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MAJOR ARTICLE

Genistein Inhibits *Clostridioides difficile* Infection via Estrogen Receptors and Lysine-Deficient Protein Kinase 1

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Background. *Clostridioides difficile* infection (CDI) is a debilitating nosocomial disease. Postmenopausal women may have an increased risk of CDI, suggesting estrogen influence. Soybean products contain a representative estrogenic isoflavone, genistein.

Methods. The anti-inflammatory and antiapoptotic effects of genistein were determined using primary human cells and fresh colonic tissues. The effects of oral genistein therapy among mice and hamsters were evaluated.

Results. Within 10 days of CDI, female c57BL/6J mice in a standard environment (regular diet) had a 50% survival rate, while those with estrogen depletion and in an isoflavone-free environment (soy-free diet) had a 25% survival rate. Oral genistein improved their 10-day survival rate to 100% on a regular diet and 75% in an isoflavone-free environment. Genistein reduced macrophage inflammatory protein-1α (MIP-1α) secretion in fresh human colonic tissues exposed to toxins. Genistein inhibited MIP-1α secretion in primary human peripheral blood mononuclear cells, abolished apoptosis and BCL-2–associated X (BAX) expression in human colonic epithelial cells, and activated lysine-deficient protein kinase 1 (WNK1) phosphorylation in both cell types. The anti-inflammatory and antiapoptotic effects of genistein were abolished by inhibiting estrogen receptors and WNK1.

Conclusions. Genistein reduces CDI disease activity by inhibiting proinflammatory cytokine expression and apoptosis via the estrogen receptor/G-protein estrogen receptor/WNK1 pathways.

Keywords. apoptosis; cytokine; gender; hormone; isoflavone.

Clostridioides difficile infection (CDI) is a common nosocomial infection among patients who received prolonged antibiotic treatment. *C. difficile* bacterium produces toxin A, toxin B, and binary toxin. According to the Centers for Disease Control and Prevention, almost half a million cases of CDI occur annually in the United States. Symptoms of CDI include severe diarrhea, fever, abdominal pain, loss of appetite, and nausea. Age, hospitalization, immunocompromised status, and previous *C. difficile* exposure are risk factors for CDI [1].

The variable severity of CDI is generally attributed to strain differences and host health. Toxin A^+B^+NAP1/B1/ribotype 027 is a hypervirulent strain in North America, causing multiple outbreaks [2]. Toxin A^-B^+ribotype 017 strain is prevalent in Asia [3]. Some patients are unresponsive to currently available therapies and eventually require invasive surgery [4]. Thus, CDI is a growing threat to the health care system and its patients.

Additionally, about 15%–35% of CDI cases will recur in 2–8 weeks [1]. The standard antibiotic (vancomycin) treatment is associated with a high recurrence rate. Fidaxomicin is noninferior to vancomycin in efficacy but is cost-ineffective [5]. Bezlotoxumab may prevent recurrent CDI but with a modest sustained cure rate (64%) [6]. Fecal microbiota transplantation has a 90% curative success rate, but adverse effects can occur [7]. Current therapeutic options are not ideal and require optimization.

Postmenopausal women may have an increased risk for CDI-related hospitalizations [8], suggesting the hormonal influence of estrogen. However, the molecular relationship between estrogen and CDI is unknown. As many women experience hot flashes and other discomforts during menopause, phytoestrogens, such as soy isoflavones, are widely used as supplements to ameliorate menopause-related discomfort. Dietary isoflavone intake can also reduce cardiovascular disease risk in postmenopausal women [9]. People in East Asia are more exposed to soy isoflavones than those in Western societies due to their high dietary soy food intake, which might be sufficient to affect health outcomes [10].

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Soy isoflavones consist predominantly of genistein and daidzein and a smaller proportion of glycitein (US Department of Agriculture Database for the Isoflavone Content of Selected Foods, 2008). The pharmacological effects of genistein on humans are well characterized [11]. Genistein, as a phytoestrogen, mediates its effects via nuclear estrogen receptor (ER) and membrane-bound G-protein estrogen receptor (GPER/GPR30) [12, 13]. Although a high dose of genistein induces cell death due to topoisomerase II inhibition [14], low concentrations of genistein are cytoprotective [15]. A phase 1 clinical trial showed no sign of apoptosis or DNA damage in postmenopausal women exposed to a dose of genistein 10 times that of dietary intake (600 mg/day) for 84 consecutive days [16]. The National Toxicology Program also concluded that there is minimal concern for adverse effects in infants who consume soy infant formula [17].

CDI causes severe diarrhea and colitis. Genistein reduces inflammation and ameliorates colitis [17]. Therefore, we hypothesize that genistein should inhibit CDI. This study investigated the influence of genistein on CDI using female models, including fresh human colonic explants, primary human colonic epithelial cells, peripheral blood mononuclear cells (PBMCs), and small animals.

METHODS

Chemicals

Chemical information is shown in Supplementary Table 1.

Human Colonic Explants

Fresh human colonic explants were obtained from the University of California, Los Angeles (UCLA) Surgical Pathology Department during surgery from noncancerous regions of the colon of female colon cancer patients and cut into 3 × 3 mm pieces, as described previously [18]. The baseline characteristics of the patients are shown in Supplementary Table 1. The Institutional Review Board (IRB) approved the tissue collection (IRB-001499). No patient-identifiable information was obtained. The informed consent was waived. All methods were compliant with relevant guidelines and regulations.

Animal Experiments

Infected animals with ≥20% weight loss from baseline level reached a humane intervention end point and were humanely euthanized. We monitored the animals frequently and ensured animals were not dying without attention. Some animals received oral administration of saline to avoid dehydration, as recommended by veterinarians. To minimize pain and distress, we humanely euthanized the sick animals when they reached a humane intervention end point. All procedures of animal experiments were approved by the UCLA's animal research committee.

Animal studies, approved by the UCLA Institutional Animal Research Committee (No. 2007-116) and compliant with the ARRIVE guidelines, used 8-week-old male and female C56BL/6J mice of around 20–22 g body weight (No. 000664, Jackson Laboratories) and 6-week-old female Golden Syrian Hamsters of about 100 g body weight (No. 049, Charles River Laboratories). Multiple batches of animals were used in this study.

Animals were randomly assigned to cages by animal facility staff in a blind manner and maintained at the UCLA animal facility under standard environmental conditions with a 12/12-hour light/dark period, 25°C room temperature, disposable polypropylene cages with HEPA-filtered air circulation, autoclaved white paper bedding, sterile water, and rodent chow (regular No. 7013 or soy-free No. 2016C; Envigo) ad libitum. At the UCLA biohazard animal facility, all mice were consistently housed in a designated room, while all hamsters were consistently housed in another room. All interventions were performed during the light cycle.

C. difficile strain A^+B^+VPI 10463 (American Type Culture Collection [ATCC] 43255), A^+B^+ribotype 027 (ATCC BAA-1805), and A^-B^+ribotype 017 (ATCC 43598) were cultured in Difco cooked meat media (No. 226730; BD) at 37°C in anaerobic conditions [19].

