SUPPLEMENTAL MATERIAL

Roles of phytoestrogen in the pathophysiology of intracranial aneurysm

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Supplemental Materials & Methods

Mouse model of intracranial aneurysms

Experiments were conducted following guidelines approved by the Institutional Animal Care and Use Committee. Details of the intracranial aneurysm mouse model were previously described.[11, 27-31] We used 8-10-week-old C57BL/6J and ER-β knockout mice (Jackson Laboratory, Bar Harbor, Maine). Intracranial aneurysms were induced by combining systemic hypertension and a single injection of elastase (35.0 milli-units, 2.5 μL over 10 minutes) into the cerebrospinal fluid at the right basal cistern (2.6 mm posterior to bregma, 1.5 mm lateral, and 5.7 mm depth).[11, 27-31] Bilateral ovariectomy and left nephrectomy were performed three weeks before elastase injection. This dose of elastase was chosen so that we can achieve 60-90% of the incidence of aneurysm formation, based on our previous studies.[27-31] We used the same lot of elastase for injections of all study groups. We performed a series of test injections for every 10 -15 mice to ensure the correct needle placement as previously described.[62] To induce systemic hypertension, we used deoxycorticosterone acetate (DOCA)-salt hypotension as previously described.[29] DOCA-salt hypertension is a classical method for inducing hypertension that has been successfully used in various species.[29, 62] DOCA-salt hypertension requires left nephrectomy followed by implantation of DOCA pellet one week later; 1% sodium chloride drinking water was started on the same day as the DOCA pellet implantation as previously described.[29, 62] Angiotensin II was not used, as it is not part of the DOCA-salt hypertension.

Evaluation of aneurysm formation and rupture

To detect aneurysmal rupture, two blinded observers performed neurological examinations daily as previously described.[29] Neurological symptoms were scored as follows: 0: normal function; 1: reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (≈10% weight loss) over 24 hours; 2: flexion of the torso and forelimbs on lifting the whole animal by the tail; 3: circling to 1 side with a normal posture at rest; 4: leaning to 1 side at rest; and 5: no spontaneous activity. Mice were euthanized when they developed neurological symptoms (score, 1-5). Aneurysms are defined as a localized outward bulging of the vascular wall (>150% of the control artery).[27, 29] When mice develop neurological symptoms associated with aneurysmal rupture (neurological score: 1-5), we euthanize them immediately (within 4 hours). In both euthanized and dead mice, we inspect the brain samples and verify the presence of aneurysm and hematoma from subarachnoid hemorrhage by examining the Circle of Willis and its major branches under a dissecting microscope (10X).[27, 29] Our study confirmed the specificity and sensitivity of this approach in detecting aneurysmal rupture.[27, 29] Because our previous studies using this model showed that aneurysmal rupture occurs within 3 weeks of aneurysm induction, asymptomatic mice were euthanized 21 days after aneurysm induction as previously described.[10, 28, 30, 31] The brain samples were perfused with phosphate-buffered saline, followed by a gelatin-containing 1% of bromophenol blue dye to visualize cerebral arteries. Two blinded observers assessed the formation of intracranial aneurysms by examining the Circle of Willis and its major branches under a dissecting microscope (10X).

Real-time PCR detection of cytokines

We collected total RNA samples from cerebral arteries (Circle of Willis and its major branches) 3 or 5 days after aneurysm induction, as previously described.[31, 33] We measured mRNA

