.2033±0.002) was less than in the estrogen group (1.7699±0.003, $p < 0.01$).

Conclusions: Isoflavone therapy in the ovariectomized rabbit model attenuated balloon catheter-induced intimal and medial layer hyperplasia in the carotid arteries. Down-regulation of the estrogen-α receptors may be involved in the hyperplasia-preventative effect.

Key words: isoflavone; hyperplasia; estrogen receptors; rabbits.

Introduction

Soybean isoflavone, which mainly contains two chemical compounds of genistein and daidzein, has been shown to possess a number of cardioprotective effects (1). High soy consumption is associated with a lower risk of coronary artery disease, which has been attributed to a reduction in LDL cholesterol, an inhibition of pro-inflammatory cytokines and cell adhesion proteins, and an increase in nitric oxide production (1). In post-menopausal women, consuming an isoflavone-enriched low-fat meal acutely increased endothelium-dependent vasodilation (2). In patients with a history of coronary artery disease, stroke or diabetes, a greater isoflavone intake was associated with better vascular endothelial function and lower carotid atherosclerotic burden (3). Dietary supplementation of an isoflavone metabolite, trans-tetrahydrodaidzein, was associated with a reduced arterial stiffness in overweight men and postmenopausal women (4). Several *in vitro* and *in vivo* experimental studies showed that isoflavone leads to dilatation in coronary arteries (5-7), aorta (8), and carotid or cerebral arteries (9, 10). However, a recent study in ovariectomized rats showed that although high soy diets increased expression of endothelial nitric oxide synthase mRNA in the brain and cerebrovasculature, the diet had no effect on middle cerebral artery structure or vascular responses to acetylcholine, serotonin, or phenylephrine (11).

The effect of isoflavone on the proliferation of endothelium and neointimal hyperplasia is not well understood. An earlier study in rabbits showed that isoflavone trans-tetrahydrodaidzein reduced neointimal smooth muscle cell proliferation after catheter-induced injuries to the carotid arteries (11). In mice, an isoflavone known as dihydrodaidzein suppressed the neointimal proliferation in the iliac arteries (12). The molecular mechanisms

of isoflavone's action on intimal proliferation are largely unknown, and it is unclear if the effect of isoflavone on intimal proliferation is also present under post-menopausal conditions. The purpose of this study is to investigate the effect of isoflavone on the intimal hyperplasia in an ovariectomized rabbit model, and to assess the role of estrogen in the isoflavone-induced vascular protection.

Materials and methods

Animal model

This study was approved by the institution review board of Liaocheng People's Hospital. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health. Forty-eight healthy New Zealand female rabbits (weight 2.5 - 2.8 kg, age 4-6 months) were divided into control (n=12), ovariectomy control (n=12), estrodiol (n=12) and isoflavone group (n=12). At day 0, three groups of animals had ovariectomy procedures. At day 14, the three groups of animals were re-anaesthetised for carotid injury. An additional 'control' group entered the study design on day 14, and these animals also had the carotid procedure. Then, all four groups proceeded for 28 days (with two of the groups receiving daily drug treatments: isoflavone or estrogen) commenced on the day of carotid injury procedure. At day 42, all animals were killed and tissue removed for measurements.

In the control group, no ovariectomy was performed, balloon-catheter-induced injuries were created in the right carotid artery but no drug therapies were given. In the ovariectomy control group, right carotid artery injuries were created and bilateral ovariectomy was

performed, but no drug therapies were prescribed. Carotid injuries and ovariectomy were conducted in both estrodiol (nilestriol, 5 mg/kg daily for up to 28 days via a gastric tube, Hualian Pharmaceuticals, Shanghai, China) and isoflavone (120mg/kg daily for up to 28 days via a gastric tube, Zhongxin Biotech Co., Shanxi, China) groups.

For the ovariectomy control, estradiol and isoflavone groups, ovariectomy was performed under general anesthesia (ketamine, 20mg/kg, iv). A midline incision was made in the lower abdomen of the animals, bilateral ovaries were identified and removed. The incision was closed by layers. Following the surgery, penicillin (800 000 units) was intramuscularly injected once daily for three days to prevention infection.

For all animals, under general anesthesia (ketamine, 20mg/kg, iv), a deflated 3 French Fogarty balloon catheter (Cordis, 2.5mm×8mm) was inserted into the right carotid artery. The balloon was inflated at 6 ATM and passed 3 times along a 3 cm length of the artery under the guidance of a colored Doppler ultrasonography (GE LOGIQ 9, probe frequency 10MHz). These manipulations created injuries to the arterial walls and the endothelium (13). The balloon catheter was then withdrawn and the arterial incision was closed with small nylon sutures. With ovariectomy control, estradiol and isoflavone groups, the carotid procedures were performed two weeks after the ovariectomy.

Colored Doppler ultrasonography was performed on day 28 following the balloon-induced carotid injuries to measure the thickness of the intima and the medial areas of the middle part of the injured segment. The diameter of the right carotid lumen was also measured from the middle part of the injured segment. The diameter of the non-injured left carotid artery lumen was also measured for comparison.