Power Analysis and Statistical Analysis

We needed to include 6 mice per group to achieve statistical significance of colonic CC chemokine ligand 3 (Ccl3) mRNA expression difference of CDI and CDI plus genistein (2.8- vs 1.6-fold) with SD = 0.5, $\alpha = .05$, and power = 80%. Animal studies were carried out 2–3 times to ensure reproducibility. In vitro experiments were performed at least 3 times to ensure reproducibility. N-numbers and the number of rounds of experiments are shown in the figure legends.

Unpaired Student *t* tests were used for 2-group comparisons of continuous data, and 2-way analyses of variance were used for multiple-group comparisons (Prism 9). Results were expressed as mean ± standard deviation. Significant *P* values are shown in each figure.

RESULTS

Endogenous Estrogen and Dietary Soy Isoflavones Reduced CDI Severity in Mice

To determine the role of endogenous estrogen in female mice with CDI, we injected 4-vinylcyclohexene diepoxide (VCD) to deplete estrogen (17β-estradiol) and simulate the postmenopausal state in female mice [20], followed by *C. difficile* inoculation (Figure 1*A*). Male mice and VCD-treated female mice had significantly lower circulating 17β-estradiol levels than untreated female mice (Figure 1*B*). Interestingly, endogenous estrogen might positively regulate CDI in female mice as VCD-treated female mice (25%) had a lower 10-day survival rate than young estrogen-intact female mice (50%) (Figure 1*C*).

Regular NIH-31 rodent diet contains 5% soy meal, which provides a significant source of dietary genistein. For comparison, we used a soy-free rodent diet to differentiate the

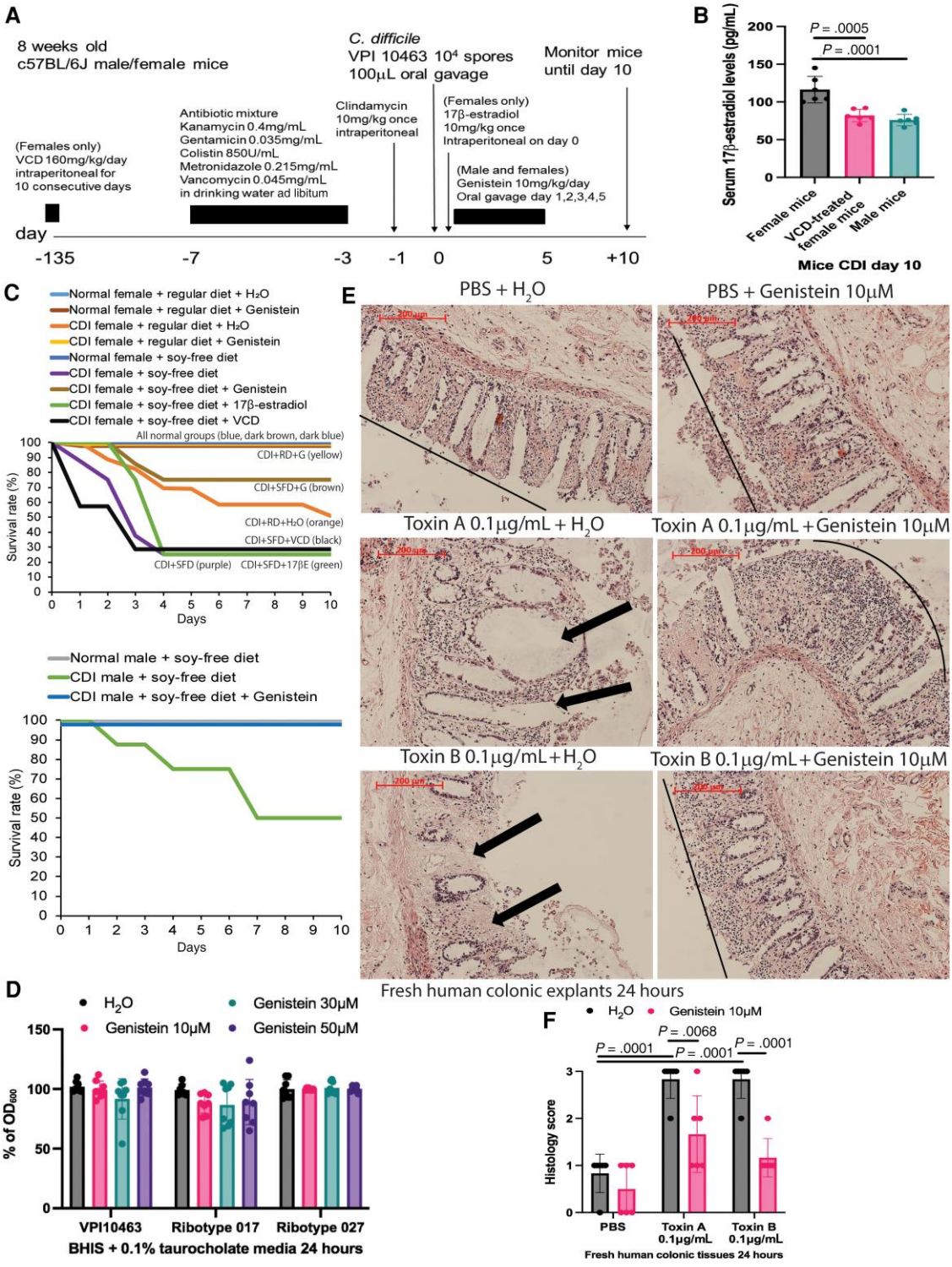

Figure 1. Estrogen reduced CDI severity in mice. *A*, Experimental plan of mouse primary CDI. Mice were infected with *Clostridioides difficile* VPI 10463. Some mice were treated with VCD, 17β-estradiol, regular diet, soy-free diet, and genistein. *B*, Serum 17β-estradiol levels in mice. *C*, Survival rate of female mice. Female mice were fed a regular or soy-free rodent diet from day −7 to day 10. Male mice were given a soy-free diet from day −7 to day 10. Mice were monitored daily for survival and body weight. Female mice on a soy-free diet or following estrogen depletion were more susceptible to CDI than estrogen-intact female mice on a regular diet; n = 8 mice per group. All results were pooled from 2 rounds of experiments. *D*, Antibacterial activity assays. Genistein did not affect *C. difficile* germination and growth. Results were pooled from 8 rounds of experiments. *E*, Hematoxylin and eosin-stained images of fresh human colonic tissues. Fresh human colonic tissues in serum-free RPMI1640 media were pretreated with water or 10 µM genistein for 30 minutes, followed by toxins (0.1 µg/mL) for 24 hours. Black lines indicated the locations of the top lining of colonic mucosa in water control and toxin- and genistein-treated groups. Arrows indicated sites of toxin-mediated disruption of mucosal integrity and thickness. Magnification ×100. *F*, Histology scores (epithelial tissue damage); n = 6 female patients. All error bars indicate standard deviation. Abbreviations: BHIS, brain heart infusion solution; CDI, *C. difficile* infection; OD, optical density; PBS, phosphate-buffered saline; VCD, 4-vinylcyclohexene diepoxide.

influence of dietary soy isoflavones on mice with CDI. After infection, female mice on the soy-free diet had a substantially lower survival rate (25%) than those on a regular diet (50%) (Figure 1*C*). However, a single intraperitoneal injection of 17β-estradiol delayed the onset of mortality by 2 days in estrogen-intact female mice on a soy-free diet (Figure 1*C*). Male mice with CDI had a 50% survival rate (Figure 1*C*).