expression levels of inflammation-related cytokines (IL-1β [interleukin-1β], IL-6 [interleukin-6], MCP-1 [monocyte chemoattractant protein-1], MMP-9 [Matrix metallopeptidase 9], and TNF-α [tumor necrosis factor-α]). The following primers were used: IL-1β, forward 5'-TGG GCC TCA AAG GAA AGA AT-3', reverse 5'-CAG GCT TGT GCT CTG CTT GT-3'; IL-6, forward 5'-CCG GAG AGG AGA CTT CAC AG-3', reverse 5'-GGA AAT TGG GGT AGG AAG GA-3'; MCP-1, forward 5'-AGG TCC CTG TCA TGC TTC TG-3', reverse 5'-GCT GCT GGT GAT CCT CTT GT-3'; MMP-9, forward 5'-AGA CCT GAA AAC CTC CAA CCT CAC-3', reverse 5'-TGT TAT GAT GGT CCC ACT TGA GGC-3'; TNF-α, forward 5'-CCA GAC CCT CAC ACT CAG ATC-3', reverse 5'-CAC TTG GTG GTT TGC TAC GAC-3' (Integrated DNA Technologies, Coralville, IA).

RNA was extracted using the RNeasy Mini Kit (Qiagen, CA) and transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen). The mRNA expression levels were determined using SYBR Green technology (Applied Biosystems, CA). Quantitative values were obtained from the threshold cycle value (CT), and the data were analyzed by the $2^{-\Delta\Delta CT}$ method. Gglyceraldehyde-3-phosphate dehydrogenase expression was quantified and used as an internal RNA control.

Blood pressure monitoring and timeline

Blood pressure were monitored using the tail cuff method (ML125M, ADinstruments) as previously reported.[62] We measured blood pressure on mice anesthetized with isoflurane. We have previously shown that isoflurane anesthesia does not mask the effects of anti-hypertensive agents.[63] A baseline measurement of blood pressure was done right before the starting of the diet switch (3 weeks before the elastase injection). We repeatedly measured the blood pressure until the end of 3 weeks after elastase injection. Blood pressure data for all experimental groups are presented in the supplemental **Figure I**.

Plasma equol detection

Chemicals and reagents

Equol (98.0% purity) was purchased from Tokyo Chemical Industry (Portland, OR). Stable isotope labeled internal standard ((R,S)-equol-d4, mixture of diastereomers, 99% purity) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). All other chemicals and reagents LC-MS grade methanol and acetonitrile were purchased from VWR International (Radnor, PA). Water was filtered and deionized with a MILLI-Q. IQ 7000 water purification system utilizing LC-Pak Polisher (Millipore Sigma, Burlington, MA) and used throughout in all aqueous solutions. Drug-free (blank) mouse plasma from BALB/c mice (with K_2-EDTA anticoagulant) was purchased from Innovative Research Inc. (Novi, MI).

Chromatographic and mass-spectrometric conditions

Instrumentation

All LC–MS/MS analyses were performed on a Sciex (Foster City, CA) QTRAP 6500+ LC–MS/MS system, which consists of Sciex Exion ultra-high performance liquid chromatography (UHPLC) coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer. The UHPLC system is equipped with two Exion LC AD UHPLC pumps (1300 bar), Exion LC AD UHPLC cooled autosampler (rated to 1300 bar), a Controller module for Exion LC systems,

Exion LC AC column oven, and two Exion LC 5 Channel Solvent Degasser unit. Analyst®1.7 software was used for data processing and quantitation.

Liquid chromatography

The best chromatographic separation was achieved on a Phenomenex Kinetex Biphenyl column (2.1 mm × 50 mm, 1.7 µm) using an isocratic elution consisting of mobile phase A (water) and mobile phase B (1:3 acetonitrile:methanol (v/v)), at a flow rate of 0.5 mL/min. The column oven temperature was maintained at 40 °C. To minimize the carryover, external wash was implemented with methanol.

Mass spectrometry (MS)

The QTRAP 6500+ mass spectrometer was operated in electrospray negative ionization using multiple reaction monitoring mode (MRM). The MS parameters were optimized to obtain the most sensitive and specific MS transitions for equol and its internal standard by direct infusion of 100 nM of the standard solutions into the ion source with a syringe pump. The Turbo ion-spray voltage was set at -4500 V, and the source temperature was set at 550 °C. Curtain gas, nebulizing gas and drying gas were optimized and delivered at 35, 80 and 60 psi, respectively. Decluttering potential, collision energy, collision cell exit potential, and entrance potential were optimized at -80, -25, -10, and -10 V, for both equol and its internal standard. The MS spectra of the parent and product ions for equol and its internal standard, under negative electrospray ionization mode, are presented in **Figure IIIA and IIIB**, respectively. The most sensitive MS transition for equol was 241 → 119 *m/z*. The most sensitive MS transitions for the internal standard was 245 → 123 *m/z*. Sample chromatograms of equol and equol-d4 are shown in **Figure IIIC.**