On day 28 of the experiment, all the animals were sacrificed with air injected into the ear veins. The right (injured) carotid arteries and the contralateral non-injured arteries were removed and washed in icy saline. Each artery was cut into two segments. The proximal segment was divided into three 5 mm pieces, fixed with 4% paraformaldehyde and embedded in paraffin. The distal segment was immediately frozen in liquid nitrogen and stored at −80°C. In addition, a 2 cm long abdominal aorta was harvested and stored at −80°C for Western blotting analysis of estrogen receptors.

Histological analysis of lesions

Paraffin-embedded arteries were cross-sectioned into 3 μm slices and were then stained with hematoxylin and eosin (H&E). Sections were counter-stained with hematoxylin. Digital micrographs of sections were captured using Olympus DP70 microscope. The lumen and internal elastic lamina (IEL) and the external elastic lamina (EEL) were identified, using the previously reported methods (13). The intimal and medial areas were defined as IEL area minus luminal area and EEL area minus IEL area, respectively (13). The ratio of intimal and medial area (I/M ratio) was calculated.

Western blotting for estrogen receptor-α (ERα) and ERβ analysis

The following primary antibodies were used: polyclonal mouse anti-rabbit ERα and ERβ (Santa Cruz), and goat anti-rabbit IgG (Santa Cruz). Abdominal aortic tissues were prepared by homogenization in PE buffer (10mM Hepes；1.5mM MgCl2；10mM KCl; 0.5mM DTT; 0.05％NP-40, pH7.9). After sonication and centrifugation, supernatants were collected and

dissolved in a buffer (5mM Hepes, 1.5mM MgCl2； 0.2mM EDTA, 0.5mM DTT, 26％ Glycerol, pH7.9). After centrifugation, protein concentrations were determined by the Bio-Rad method. Supernatants were stored at −80°C until analysis. The samples were diluted in SDS sample buffer and denatured before loading on a SDS-polyacrylamide gel (8% Tris-glycine; NOVEX, San Diego, CA). One hundred micrograms of total protein were loaded into each lane. Anti-rabbit polycolonal ERα and ERβ antibodies (1:300) were added before incubation at 4°C for 24 hours. The samples were washed three times with TBST, before HRP-labelled goat anti-rabbit IgG (1:20000) were added. Autoradiograms were scanned and the bands corresponding to ER protein were analyzed by densitometry. Quantitative analyses were performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

SPSS v13.0 was used for the statistical analysis. Quantitative variables are expressed as mean ± standard deviation (SD). Comparisons of medial wall thickness, the I/M ratio and optical density of ERα and ERβ protein expression among groups were performed by one-way ANOVA. *P* value < 0.05 was considered statistically significant.

Results

Colored Doppler ultrasound examination

All animals survived the right carotid artery or ovariectomy procedures and completed the 28-day study. Colored Doppler ultrasound examination of the injured right carotid artery 28 days after the balloon-induced injury showed thickening in the intimal and medial layers,

with various degrees of lumen stenosis in the control and ovariectomy control groups. The mean values of the medial layer thickness in the four groups are shown in Table 1. The mean medial layer thickness of the injured carotid arteries in the estrogen and isoflavone groups were less than in the control and ovariectomy control group (p <0.01).

There was no stenosis in the contralateral non-injured carotid artery in the four groups animals, and the mean medial layer thickness of these non-injured carotid arteries in the control, ovariectomy control, estrogen and isoflavone group was 0.25 ± 0.03, 0.24 ± 0.04, 0.27 ± 0.06, and 0.25 ± 0.07 mm, respectively (p >0.05).

Histological analysis

Under magnification (×200), the lumen of the contralateral non-injured left carotid artery was patent in all animals. There was a layer of endothelium attached to the intact elastic layer. There was intimal thickening in the injured right carotid arteries with abundant smooth muscle cells and frequent ruptures of the internal elastic layer (Fig 1A and 1B). There was also a thickening in the medial layer (Fig 1A to 1D). The I/M ratios in the estrogen and isoflavone groups were lower than in the control and ovariectomy control groups (p <0.01, Table 1). However, there was no statistically significant difference in the I/M ratio between the estrogen and isoflavone group, or between the ovariectomy control and the control group (p >0.05).

Western blotting

Western blotting results are shown in Fig 2 and Table 3. The optic density of ERα protein expression in the isoflavone group was lower than in the estrogen or ovariectomy control or the control group (p <0.01). The *ERβ* expression in the isoflavone and in the ovariectomy control group was also lower than in the estrogen group (p <0.01).