Oral Genistein Treatment Improved CDI Survival in Mice

To validate the protective effects of genistein, a representative soy isoflavone, we administered genistein (10 mg/kg/day via oral gavage) to the mice on days 1, 2, 3, 4, and 5 postinfection. This dose has been shown to effectively ameliorate dextran sulfate-mediated colitis in mice [17]. Daily oral genistein treatment improved the survival rate among female mice on both regular and soy-free diets (Figure 1*C*). The same genistein treatment also prevented mortality among the infected male mice (Figure 1*C*). Neither VCD treatment, diet, nor genistein, affected body weight changes in male and female mice with CDI (Supplementary Figure 1A and 1B).

Our liquid chromatography/mass spectrometry analysis detected fecal genistein in control uninfected female mice on a regular diet (Supplementary Figure 1C and 1D). Oral genistein increased fecal genistein levels by 18-fold and produced low fecal levels of its metabolites (Supplementary Figure 1E).

Genistein Protected Against Toxin-Mediated Cell Death

Genistein showed no antibacterial effects against the *C. difficile* bacterium (Figure 1*D*). However, genistein pretreatment reduced epithelial layer disruption, as reflected by a lowered histology score, in fresh human colonic explants exposed to toxins A and B (Figure 1*E* and 1*F*). This observation suggests that genistein protected host cells against toxins. Genistein also diminished toxin-induced actin cytoskeletal disruption in human colonic fibroblasts, a key event in cell rounding (Supplementary Figure 2A). Another soy isoflavone, daidzein, and its metabolite S-equol, failed to prevent toxin-mediated cell rounding (Supplementary Figure 2B).

C. difficile toxin-induced cell rounding is known to be followed by apoptosis in human intestinal epithelial cells [21]. Genistein prevented toxin A- and B-mediated apoptosis in human colonic epithelial cells (Figure 2*A*). Both genistein-4′,7-diglucuronide and genistein-7-β-D-glucuronide-4′-sulfate are major circulating metabolites in humans [22], but neither showed antiapoptotic effects (Figure 2*A*). E2 estrogen (17β-estradiol), daidzein, and S-equol were also not antiapoptotic (Figure 2*A*). The optimal antiapoptotic concentration of genistein is 10 μM (Figure 2*B*).

Genistein Inhibited Apoptosis via ER, Lysine-Deficient Protein Kinase 1 Phosphorylation, and BCL-2-Associated X Protein Inhibition in Colonic Epithelial Cells

The antiapoptotic mechanism of genistein was further explored using qualitative protein arrays. Both toxins A and B increased proapoptotic BCL-2-associated X protein (BAX) protein expression in primary human colonic epithelial cells, which was reduced by genistein pretreatment (Figure 2*C*). Genistein also induced lysine-deficient protein kinase 1 (WNK1) phosphorylation in toxin-exposed primary human colonic epithelial cells (Figure 2*D*).

Additional quantitative validation by enzyme-linked immunosorbent assay (ELISA) indicated that genistein at 10 μM was optimal for inhibiting BAX protein expression and inducing WNK1 phosphorylation in toxin-exposed primary human colonic epithelial cells (Supplementary Figure 3A and 3B). Interestingly, WNK1 inhibition dephosphorylated WNK1, increased BAX expression, and promoted toxin B-mediated apoptosis (Supplementary Figure 3A–3C).

The genistein-mediated BAX suppression, WNK1 phosphorylation, and apoptosis inhibition were all reversed by the WNK1 inhibitor (WNK463) and an ER inhibitor (tamoxifen) (Figure 2*E* and 2*F*). The antiapoptotic effect of genistein was also abolished by a BAX inducer (BTSA1) (Figure 2*F*). On the other hand, GPER did not mediate the antiapoptotic effect of genistein, as the GPER inhibitor (G15) failed to reverse the genistein-mediated inhibition of apoptosis (Figure 2*F*). These results suggested that the ER-↑pWNK1-↓BAX cascade mediated genistein's antiapoptotic effect.

Genistein also protected epithelial barrier functions by reversing the toxin A- and B-mediated reduction of transepithelial electrical resistance through a human colonic epithelial Caco2 monolayer (Supplementary Figure 3D) and by preventing toxin B-mediated human colonic organoid disruption (Supplementary Figure 3E).

Genistein Inhibited Macrophage Inflammatory Protein-1α Secretion via ER, GPER, and WNK1 in Immune Cells

We previously demonstrated that macrophage inflammatory protein 1α (MIP-1α) mediates CDI infection [18]. Genistein inhibited toxin A- and toxin B-mediated MIP-1α secretion in fresh human colonic tissues and human primary PBMCs (Figure 3*A* and 3*B*). Neither genistein metabolites (genistein-7β-D-glucuronide-4-sulfate and genistein diglucuronide), soy isoflavone (daidzein), nor the daidzein metabolite (S-equol) affected toxin-induced MIP-1α secretion in PBMCs (Figure 3*C*). For comparison, 17β-estradiol protected against toxin A- but not toxin B-mediated MIP-1α secretion (Figure 3*C*).

Like human primary colonic epithelial cells (HPECs), genistein at 10 μM activated WNK1 phosphorylation in PBMCs (Figure 3*D*). The genistein-mediated inhibition of MIP-1α secretion was reversed by tamoxifen, G15, and WNK463 (Figure 3*E*), suggesting the involvement of ER, GPER, and WNK1. However, these inhibitors alone did not affect MIP-1α secretion in toxin-exposed PBMCs (Figure 3*F*).

Oral Intake of Genistein Reversed Colonic Injury in Mice With CDI

Oral genistein treatment effectively lowered CDI mortality among female mice (Figure 1*C*). Therefore, it was justified to

