BASIC AND TRANSLATIONAL SCIENCES

Roles of Phytoestrogen in the Pathophysiology of Intracranial Aneurysm

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BACKGROUND AND PURPOSE: The incidences of intracranial aneurysm and aneurysmal subarachnoid hemorrhage are high in postmenopausal women. Although population-based studies suggest that hormone replacement therapy is beneficial for postmenopausal women with intracranial aneurysms, estrogen replacement may no longer be recommended for the prevention of chronic diseases given its association with adverse outcomes, such as cancer and ischemic stroke. The isoflavone daidzein and its intestinal metabolite equol are bioactive phytoestrogens and potent agonists of estrogen receptors. Given their estrogenic properties, we investigated whether the isoflavones daidzein and equol are protective against the formation and rupture of intracranial aneurysms in a mouse model of the postmenopausal state.

METHODS: We induced intracranial aneurysms in ovariectomized adult female mice using a combination of induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid. We fed the mice with an isoflavone-free diet with/without daidzein supplementation, or in a combination of intraperitoneal equol, or oral vancomycin treatment. We also used estrogen receptor beta knockout mice.

RESULTS: Both dietary daidzein and supplementation with its metabolite, equol, were protective against aneurysm formation in ovariectomized mice. The protective effects of daidzein and equol required estrogen receptor-β. The disruption of the intestinal microbial conversion of daidzein to equol abolished daidzein's protective effect against aneurysm formation. Mice treated with equol had lower inflammatory cytokines in the cerebral arteries, suggesting that phytoestrogens modulate inflammatory processes important to intracranial aneurysm pathogenesis.

CONCLUSIONS: Our study establishes that both dietary daidzein and its metabolite, equol, protect against aneurysm formation in ovariectomized female mice through the activation of estrogen receptor-β and subsequent suppression of inflammation. Dietary daidzein's protective effect required the intestinal conversion to equol. Our results indicate the potential therapeutic value of dietary daidzein and its metabolite, equol, for the prevention of the formation of intracranial aneurysms and related subarachnoid hemorrhage.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: daidzein ∎ intracranial aneurysm ∎ mice ∎ receptors, estrogen ∎ subarachnoid hemorrhage

The pathogenesis of intracranial aneurysms is poorly understood. The incidences of intracranial aneurysm and aneurysmal subarachnoid hemorrhage are high in postmenopausal women, suggesting estrogen may be protective against aneurysm formation or rupture.[1–4] Moreover, population-based studies suggest an association between estrogen-containing hormone replacement therapy and a reduced incidence of aneurysmal subarachnoid hemorrhage.[5–9] In animal models of intracranial aneurysms,

Nonstandard Abbreviations and Acronyms	
ER	estrogen receptor
IL	interleukin
MCP	monocyte chemoattractant protein
MMP	matrix metallopeptidase
TNF	tumor necrosis factor

estrogen has been shown to prevent aneurysm formation and rupture, primarily through activation of ER (estrogen receptor)-β and modulation of inflammatory processes.[10–12] Although these findings suggest the potentially protective effect of estrogen hormone replacement therapy against the formation and rupture of intracranial aneurysm in postmenopausal women, hormone replacement therapy is associated with an increased risk of other significant adverse outcomes, including cancer and ischemic stroke.[13–16] Therefore, hormone replacement therapy is contraindicated for the prevention of chronic diseases.[17]

Isoflavones are plant-based, diet-derived compounds that structurally resemble estradiol and exert estrogenic activities with tissue and receptor specificity.[18–20] Regular consumption of isoflavones has been shown to alleviate the vasomotor symptoms of estrogen deficiency and associated with the reduced incidence of estrogen-dependent diseases in postmenopausal women.[21–23] Daidzein is one of the common isoflavones in diets—found in legumes, peas, and beans—and is a potent phytoestrogen that acts as an ER agonist.[20,24,25] Daidzein is converted to equol by gut microbiota.[20,26] After being absorbed from the gut, equol exerts estrogenic activity on various tissues. Equol is more bioactive and estrogenic than its precursor daidzein.[25] More importantly, equol preferentially binds to ER-β,[20] a receptor subtype responsible for the protective effects of estrogen against the formation and rupture of intracranial aneurysm in ovariectomized female mice, a model of the postmenopausal state.

Given their estrogenic properties, we hypothesized that phytoestrogens may prevent intracranial aneurysm formation and subarachnoid hemorrhage through activation of ER-β and modulation of inflammatory processes in postmenopausal women who are at increased risk for aneurysmal formation and rupture. As a first step, we investigated whether the phytoestrogens daidzein and equol are protective against the formation and rupture of intracranial aneurysms in ovariectomized female mice.

METHODS

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Mouse Model of Intracranial Aneurysm

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- to 10-week-old C57BL/6J and ER-β knockout mice (Jackson Laboratory, Bar Harbor, Maine). All mice used in the experiments are summarized in Table I in the Data Supplement.