Discussion

In this study the effect of isoflavone on balloon injury-induced vascular hyperplasia was assessed from two different aspects in an ovariectomized rabbit model. First, the lumen and the wall thickness of the injured carotid artery were measured with coloured Doppler ultrasound. Four weeks after isoflavone treatment, the thickness of the medial layer in the injured carotid artery was 20 ± 4% less than in the ovariectomy control group, indicating that isoflavone suppressed injury-induced medial layer hyperplasia. Histological analysis showed that the intimal/medial ratio of the injured carotid artery in the isoflavone group was lower than in the ovariectomy control, confirming the above ultrasonographic findings. Furthermore, there was no statistically significant difference in the medial layer thickness and the intimal/medial ratio between the isoflavone and estrogen groups, suggesting this phytoestrogen may have similar effect to physiological estrogens in preventing injury-induced vascular remodelling in ovariectomized rabbits.

Although the results of our study are consistent with a previous report where isoflavone was found to suppress neointimal proliferation in the carotid arteries (11), there are some major differences. In the previous report, an isoflavone known as trans-tetrahydrodaidzein (trans-THD) only reduced the neointimal smooth muscle cell proliferation (11). It did not

affect medial cell proliferation (11). The isoflavone used in the present study contains both daidsein and genistein. After 28 days of treatment with isoflavone, both intimal and medial proliferation was suppressed. Whether these differences in the vascular responses are due to the differences in animal models (ovariectmized rabbits in the present study) or the types of isoflavones, or both, remains unclear.

A previous study on rat carotid artery showed that estrogen acts on the estrogen receptors on the vascular smooth muscle cells to elicit vascular dilatation (14). There has been a paucity of data on the effect of isoflavone therapy on estrogen receptor expression. In the present study, after 28 days treatment with isoflavone, there was a reduced expression of estrogen-α receptors in the abdominal aorta. The mean optimal density of estrogen-α receptors of the isoflavone-treated animals was less than in the ovariectomy control or the estrogen-treated animals. In addition, there was no statistically significant difference in the mean optical density of estrogen-α receptors between the ovariectomy control and the estrogen group. These results suggest isoflavone has a down-regulating effect on the estrogen-α receptors in the vascular walls. Furthermore, the optical density of estrogen-β receptors in the isoflavone group was lower than in the estrogen group, and there was no statistically significant difference in the estrogen-β receptors between the isoflavone and ovariectomy control group. These results indicate that isoflavone had little effect on the estrogen-β receptors, whereas estrogen may have an up-regulating effect on these receptors in the vascular walls.

Although this study showed that isoflavone has a down-regulating effect on estrogen-α receptors in the abdominal aorta, it is unclear as to what role this down-regulation has played

in the preventative effect of isoflavone on the injury-induced hyperplasia of the carotid artery. Estrogen, which up-regulated estrogen-β receptors in this rabbit model, had no significant effect on the estrogen-α receptors and yet the medial layer thickness and I/M ratio of the injured carotid arteries were similar between the estrogen and isoflavone groups. It is possible that isoflavone exerted its hyperplasia-preventative effect through down-regulating estrogen-α receptors, and estrogen prevented hyperplasia through its up-regulating effect of estrogen-β receptors. However, this hypothesis needs to be further investigated by measuring the estrogen receptors in the injured and non-injured carotid arteries as well as in other large arteries such as the abdominal aorta.

Conclusions

This study on an ovariectomized rabbit model demonstrated that soybean isoflavone prevented the intimal and medial layer hyperplasia induced by balloon catheter injuries in the carotid arteries. Isoflavone therapy was also associated with a reduced expression of estrogen-α receptors in the large arteries such as abdominal aorta. Further studies are required to clarify the role of estrogen-α receptor down-regulation in the hyperplasia-preventative effect following isoflavone therapy.

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Table 1. Medial layer thickness and I/M ratio of the injured right carotid artery 28 days after the vascular injury

Groups	***Medial thickness (mm)***	***I/M ratio***
Control (n=12)	0.36±0.04	46.56±5.64
Ovariectomy control (n=12)	0.35±0.04	48.94±8.92
Estrogen (n=12)	0.28±0.03*	16.96±3.84*
Isoflavone (n=12)	0.28±0.03*	16.85±3.79*

I/M: intimal and medial layer (I/M) ratio. *p <0.01 compared with control and ovariectomy group.

Table 2. Optical density of ERα and ERβ protein expression 28 days after the vascular injury

Groups	*ERα*	*ERβ*
Control (n=12)	0.526±0.001	1.410±0.004
Ovariectomy control (n=12)	0.590±0.001	1.2033±0.002**
Estrogen (n=12)	0.633±0.002	1.7699±0.003
Isoflavone (n=12)	0.317±0.002*	1.350±0.002**

*p <0.01 compared with estrogen and control group. **p <0.01 compared with estrogen group.

Figure legends

Fig 1. Hematoxylin and eosin staining of the injured right carotid artery from ovariectomy, control, isoflavone, and estrogen group, 28 days after the balloon-induced vascular injury. A: control; B: ovariectomy control; C: estrogen; D: isoflavone. Significant intimal thickening was observed in A and B (arrows). Arrows indicate intima and arrow heads indicate medial layer of the carotid artery.

Figure 2. Western blotting of estrogen receptors α and β. Band 1: ovariectomy control; band 2: isoflavone; band 3: control; and band 4: estrogen.