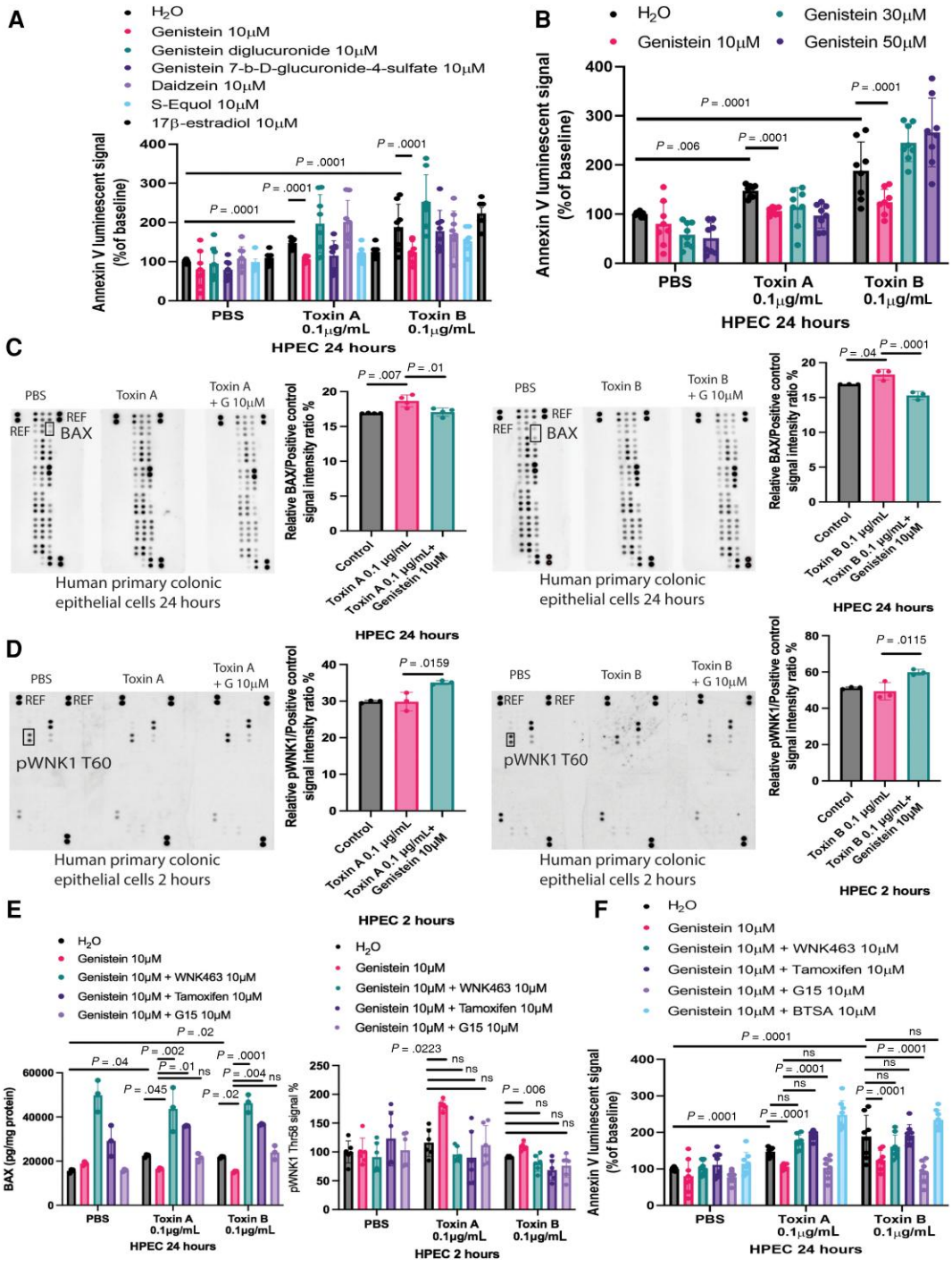

Figure 2. Genistein reduced colonic epithelial cell apoptosis via WNK1 phosphorylation. *A* and *B*, Apoptosis assays. HPECs were pretreated with genistein, metabolites, or estrogen for 30 minutes, followed by toxins. The annexin V luminescent signal above 100% indicated the occurrence of apoptosis. Toxins increased luminescence signal (apoptosis), which was prevented by genistein (10 μM) but not other metabolites. Results were pooled from 8 independent experiments. *C*, Apoptosis arrays. HPEC lysates were loaded onto array membranes. BAX protein (in rectangles) and reference signals were quantified. Genistein inhibited toxin-induced BAX protein expression. Results were pooled from 3–4 rounds of array experiments. *D*, Phosphokinase arrays. HPEC lysates were loaded to array membranes. Phospho-WNK1 T60 protein (in rectangles) and reference signals were quantified. Genistein induced WNK1 phosphorylation in HPEC. Results were pooled from 3 rounds of array experiments. *E*, BAX and pWNK1 ELISA. HPEC were pretreated with inhibitors and genistein for 30 minutes and incubated with toxins for 2–24 hours. Genistein-mediated BAX inhibition and WNK1 phosphorylation was reversed by WNK1 inhibitor (WNK463), ER inhibitor (tamoxifen), but not GPER inhibitor (G15). *F*, Apoptosis assay. HPEC were pretreated with genistein, metabolites, and inhibitors, followed by incubation with toxins and RealTime-Glo Annexin V Apoptosis Assay reagents for 24 hours. The Annexin V luminescent signal above 100% indicated the occurrence of apoptosis. Genistein (10 μM) inhibited apoptosis, which was reversed by the WNK1 inhibitor (WNK463), ER inhibitor (tamoxifen), and BAX1 activator (BTSA1). The GPER inhibitor (G15) did not affect genistein-mediated modulation in BAX expression and apoptosis. Results were pooled from 3 BAX ELISA, 6 pWNK1 ELISA, and 8 apoptosis assays. All error bars indicate standard deviation. Abbreviations: BAX, BCL-2–associated X; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; GPER, G-protein estrogen receptor; HPEC, human primary epithelial cell; ns, not significant; PBS, phosphate-buffered saline; REF, reference; WNK1, lysine-deficient protein kinase 1.