Intracranial aneurysms were induced by combining systemic hypertension and a single injection of elastase (35.0 milli-units) into the cerebrospinal fluid at the right basal cistern.[11,27–31] To induce systemic hypertension, we used the deoxycorticosterone acetate-salt hypertension method.[11,28–31] Bilateral ovariectomy and left nephrectomy were performed 3 weeks before elastase injection.

Drug Treatment and Dietary Supplement

We replaced standard chow with an isoflavone-free diet (AIN-93G, no soybean oil, D10012G, Research Diets, Inc) at the same time as ovariectomy/nephrectomy. Systemic treatment with equol (0.5 mg/kg·per day, in 20% dimethyl sulfoxide in polyethylene glycol-300) was delivered via an implanted mini-osmotic pump (Model 1004, Alzet) for 4 weeks starting 1 week before elastase injection. For the vehicle-treated group, mini-osmotic pumps were filled with 20% dimethyl sulfoxide in polyethylene glycol-300. In dietary daidzein treatment experiments, the isoflavone-free diet was supplemented with daidzein (0.1%) for 4 weeks starting 1 week before elastase injection. Oral vancomycin (50 mg/kg·per day) started at the same time as dietary daidzein was used to block the conversion of daidzein to equol by the gut microbiota following the protocol established by Blair et al.[32]

Evaluation of Aneurysm Formation and Rupture

Two observers who were blinded to the treatments performed neurological examinations daily to detect aneurysmal rupture using a neurological scoring system as previously described.[10,28,30,31] Mice were euthanized when they developed neurological symptoms (score 1–5, for details, see Data Supplement). Asymptomatic mice were euthanized 21 days after aneurysm induction as previously described.[10,28,30,31]

Plasma Equol Measurement

We used a Sciex (Foster City, CA) QTRAP 6500+ LC–MS/MS system, which consists of the Sciex Exion ultra-high performance liquid chromatography coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer (please see Data Supplement).

Real-Time Polymerase Chain Reaction 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 [interleukin]-1β, IL-6, MCP-1 [monocyte chemoattractant protein], MMP-9 [matrix metallopeptidase], and TNF-α [tumor necrosis factor]; Data Supplement).

Statistical Analysis

Fisher exact test was used to analyze the incidences of aneurysm formation and subarachnoid hemorrhage. The survival rate was evaluated by log-rank (Mantel-Cox) test. We used multiple *t* test with post hoc Holm-Sidak method for the analysis of real-time polymerase chain reaction data. $P<0.05$ were considered statistically significant. Data are expressed as means±SD.

RESULTS

Equol Reduced the Formation of Aneurysms in Ovariectomized Female Mice

Figure 1 shows representative unruptured and ruptured aneurysms from the mouse model used in the current study. To assess the potential role of phytoestrogens in the pathophysiology of intracranial aneurysms, we tested the effect of equol, the intestinal metabolite of daidzein, on the formation and rupture of intracranial aneurysm. Three weeks before aneurysm induction, female mice underwent bilateral ovariectomy and were started on an isoflavone-free diet. Systemic treatment with equol (0.5 mg/kg per day) or vehicle began 1 week before aneurysm induction and was continued for 4 weeks (Figure 2A).

Equol treatment significantly reduced the incidence of aneurysm formation compared to vehicle (vehicle versus equol; 89% versus 53%; n=16/18 versus n=8/15. $P<0.05$; Figure 2B). There was a trend for equol-treated mice to have lower incidence of aneurysmal rupture than vehicle-treated mice (vehicle versus equol; 75% versus 50%; n=12/16 versus n=4/8. $P=0.2$; Figure 2C). There was no difference in the blood pressure between the 2 groups (Figure I in the Data Supplement).

Equol's Protective Effect Against Aneurysm Formation Required ER-β

We previously found that ER-β activation is necessary for estrogen-mediated prevention of intracranial aneurysm formation in ovariectomized female mice.[10] To determine if equol's protective effect also requires ER-β, we treated ovariectomized ER-β knockout mice with equol and assessed for incidence of aneurysm formation and rupture (Figure 3A).