Sample preparation

Stock solutions, calibration standards, and quality control (QC) samples

Equol stock solution was prepared in 40% acetonitrile at a final concentration of 1 mM, whereas its internal standard stock solution was prepared in methanol at a final concentration of 1 mM, and stored at -20 °C. Equol working solutions were prepared fresh by serial dilutions of the stock solution with dilution solution (40% acetonitrile in water) on each day of analysis. Equol internal standard working solutions were prepared in methanol at the concentration of 250 nM. For the analysis of equol in plasma samples, the calibration standards were prepared by spiking equol working solution with blank mouse plasma to make the final concentrations at 1, 2, 5, 20, 50, 200, 500, 1000, 2500, and 5000 nM. QC samples were prepared in blank mouse plasma, at the concentrations 3 nM (LQC), 150 nM (MQC), 4000 nM (HQC). All standards and QC samples were prepared fresh daily.

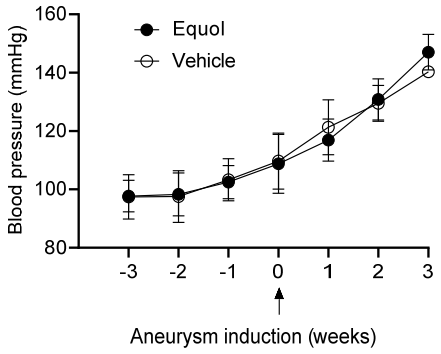
Determination of total plasma concentrations of equol

Frozen plasma samples were thawed at room temperature. An aliquot of 10 µL plasma was transferred into a micro centrifuge tube, as well as 30 µL methanol containing 250 nM internal

standard was added. The mixture was vortex-mixed for 5 s and centrifuged at 12,000 *g* at 4 °C for 10 min (Eppendorf centrifuge, model 5427R). Around 30 µL of supernatant was transferred to a polypropylene auto-sampler vial. Then, 5 µL was injected into the LC–MS/MS system.

Supplemental Figures and Figure Legends

A



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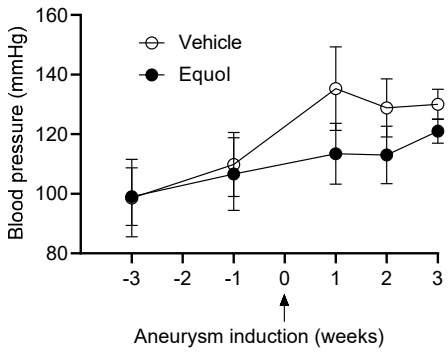

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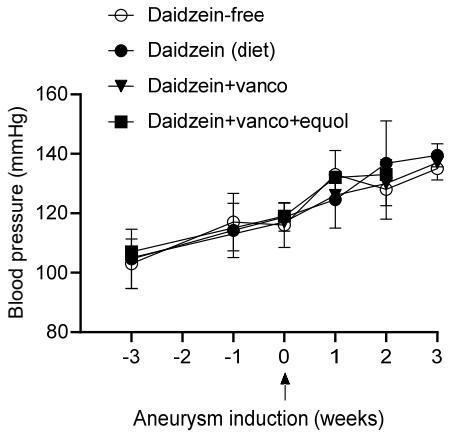

Figure I. **Blood pressure monitoring and timeline.** Blood pressure were measured using the tail cuff method (ML125M, ADinstruments). Mice were anesthetized with isoflurane. A baseline measurement of blood pressure was done right before starting the diet switch (3 weeks before the elastase injection). We repeatedly measured the blood pressure until 3 weeks after elastase injection. **A.** Blood pressure data for mice in **Figure 2**. **B.** Blood pressure data for mice in **Figure 3**. **C.** Blood pressure data for mice in **Figure 5/6**.