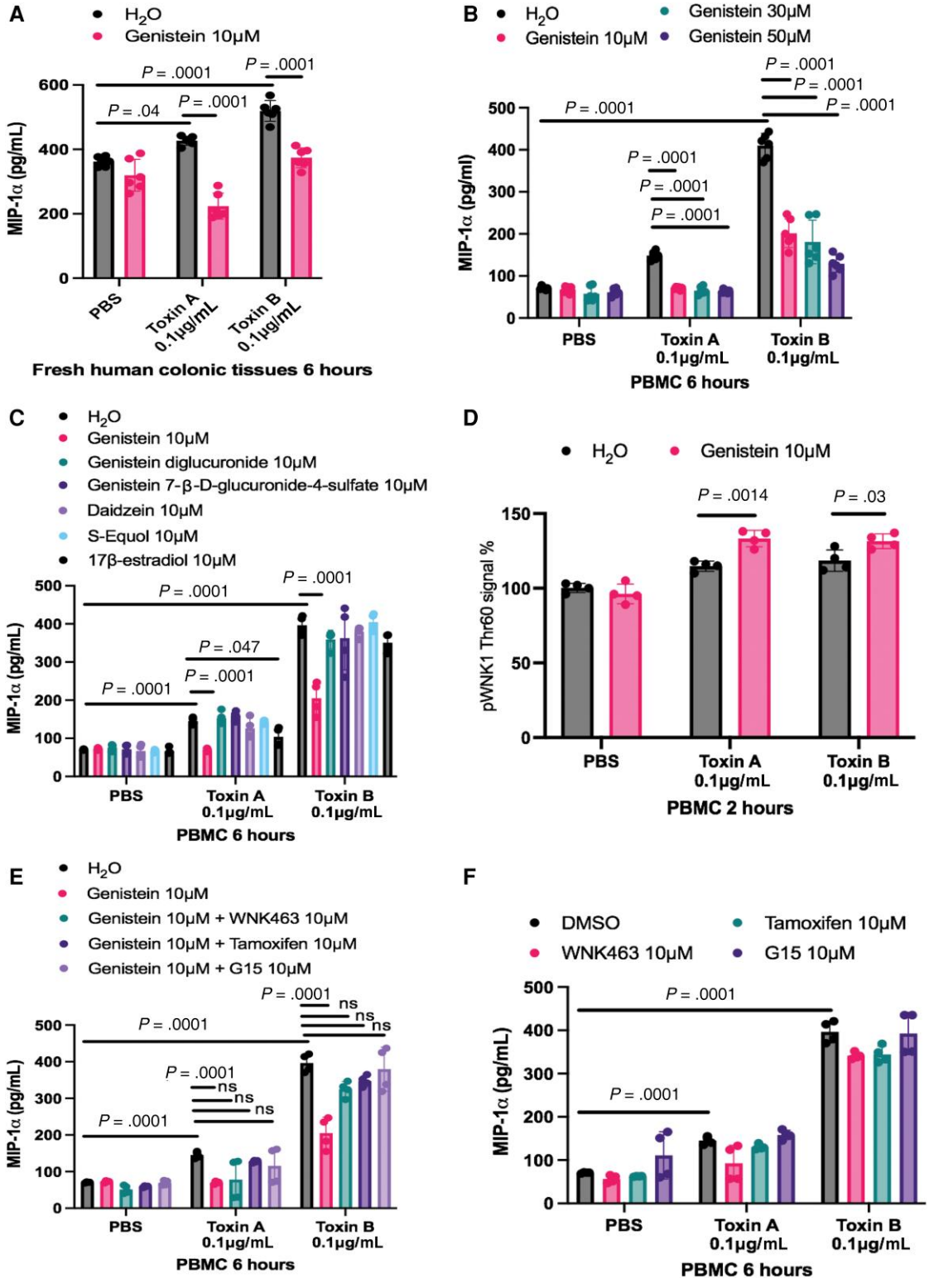

Figure 3. Genistein inhibited MIP-1α expression via WNK1. *A*, MIP-1α ELISA. Fresh human colonic tissues from 6 female patients were treated with genistein for 30 minutes, followed by toxins for 6 hours. Genistein diminished MIP-1α secretion. *B*, *C*, *E*, and *F*, MIP-1α ELISA. PBMCs from 3 female donors were treated with genistein, estrogen, metabolites, and inhibitors (WNK463 against WNK1, tamoxifen against ER, and G15 against GPER) for 30 minutes, followed by toxins for 6 hours. Genistein (10–50 μM) diminished MIP-1α secretion, which was reversed by inhibitors of ER, GPER, and WNK1. *D*, Phosphorylated WNK1 ELISA. PBMCs from 3 female donors were treated with or without genistein for 30 minutes, followed by toxins for 2 hours. The WNK1 phosphorylation in the cell lysates was measured by ELISA. Results were pooled from 4–6 independent experiments. All error bars indicate standard deviation. Abbreviations: DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; GPER, G-protein estrogen receptor; MIP-1α, macrophage inflammatory protein-1α; PBMC, peripheral blood mononuclear cells; WNK1, lysine-deficient protein kinase 1.

explore the therapeutic mechanism of genistein in CDI among female mice by treating the infected female mice with genistein (10 mg/kg/day) by oral gavage (Figure 4*A*). Genistein treatment did not affect fecal toxin levels (Figure 4*B*).

WNK1 and BAX are involved in the protective effects of genistein as oral administration of WNK463 and BTSA1 increased the mortality of the genistein-treated infected mice (Figure 4*C*). ER and GPER also partially mediated the therapeutic effects of genistein because oral administration of tamoxifen and G15 partially abolished the survival protection of genistein among the infected mice (Figure 4*C*). Neither genistein nor the drug treatments significantly influenced body weights among the infected mice (Figure 4*D*).

CDI generally caused epithelial disruption and neutrophil infiltration in the colonic mucosa of the infected mice, which was ameliorated by oral genistein treatment (Figure 4*E*). Oral treatment with inhibitors of ER, GPER, and WNK1 and activators of BAX abolished the protective effects of genistein (Figure 4*E* and 4*F*). However, these agents alone did not further worsen the colonic injury or affect body weight (Supplementary Figure 4A). Furthermore, ER and GPER regulated WNK1 signaling as their inhibitors reversed genistein-induced colonic WNK1 phosphorylation in the infected mice (Figure 5*A*).

In the infected mice, genistein reduced the high colonic proinflammatory Ccl3 and proapoptotic Bax mRNA expression (Figure 5*B* and 5*C*). The genistein-mediated Ccl3 inhibition was reversed by inhibitors of WNK1, ER, and GPER (Figure 5*B*). On the other hand, the genistein-dependent suppression of Bax was reversed by WNK1 and ER but not GPER inhibitors (Figure 5*C*). These findings address the importance of WNK1 and estrogen receptors for regulating genistein-dependent targets (Ccl3 and Bax) and colitis.

Genistein Prevented CDI Recurrence

We further determined the genistein effect on CDI recurrence (Figure 5*D*). As observed previously [23], vancomycin initially prevented CDI mortality, but the infected mice showed relapsing mortality after tapering vancomycin (Figure 5*E*). Interestingly, genistein prevented relapsing CDI mortality (Figure 5*E*) without affecting body weight among the vancomycin-treated infected mice (Figure 5*F*).

Similarly, *C. difficile* reinfection caused transient weight loss that was not observed in mice with genistein pretreatment (Supplementary Figure 4B and 4C). Flow cytometry indicated that the lowered circulating lymphocyte count in the genistein-treated infected mice (Supplementary Figure 4D) might be associated with reduced CDI activity. Interestingly, genistein increased CD8 memory T-cell count in the intraepithelial lymphocyte compartment (Supplementary Figure 4E), which may help protect against reinfection [24].

Genistein Ameliorated Primary and Relapsing Hypervirulent CDI in Hamsters

A clinical study showed that toxin B inhibition is crucial for ameliorating CDI [6]. Toxin A, but not toxin B, causes injury in the mouse colon [18], while both toxins mediate cecitis in hamsters [18]. To further validate the therapeutic effect of genistein against CDI caused by hypervirulent strains, we infected toxin B-responsive hamsters with hypervirulent *C. difficile* strains, followed by oral genistein treatment (Figure 6*A*).

The A^-B^+ribotype 017 only caused minor mortality, while the A^+B^+ribotype 027 caused severe mortality among infected hamsters (Figure 6*B*). All treatments had minimal impact on body weight (Figure 6*B*). CDI led to cecitis with disrupted cecal mucosal epithelium and thickened submucosa due to immune cell infiltration, as quantitatively reflected by increased histology scores (Figure 6*C*). Ribotype 027 infection led to slightly higher cecal histology scores and MIP-1α levels than ribotype 017 infection (Figure 6*C*).

Oral genistein treatment reduced mortality, ameliorated cecal injury, lowered histology scores, and reduced cecal MIP-1α levels in the infected hamsters without affecting cecal toxin levels (Figure 6*B*–6*D*). This survival protection was WNK1 dependent, as WNK463 reduced the survival rate in genistein-treated hamsters (Figure 6B). Oral genistein also partially prevented recurrence-associated mortality in ribotype 027-infected hamsters after tapering vancomycin (Figure 6*E*).

Intestinal Microbiome Characteristics in Infected Animals

Shotgun metagenome sequencing showed that genistein reduced the elevated fecal alpha diversity in infected mice but not infected hamsters (Supplementary Figure 5A–5C). Genistein also altered fecal microbial composition in mice but not hamsters, as shown by beta diversity (Supplementary Figure 6*A*–6*C*). However, genistein did not affect the fecal and cecal abundance of *C. difficile* and other dominant bacterial species in the infected mice and hamsters, respectively (Supplementary Figure 7A–7C).