Unlike our results for ovariectomized wild-type mice, we found no significant difference in the incidence of aneurysm formation between vehicle and equol-treated groups in ovariectomized ER-β knockout mice (vehicle versus equol; 77% versus 80%; n=10/13 versus n=8/10. $P=1$, Figure 3B). Likewise, there was no difference in the incidence of rupture between vehicle and equol-treated groups (vehicle versus equol; 70% versus 63%; n=7/10 versus 5/8. $P=0.4$, Figure 3C). Taken
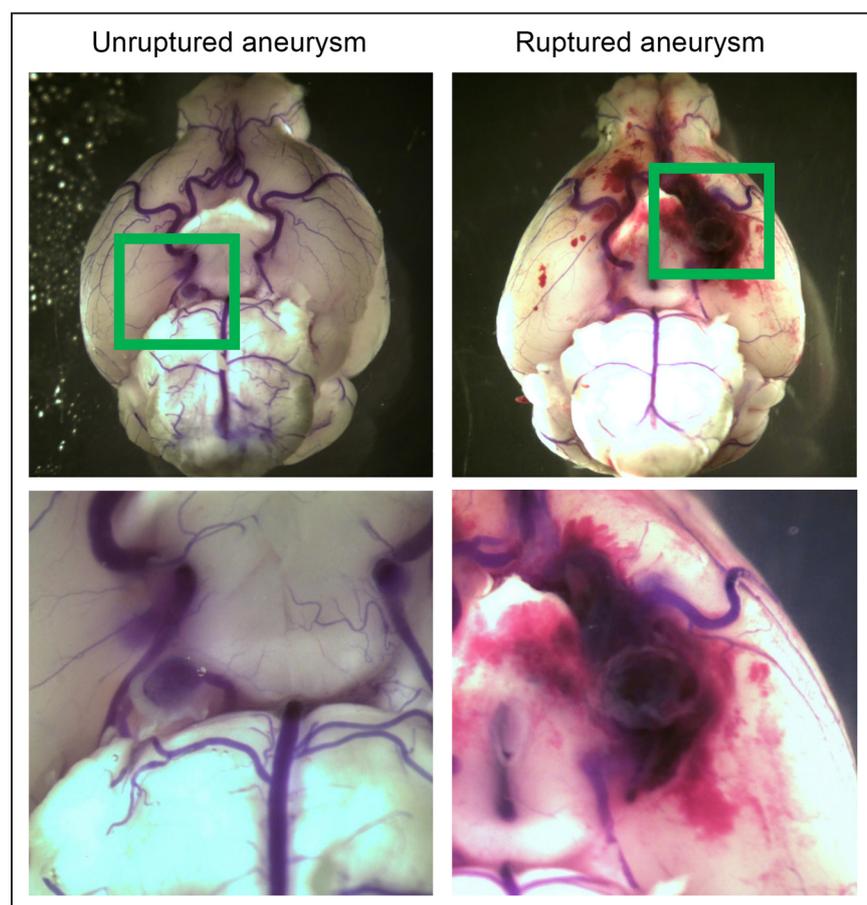

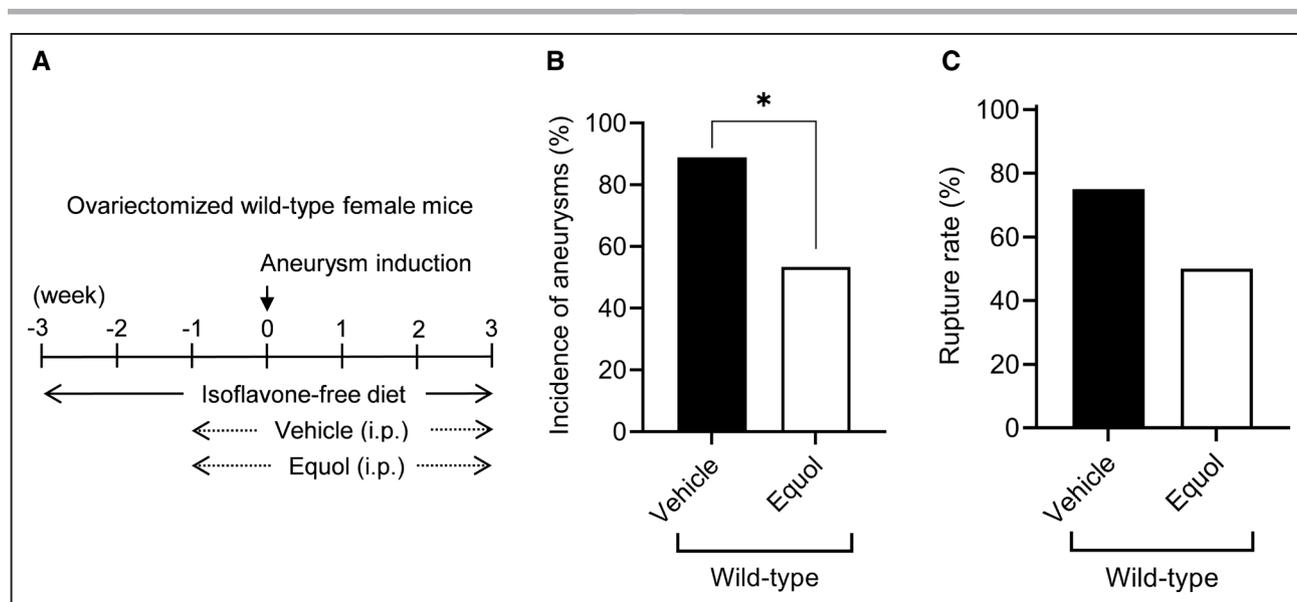**Figure 1.** Representative images of unruptured and ruptured aneurysms.