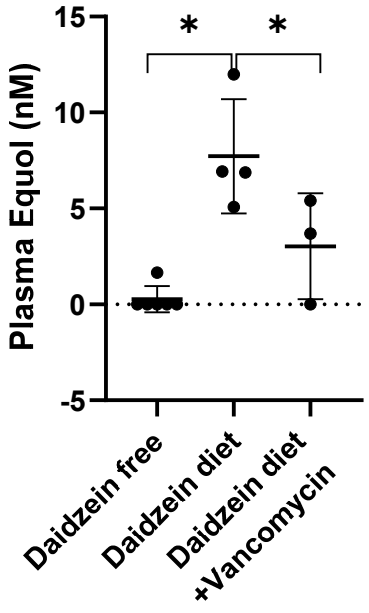

Figure II. Plasma equol level detected by LC-MS. Feeding with the daidzein-containing diet for two weeks resulted the plasma concentration of equol at 7.72 ± 2.98 (nM), while the plasma equol concentration in the daidzein-free diet group was under the detection level. More importantly, oral vancomycin treatment significantly reduced the plasma equol concentration ($P < 0.05$, One-way ANOVA with Tukey's multiple comparisons test).

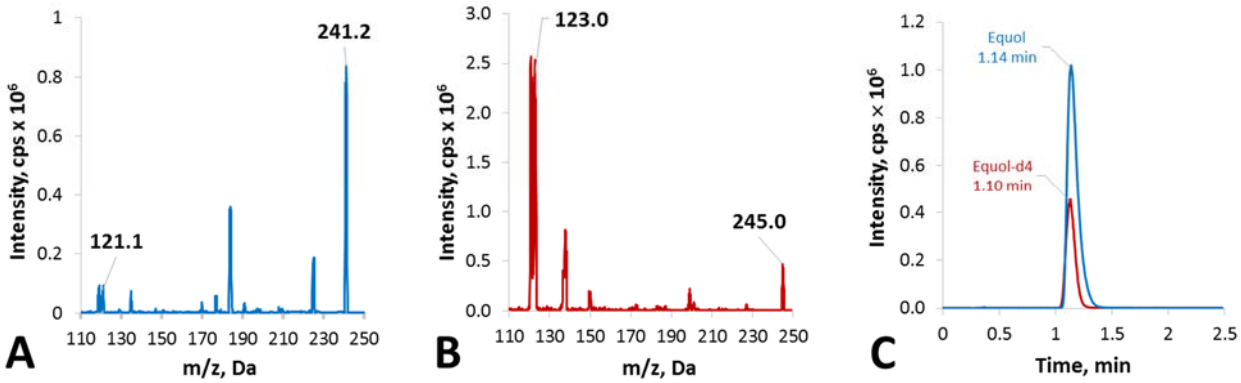

Figure III. The MS spectra of the parent and product ions for equol and its internal standard, under negative electrospray ionization mode, are presented in **A** and **B**, respectively. Sample chromatograms of equol and equol-d4 are shown in **C.**

Table I. Mice included/excluded in Figures

Figures	Groups	Included	Excluded*
Figure 2 Wild-type mice	Vehicle-treated Equol-treated	18 15	2 3
Figure 3 ERβ KO mice	Vehicle-treated Equol-treated	13 10	0 0
Figure 4 RT-PCR	3-days Vehicle-treated Equol-treated 5-days Vehicle-treated Equol-treated	 7 5 9 11	 0 0 0 0
Figure 5/6 Wild-type mice	Daidzein-free diet Daidzein-diet Daidzein+vancomycin Daidzein+vancomycin+equol	16 19 14 13	6 3 1 1

*Reasons for exclusion include intracerebral hemorrhage, unknown SAH, died in the cage and cannibalized, or accidental death after anesthesia.

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	N/A
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	No

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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