DISCUSSION

This study is the first to demonstrate the anti–MIP-1α and anti-apoptotic effects of genistein therapy. Genistein reduced CDI disease activity by inhibiting proinflammatory cytokine expression and apoptosis via the ER/GPER/WNK1 pathways.

The increased mortality in VCD-treated infected mice (Figure 1*C*) and elevated hospitalization risk among postmenopausal women with CDI consistently indicated the importance of endogenous estrogen [8]. VCD treatment and ovariectomy are commonly used for simulating menopause. However, we preferred the VCD approach as the reduction in estrogen level is gradual, while the androgen-secreting capability of the ovary is preserved [20].

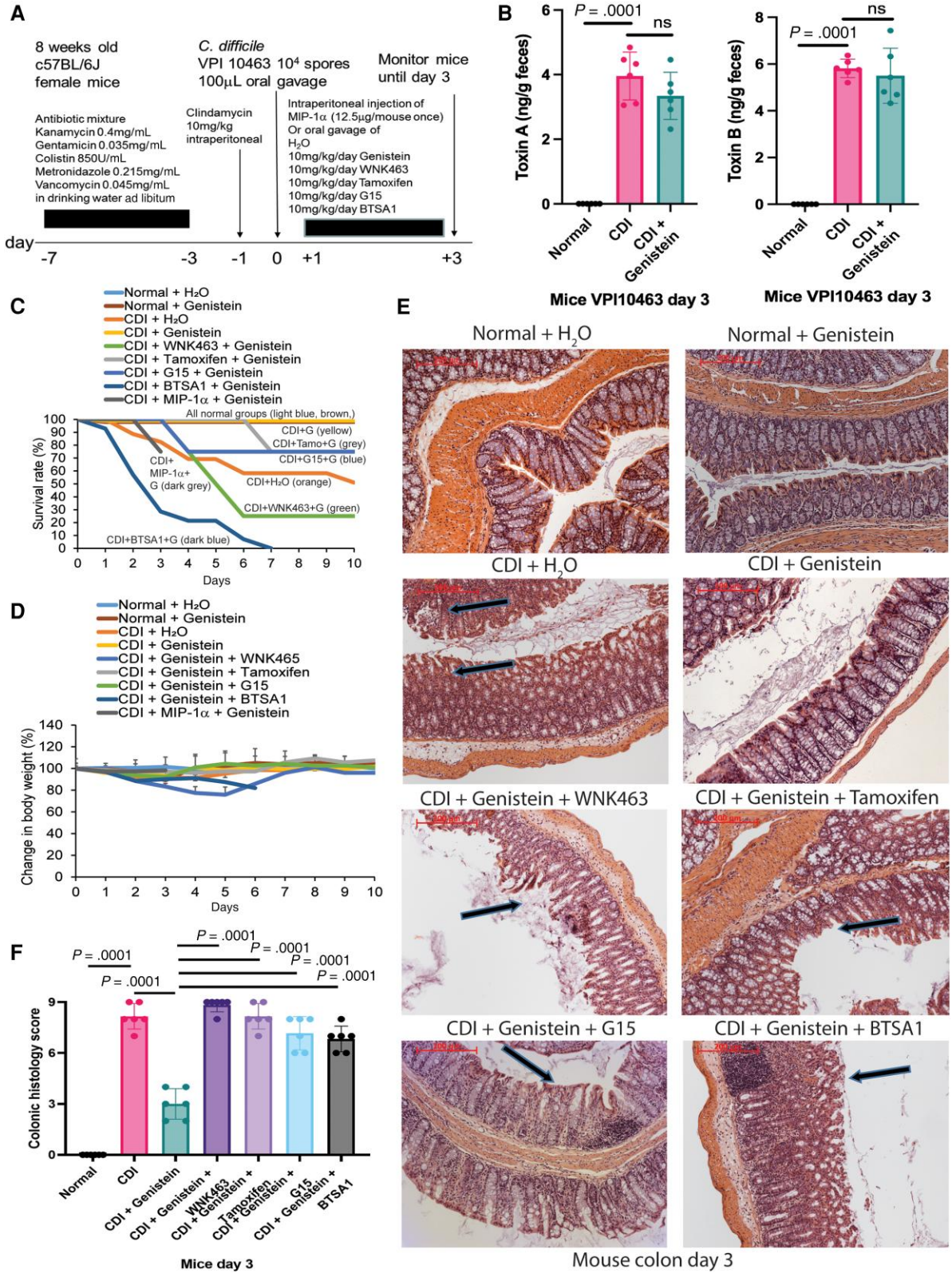

Figure 4. Genistein reduced CDI severity in mice. *A*, Experimental plan of mouse primary CDI. Mice were infected with *Clostridioides difficile* VPI 10463. *B*, Fecal toxin levels. *C*, Survival rate. Inhibitors of WNK1, ER, and GPER and activator of BAX reversed the prosurvival effect of genistein. *D*, Changes in body weight. *E*, Hematoxylin and eosin-stained images of colonic tissues. Arrows indicated disrupted colonic mucosa and immune cell infiltration. Magnification ×100. *F*, Colonic histology score. Inhibitors of WNK1, ER, and GPER and activator of BAX reversed the protective effect of genistein; n = 6 female mice per group. All results were pooled from 2 rounds of experiments. All error bars indicate standard deviation. Abbreviations: BAX, BCL-2–associated X; CDI, *C. difficile* infection; ER, estrogen receptor; GPER, G-protein estrogen receptor; MIP-1α, macrophage inflammatory protein-1α; ns, not significant; WNK1, lysine-deficient protein kinase 1.