Figure 2. Equol reduced the formation of intracranial aneurysms in ovariectomized female mice.
A, Schematic diagram of experimental protocols for ovariectomized wild-type female mice. **B** and **C**, Equol treatment significantly reduced the incidence of aneurysms (**B**; *P<0.05).

together, these data demonstrate that equol's protective effect against aneurysm formation requires ER-β activation. There was no difference in the blood pressure between the groups at any time point (Figure I in the Data Supplement).

Equol Decreased mRNA Expression of Proinflammatory Cytokines in Cerebral Arteries

We previously found that the anti-inflammatory effects of ER-β activation mediate estrogen's protection against aneurysm formation.[10] Therefore, we investigated whether systemic treatment reduces the expression of anti-inflammatory cytokines in cerebral arteries.

There was a trend for IL-1β and IL-6 levels 3 days after aneurysm induction in the equol-treated mice to be lower than those in controls (upper, Figure 4). At 5 days of postaneurysm induction, mRNA levels of IL-6 were significantly lower in equol-treated mice compared with vehicle-treated mice (adjusted P value, P<0.05, multiple t test). There was a trend for reduced IL-1β (P=0.17) and TNFα (P=0.15) levels in the equol-treated mice compared with vehicle-treated mice (lower, Figure 4).

Dietary Daidzein Reduced Aneurysm Formation in Ovariectomized Female Mice

Next, we tested whether equol's dietary precursor, daidzein, also protects against the formation of aneurysms in ovariectomized female mice. An isoflavone-free diet was started 3 weeks before aneurysm induction. In the experimental group, daidzein was added to the diet 1 week before aneurysm induction; the control group received the same amount of isoflavone-free chow (Figure 5A).

Similar to the systemic equol treatment, dietary daidzein reduced the incidence of aneurysm formation (daidzein-containing diet versus daidzein-free diet: 53% versus 88%; n=10/19 versus n=14/16. P<0.05; Figure 5B). There was no difference in aneurysmal rupture between the 2 groups (daidzein-containing diet versus daidzein-free diet: 70% versus 79%; n=7/10 versus n=11/14; P=0.5, Figure 5C).

Daidzein's Protective Effect Against Aneurysm Formation Was Dependent on Intestinal Conversion to Equol by Gut Microbiota

Equol is produced by gut microbiota from its dietary precursor, daidzein.[34–37] Oral vancomycin treatment reduces the conversion of daidzein to equol by 99% by disrupting the composition of gut microbiota,[32] whereas systemic absorption of oral vancomycin is negligible.[38,39] Therefore, we used oral vancomycin to assess whether the intestinal conversion to equol is required for the protective effect of daidzein.

An isoflavone-free diet was started 3 weeks before aneurysm induction. One week before aneurysm induction, mice were concurrently given a daidzein supplemented diet and started on oral vancomycin. As an additional control, another group of mice received oral vancomycin without daidzein to evaluate for a potential confounding effect of vancomycin. Another group received a combination of daidzein, oral vancomycin, and systemic equol to confirm equol protected against aneurysm formation, despite blocking the intestinal metabolism of daidzein (Figure 6A).

We found that oral vancomycin abolished the protective effect of daidzein on the incidence of aneurysm formation
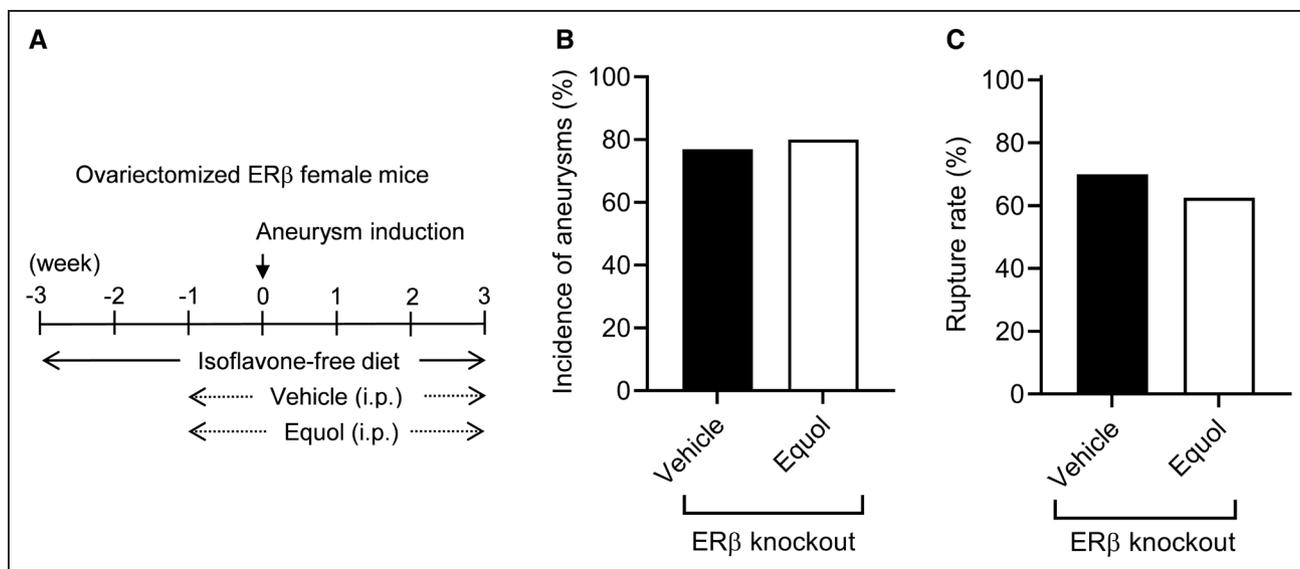
Figure 3. Equol's protective effect against aneurysm formation required ER (estrogen receptor)-β.
A, Schematic diagram of experimental protocols for ovariectomized ER-β knockout female mice. **B** and **C**, The protective effect of equol was lost in ovariectomized ER-β knockout female mice.

