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Dietary phytoestrogens inhibit experimental aneurysm formation in male mice

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ABSTRACT

Background: The purpose of these experiments was to test the hypothesis that dietary phytoestrogens would diminish experimental aortic aneurysm formation.

Materials and methods: Six-wk-old C57BL/6 mice were divided into groups, fed either a diet with minimal phytoestrogen content or a regular commercial rodent diet with high phytoestrogen content for 2 wk. At the age of 8 wk, aortic aneurysms were induced by infusing the isolated infrarenal abdominal aorta with 0.4% elastase for 5 min. Mice were recovered and the diameter of the infused aorta was measured at postoperative days 3, 7, and 14. Abdominal aorta samples were collected for histology, cytokine array, and gelatin zymography after aortic diameter measurement. Blood samples were also collected to determine serum phytoestrogens and estradiol levels. Multiple-group comparisons were done using an analysis of variance with *post hoc* Tukey tests.

Results: Compared with mice on a minimal phytoestrogen diet, mice on a regular rodent diet had higher levels of serum phytoestrogens (male, 1138 ± 846 ng/dL; female, 310 ± 295 ng/dL). These serum phytoestrogen levels were also much higher than their own endogenous estradiol levels (109-fold higher for males and 35.5-fold higher for females). Although aortic diameters of female mice were unaffected by the phytoestrogen concentration in the diets, male mice on the regular rodent diet (M+ group) developed smaller aortic aneurysms than male mice on the minimal phytoestrogen diet (M− group) on postoperative day 14 (M+ 54.8 ± 8.8% *versus* M− 109.3 ± 37.6%; $P < 0.001$). During aneurysm development (postoperative days 3 and 7), there were fewer neutrophils, macrophages, and lymphocytes in the aorta from the M+ group than from the M− group. Concentrations of multiple proinflammatory cytokines (matrix metalloproteinases [MMPs]; interleukin 1β [IL-1β]; IL-6; IL-17; IL-23; monocyte chemoattractant protein-1; regulated on activation, normal T cell expressed and secreted; interferon γ; and tumor necrosis factor α) from aortas of the M+ group were also lower than those from the aortas of the M− group. Zymography also demonstrated that the M+ group had lower levels of

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aortic MMP-9s than the M− group on postoperative day 14 ($P < 0.001$ for pro-MMP-9, $P < 0.001$ for active MMP-9).

Conclusions: These results suggest that dietary phytoestrogens inhibit experimental aortic aneurysm formation in male mice *via* a reduction of the inflammatory response in the aorta wall. The protective effect of dietary phytoestrogens on aneurysm formation warrants further investigation.

1. Introduction

Abdominal aortic aneurysms (AAAs) are a gender-related disease with a prevalence of a male to female ratio of 4:1. Estrogens play a protective role in the development of AAAs [1–3]. Phytoestrogens are plant-derived chemicals that are strikingly similar to estrogens both in structure and function. Therefore, the potential benefits and risks of phytoestrogen exposure have already attracted much attention [4–9]. Major sources of phytoestrogens include soybeans, alfalfa, and flaxseed. Phytoestrogens are selective estrogen receptor (ER) modulators and have anti-inflammatory, antioxidant, and antiproliferative properties [10–14]. Animal experiments have demonstrated that phytoestrogens can reduce plasma cholesterol and attenuate atherosclerosis [15–17]. However, little is known regarding the effects of phytoestrogens on aortic aneurysm formation. Therefore, we hypothesized that dietary supplementation with phytoestrogens might reduce the inflammation in the aortic wall and thus inhibit aneurysm formation in an experimental model.

2. Materials and methods

2.1. Experimental design

Experiment 1 (gender-based study): 32 ($n = 16$ males and 16 females) 6-wk-old wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were divided into four groups of eight mice based on dietary phytoestrogen exposure to determine the influence of phytoestrogen content on aortic aneurysm formation. Thus, four groups were evaluated: (1) male mice fed a diet with minimal phytoestrogen (M−), (2) male mice fed a regular diet (M+), (3) female mice fed a diet with minimal phytoestrogen (F−), and (4) female mice fed a regular diet (F+). The isoflavone content, one of the major classes of phytoestrogens, ranged from non-detectable to 20 mg/kg for the minimal phytoestrogen diet (2016 Teklad Global 16% Protein Rodent Diet, Madison, WI), whereas the regular diet (7012 Teklad LM485 Mouse/Rat Diet, Madison, WI) had between 300 and 500 mg/kg. The other ingredients in the both diets were similar (see www.harlan.com). Both rodent diets were commercially available and obtained on April 2011. Two weeks after mice were placed on the diets, AAAs were induced surgically [18,19].

Briefly, infrarenal abdominal aorta was isolated and infused *in situ* with porcine pancreatic elastase (0.4 U/mL; Sigma, St. Louis, MO) for 5 min at a pressure of 100 mm Hg. Elastase solution was evacuated and the mice were allowed to recover. Mice abdominal aortic diameters ($n = 8$/group) were measured immediately after infusion to ensure similar dilation. On postoperative day 14, the infrarenal abdominal aorta was dissected and the maximal aortic diameter was measured using video microscopy with NIS-Elements D.3.10 software attached to the microscope (Nikon SMX-800, Melville, NY). Aortic dilation was determined using the formula (maximal aortic diameter − internal control diameter)/maximal aortic diameter × 100%. The internal control diameter was the diameter of un-infused infrarenal aorta just above the infused section. A dilation of 50% or more was considered to be positive for AAA formation. All measurements were performed when the animal was alive. Blood samples were collected immediately after the measurement of the aorta diameter. Aorta samples were harvested for protein analysis ($n = 5$/group) and histologic studies ($n = 3$/group). Serum samples were used to determine phytoestrogens and estradiol levels.

Experiment 2 (time course study): 6-wk-old wild-type C57BL/6 male mice ($n = 24$) were divided into two groups based on diet. As mentioned previously, one group was fed with the minimal phytoestrogen 2016 diet, whereas the other group was maintained on the phytoestrogen-rich 7012 diet. At the age of 8 wk, all mice underwent elastase infusion. To evaluate AAA formation at earlier time points, six mice in each group were evaluated at postoperative days 3 and 7. After aortic diameters were measured ($n = 6$/group), samples were collected for histology ($n = 3$/group) and protein analysis ($n = 3$/group).

All experiments were conducted in accordance with the standards approved by the Animal Care and Use Committee of University of Virginia (IACUC #3848).

2.2. Mass spectrometry

Mass spectrometry was performed on serum samples to identify and quantify four common phytoestrogens in serum. Four standards were used in the test (Sigma, St Louis, MO; purity: daidzein ≥97%, genistein ≥98%, equol ≥99%, and coumestrol ≥95%). The test used a *liquid chromatography–mass spectrometry* system, which consisted of a Thermo Electron TSQ Quantum Access MAX mass spectrometer system with a Protana nanospray ion source (Thermo Scientific Inc, San Jose, CA) interfaced to a self-packed 8 cm × 75 μm (inner diameter) Phenomenex Jupiter 10 μm C18 reversed-phase capillary column (Phenomenex Inc, Torrance, CA). Samples were prepared as published [20]. Dry samples were resuspended using 5% ethanol to a volume equal to 25% of the plasma volume processed. Samples were vortexed and spun at 14K rpm. A 5-μL aliquot of each extract