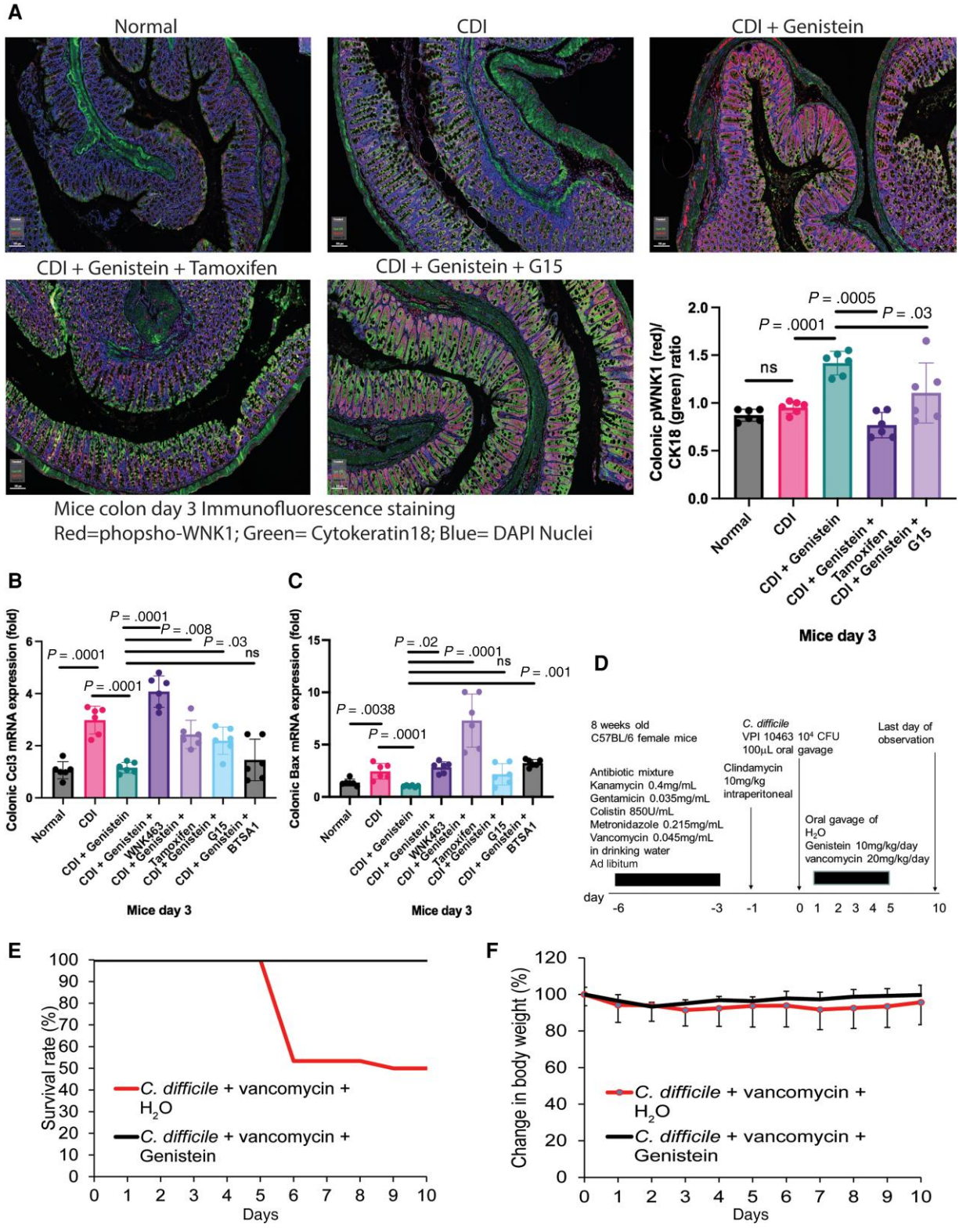

Figure 5. Genistein prevented CDI recurrence in mice. *A*, Immunofluorescence of phosphorylated WNK1 (red) and cytokeratin 18 (green) with blue nuclear DAPI staining of mouse colonic tissues. The 100-μm scale bar is located at the lower-left corner of each image. The bar chart showed that genistein increased colonic phosphorylated WNK1 signal in the infected mice, which was reversed by tamoxifen and G15. *B* and *C*, Colonic Ccl3 and Bax mRNA expression. Genistein reduced colonic Ccl3 and Bax mRNA expression. BAX activator (BTSA1) reversed the genistein-mediated inhibition of Bax mRNA expression. *D*, Experimental plan of mouse relapsing CDI. *E*, Survival rate. Genistein prevented CDI recurrence. *F*, Changes in body weight; n = 6 female mice per group. All results were pooled from 2 rounds of experiments. All error bars indicate standard deviation. Abbreviations: Bax, BCL-2–associated X; Ccl3, CC chemokine ligand 3; CDI, *C. difficile* infection; CFU, colony-forming unit; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant; WNK1, lysine-deficient protein kinase 1.