(daidzein diet versus daidzein diet with vancomycin: 53% versus 86%; n=10/19 versus n=12/14; $P<0.05$; Figure 6B), confirming daidzein's protective effect requires the intestinal conversion of daidzein to equol. When systemic equol was administered to mice receiving daidzein and vancomycin, the protective effects were rescued (daidzein diet+vancomycin versus daidzein diet+vancomycin+equol: 86% versus 46%; n=12/14 versus n=6/13. $P<0.05$; Figure 6B). Vancomycin treatment alone did not affect the formation of aneurysms (data not shown). There was no significant difference among any of the groups in aneurysm rupture rate for any treatments, including vancomycin ($P=0.88$, Fisher exact test, Figure 6C). This nonstatistical difference observed in this graph is within the margin of error for this model. There was no difference in the blood pressure between the groups at any time point (Figure I in the Data Supplement).

To assess the equol production from dietary daidzein, we measured the plasma equol concentration in mice that received daidzein-free diet, daidzein-containing diet, and daidzein-containing diet, and vancomycin treatment. We measured the plasma equol concentration after 2 weeks of the diet and vancomycin treatments. As shown in Figure II in the Data Supplement, feeding with the daidzein-containing diet for 2 weeks resulted in the plasma concentration of equol at 7.72±2.98 (nM), whereas 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, suggesting the effective blocking of the intestinal conversion of daidzein to equol (please see Data Supplement). As a comparison, plasma equol concentration reached 103±70 (nM) in mice treated with equol.

DISCUSSION

Both clinical and preclinical studies have shown the potentially protective effect of estrogen against the formation and rupture of intracranial aneurysms in postmenopausal women.[1–4,10–12] However, estrogen replacement is associated with adverse outcomes such as cancer and ischemic stroke, partly due to lack of tissue or receptor subtype specificities of estradiol.[5,6,14–16] Thus, a treatment strategy that targets the ER with the subtype specificity while not causing estrogen's adverse outcomes may be viable for the prevention of aneurysm formation and rupture in postmenopausal women.

Isoflavones, including genistein, daidzein, and glycitein, resemble estradiol and are potent ER agonists. The dietary isoflavone, daidzein, and its metabolite equol have more potent estrogenic activity than any other isoflavone or isoflavone-derived metabolite.[20,40] Diets rich in isoflavones are reported to be effective in alleviating vasomotor symptoms of menopause and may be protective against estrogen-dependent diseases.[21] Therefore, the use of phytoestrogens has the potential as a therapeutic against aneurysm formation and rupture in postmenopausal women.

Here, we have shown the isoflavone daidzein and its metabolite, equol, are protective against aneurysm formation in ovariectomized female mice. Consistent with our previous findings on estrogen deficiency,[10,11] equol's protective effect against aneurysm formation was dependent on the activation of ER-β.