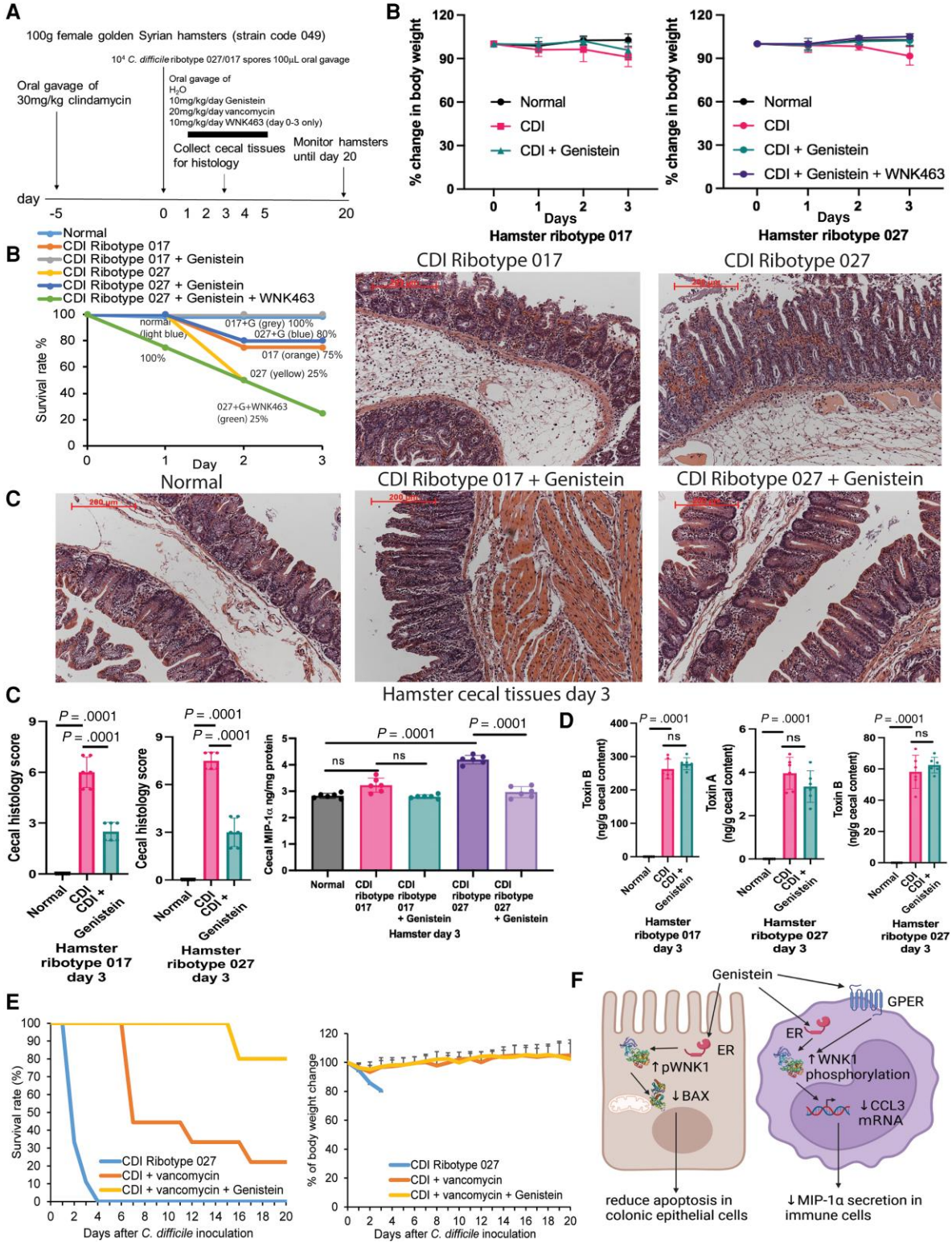

Figure 6. Genistein reduced CDI severity in hamsters. *A*, Experimental plan of hamster primary and relapsing CDI. Hamsters were infected with hypervirulent *Clostridioides difficile* strains. *B*, Survival rate and changes in body weight in primary CDI infection. Genistein improved survival; n = 10 hamsters per group. Results were pooled from 2 rounds of experiments. *C* and *D*, Cecal hematoxylin and eosin-stained images, histology scores, cecal tissue MIP-1α levels, and cecal content toxin levels. CDI caused cecitis with increased histology scores and cecal MIP-1α levels, which were reduced by genistein; n = 6 hamsters per group. Results were pooled from 3 rounds of experiments. *E*, Survival and changes in body weight in vancomycin-associated CDI recurrence; n = 10 hamsters per group. All results were pooled from 2 rounds of experiments. *F*, Mechanisms of action of genistein. The graphical image was created by Biorender. All error bars indicate standard deviation. Abbreviations: BAX, BCL-2–associated X; Ccl3, CC chemokine ligand 3; CDI, *C. difficile* infection; GPER, G-protein estrogen receptor; MIP-1α, macrophage inflammatory protein-1α; ns, not significant; ER, estrogen receptor; WNK1, lysine-deficient protein kinase 1.

Genistein has been widely used as a phytoestrogen supplement for postmenopausal women. It exists in the soy diet, commonly consumed by the Asian population. High soy consumption is sufficient to relieve hot flashes in Japanese women [25]. Interestingly, the Asian population tends to develop milder CDI symptoms than the Western population [26]. The cause of this difference is unknown but may be related to *C. difficile* strains, diet, environment, and genetics. As dietary soy isoflavones reduced the CDI severity in mice, the association between CDI severity and dietary soy consumption among the Asian population merits further investigation.

Although ER and GPER inhibitors alone did not affect BAX expression, WNK1 phosphorylation, or apoptosis in colonic epithelial cells (Supplementary Figure 3A–3C), a low concentration of genistein (10 μM) via the estrogen receptor exerted antiapoptotic effects (Figure 2*F*). Genistein at low concentrations also stimulates epithelial cell proliferation [27], presumably because WNK1 is involved in the cell survival PI3K-AKT pathway [28]. Although we could not perform extensive mechanistic experiments with hamsters due to their size, expense, and limited laboratory capacity, the central role of WNK1 in the survival protection of genistein among infected mice and hamsters was consistent (Figure 4*C* and Figure 6*B*). Genistein up to 50 μM did not induce apoptosis and BAX expression in HPEC (Figure 2*B* and Supplementary Figure 3A). However, the high concentration of genistein (100 μM) inhibits cell viability and other cellular functions [29–31].

Many reports demonstrated the diverse anti-inflammatory activities of genistein via various signaling pathways [32]. Genistein inhibits phospholipase A2 [33], cyclooxygenase 2 [34], and tyrosine kinases [35], which may protect against toxin A-mediated intestinal damage [36]. Genistein may also mediate anti-inflammatory effects via WNK1 in other conditions, as WNK1 knockdown activates cytokine production in lipopolysaccharide-treated macrophages [37].

All female sex hormones (estradiol, estrone, and estriol) cannot inhibit *C. difficile* growth [38]. The anti-inflammatory effect of 17β-estradiol in toxin A-exposed PBMC (Figure 3C) may explain the transient survival protection of estrogen in the infected female mice (Figure 1*C*). However, the low long-term survival rate of this one-time estrogen injection group suggested that persistent estrogen treatment is necessary to maintain survival in the infected mice (Figure 1*C*). In CDI patients, toxin B is the primary pathogenic factor [6]. Estrogen could not inhibit toxin B-mediated apoptosis (Figure 2*A*) and MIP-1α secretion (Figure 3*C*). Estrogen in menopausal hormone therapy may also pose a cancer risk, while soy isoflavones are safer alternatives for treating menopausal symptoms [39]. As the utility of exogenous estrogen treatment to mitigate CDI risk among postmenopausal women is unclear, genistein is superior to exogenous estrogen in protection against CDI.

Aged mice (12–14 months) develop more severe CDI colitis than young (2–4 months) mice [40]. Age is unlikely to be a confounding variable in our study because we injected 2-month-old (8 weeks) mice with VCD, followed by 135 days (4.5 months) waiting period until they reached 6.5 months. For other groups, 2-month-old female and male mice were infected and treated. Based on our experience in a previous study [41], infected male and female mice on a regular diet had a comparable survival rate of about 50%. We noted that male mice on a soy-free diet had a similar survival rate as female mice on a regular diet (Figure 1*C*), indicating no effect of dietary genistein or phytoestrogen on the survival of infected male mice.

Although another soy isoflavone, daidzein, could not relieve menopausal symptoms in postmenopausal women [42], 33% of the white population metabolizes daidzein into equol in the gut by bacteria [43]. Like genistein [44], the ERβ-binding equol is a dietary supplement used to relieve menopausal symptoms [45]. Daidzein does not inhibit *C. difficile* growth, while S-equol inhibits *C. difficile* growth and spore formation but does not reduce toxin production [38]. However, daidzein and S-equol are unlikely to confer protection against CDI because both failed to prevent toxin-mediated apoptosis, MIP-1α secretion, and cell rounding (Figure 2*A*, Figure 3*C*, and Supplementary Figure 2B).

Short-term genistein treatment did not affect the abundance of *C. difficile* and other dominant bacterial species in the infected animals (Supplementary Figure 7A–7C). We also did not find any significant enhancement of anti-*C. difficile* bacterial species in genistein-treated animals (Supplementary Figure 7A–7C). As genistein did not consistently affect gut microbiota diversities and abundances among animal models (Supplementary Figures 5–7), the association between genistein's effect and changes in the intestinal microbiome is unclear.

CDI is a life-threatening disease and survival rate is one of the metrics to evaluate disease activity. The mortality rate of CDI animals in our study was comparable to other studies [19, 41, 46, 47]. The weight loss of animals after *C. difficile* inoculation were also comparable to other studies (Figure 4*D*, Figure 5*F*, Figure 6*B* and 6*E*, and Supplementary Figure 1A and 1B) [23, 48].

In summary, dietary soy isoflavones affect survival in female mice with CDI. Oral genistein improved survival in the infected mice and hamsters. Our study is the first to demonstrate the protective roles of genistein against the detrimental effects of *C. difficile* toxins via WNK1/ER/GPER-dependent antiapoptotic effect in epithelial cells and inhibition of MIP-1α expression in PBMCs (Figure 6*F*). The significance of genistein and soy consumption in reducing CDI risk or recurrence prevention merits further investigation.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not

copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Y. X., L. F., A. C. E., B. N., A. B., and K. F. F. performed the experiments. H. F. produced *C. difficile* toxins. H. W. K. contributed to the conception and design, supervised the entire study, and wrote the manuscript. M. S. supervised and sponsored Y. X. and critically reviewed the manuscript. All authors have read, assisted with editing, and approved the final version of this manuscript.

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Data sharing. We may share additional unpublished data from the study; please contact H. W. K.

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