We found that daidzein's protective effect against aneurysm formation is primarily exerted by its bioactive, bacterially-produced metabolite equol.[20] Similar to systemically administered equol, daidzein was
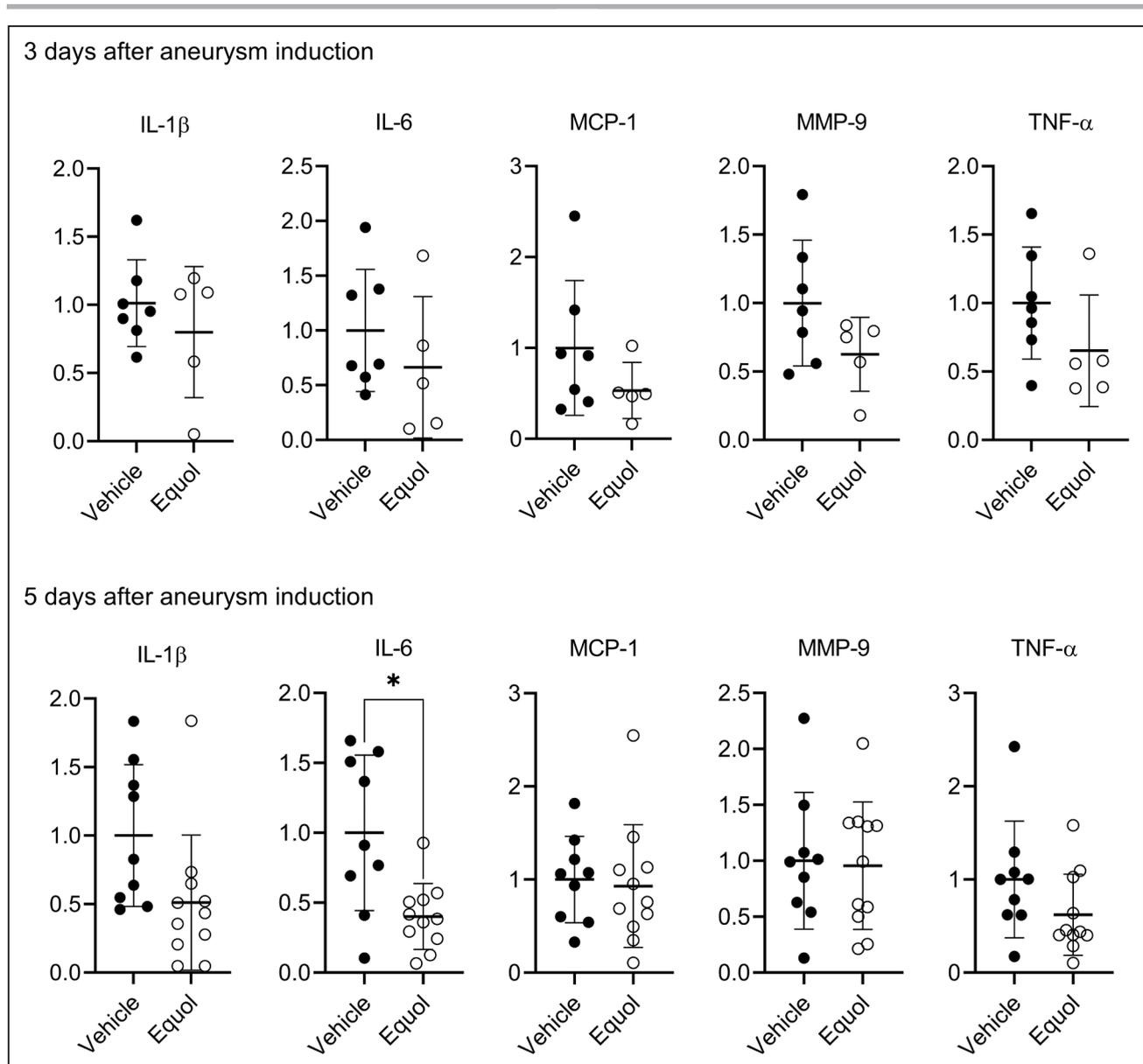
Figure 4. Equol decreased mRNA expression of proinflammatory cytokines in ovariectomized female mice.
There was a trend of reduction in IL (interleukin)-1β and IL-6 expression levels 3 d after aneurysm induction in the equol-treated mice relative to controls (**upper**). At 5 d of postaneurysm induction, mRNA level of IL-6 was significantly lower in equol-treated mice compared to vehicle (**lower**, *$P<0.05$). There was a tendency for reduced IL-1β and TNF (tumor necrosis factor)-α levels in the equol-treated mice compared with vehicle-treated animals, though this difference did not achieve statistical significance. MCP indicates monocyte chemoattractant protein; and MMP, matrix metallopeptidase.

protective against aneurysm formation. Several factors influence the bioavailability of isoflavones, including gut microbiota, bowel disease, age, and sex.[41] We found systemically administered equol resulted in higher plasma concentration than daidzein ingestion and gut absorption alone, thus providing a higher degree of protection against the formation of aneurysms. Daidzein is a natural compound, found primarily in legumes and beans and is metabolized to equol by gut microbiota.[20] We found that blocking the intestinal conversion of dietary daidzein to equol abolished daidzein's protective effect, suggesting the

importance of the intestinal conversion of phytoestrogens for their protective effects.

In our previous studies, estrogen was found to be protective against aneurysm formation in ovariectomized female mice.[10–12] Although the exact pathogenesis of aneurysm formation and rupture remains fully elucidated, numerous studies have demonstrated that inflammation is likely to play a pivotal role.[42–46] It is, therefore, possible that the protection estrogen confers may be through modulation of inflammatory processes. We previously found estrogen's protective effect required activation of ER-β and the production of nitric oxide.[10] Nitric oxide is
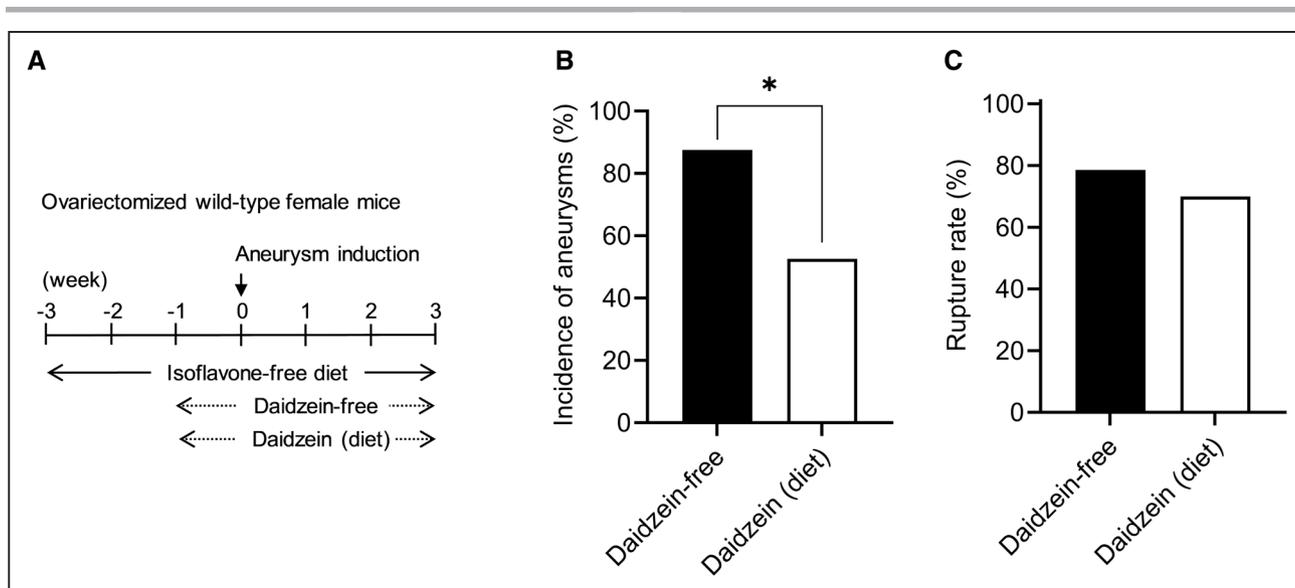
Figure 5. Daidzein reduced aneurysm formation in ovariectomized female mice.
A, Schematic diagram of experimental protocols for dietary daidzein treatment in ovariectomized wild-type female mice. **B** and **C**, There is a significant difference between daidzein-containing diet and daidzein-free diet-fed groups in the incidence of aneurysms (**B**; *$P<0.05$).

involved in acute and chronic inflammation, processes integral to the pathophysiology of intracranial aneurysm. Hoh et al. also reported a link between estrogen deficiency and inflammation, suggesting estrogen deficiency promotes aneurysm rupture by upregulating IL-17, leading to the downregulation of E-cadherin and macrophage infiltration in the aneurysm wall.[12] Consistent with these findings, we found treatment with equol significantly reduced proinflammatory cytokines, suggesting a link between estrogen and inflammation in the pathogenesis of intracranial aneurysm.

Although both equol and daidzein were protective against the formation of aneurysms, we observed only a trend for their protective effect against the development of aneurysmal rupture. It is possible that our studies were underpowered to detect the potentially protective effect of phytoestrogens against the development of aneurysmal rupture. Also, the duration of treatments may have been too short to exert protective effects. Alternatively, these findings simply reflect the weaker estrogenic potency of phytoestrogens compared to estrogens. Future studies with longer treatment duration and higher doses may be needed.

There are other limitations to our study. Although our mouse model recapitulates key features of human intracranial aneurysms, it may not completely replicate the pathogenesis of aneurysm formation and rupture in humans.[33,47,48] Additionally, ovariectomy in premenopausal young mice may not completely replicate physiological menopause.[49,50] Female mice reach reproductive maturity at approximately 7 weeks, and menopause occurs at ≈12 to 14 months.[51] Although older rodents may be used to model menopause, only 25% to 40% naturally model the human menopausal transition.[52] In contrast, ovariectomy

in young, female mice has been widely used as a model for menopause in preclinical translational research due to its simplicity and consistency.[52–54] Further studies using older postmenopausal mice are needed to confirm our findings. In addition, it needs to be tested whether the protections offered by phytoestrogen can be extended to males and premenopausal females. We recognize different lines of ER-β knockout mice have slightly different phenotypes.[55–57] Some of the key differences are noted in reproductive organs such as the prostate.[56,57] However, there is no report suggesting apparent differences in the vascular or inflammatory cells in these lines of ER-β knockout mice. Although it is unlikely that the subtle differences in reproductive organs represent significant confounding factors for our study, we cannot completely exclude such possibility. Another limitation of this study is that we did not determine the effect of vancomycin on the inflammatory cytokines. Given its poor absorption, the effect of oral vancomycin may be restricted to the gut, and a systemic effect of oral vancomycin is not expected.[38,39,58,59] With expansive experiments, we may be able to examine the effect of vancomycin on the inflammatory cytokines and the rupture rate (on which vancomycin treatment showed a slight trend of increase). However, such expansive experiments are beyond the scope of this article.

Although laboratory mice can consistently produce equol in response to soy or daidzein ingestion, not all humans are equol producers. Only 25% to 30% of White people are host to microbiota capable of converting daidzein to equol, compared with 50% to 60% of Asian people.[21,60] Ultimately, human studies are needed to confirm the contribution of isoflavone consumption to the pathogenesis of intracranial aneurysm.
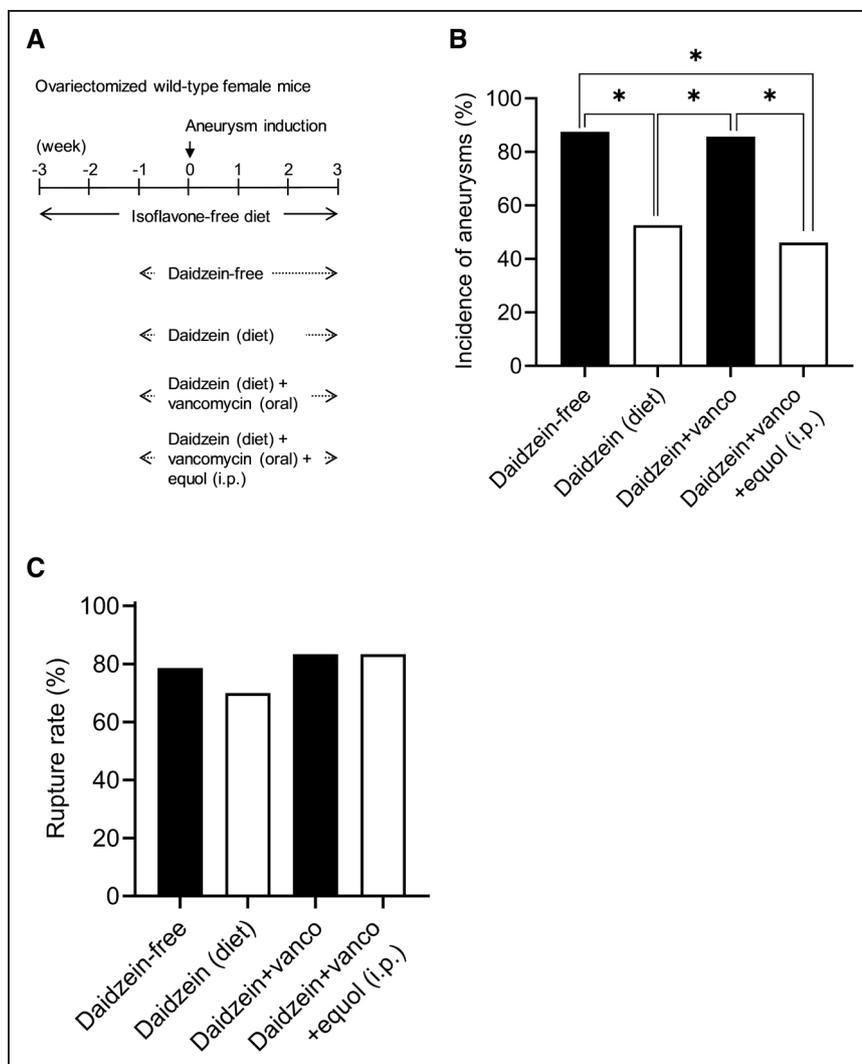
Figure 6. Daidzein's protective effect against aneurysm formation was dependent on intestinal metabolism to equol by gut microbiota.
A, Schematic diagram of experimental protocols for dietary daidzein, oral vancomycin, and intraperitoneal administration of equol in ovariectomized wild-type female mice. **B**, Oral vancomycin treatment abolished the protective effect of dietary daidzein on the incidence of aneurysm formation; systemic equol administration rescued the protective effects of dietary daidzein from oral vancomycin treatment (*$P < 0.05$). **C**, There is no significant effect of the drugs on the incidence of subarachnoid hemorrhage.

Finally, apart from their estrogenic activity, isoflavones are potent antioxidants, and as such, they may exert protective effects against aneurysm formation or subarachnoid hemorrhage independent of ER activation.[61] However, our ER-β knockout mice experiment suggests their protective effects on the formation of intracranial aneurysm are primarily mediated by ER activation.

CONCLUSIONS

Our study firmly established that both dietary daidzein and supplementation with its metabolite, equol, were protective against aneurysm formation in ovariectomized female mice. These protective effects of daidzein and equol required ER-β activation. In addition, our results illustrate the potential role of gut microbiota in the pathophysiology of intracranial aneurysms because the metabolism of the isoflavone daidzein to the biologically active phytoestrogen equol is dependent on gut microbiota.[33] Our results indicate the potential therapeutic value of dietary daidzein and its metabolite, equol, for the prevention of the formation of intracranial aneurysms.

Affiliations

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Disclosures

None.

Supplemental Material

Expanded Materials and Methods
Online Figures I–III
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