Drug Design, Development and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Caffeic Acid Protects Against Ulcerative Colitis via Inhibiting Mitochondrial Apoptosis and Immune Overactivation in *Drosophila*

Minghui Xiu[1,2,*], Botong Li[3,*], Li He[1], Yan Shi[3], Yongxuan Zhang[1], Shihong Zhou[1], Yongqi Liu[2,3], Ningbo Wang[4], Jianzheng He[2,3,5]

[1]College of Public Health, Gansu University of Chinese Medicine, Lanzhou City, Gansu Province, People's Republic of China; [2]Key Laboratory of Dunhuang Medicine, Ministry of Education, Lanzhou City, Gansu Province, People's Republic of China; [3]Provincial-Level Key Laboratory for Molecular Medicine of Major Diseases and the Prevention and Treatment with Traditional Chinese Medicine Research in Gansu Colleges and University, Gansu University of Chinese Medicine, Lanzhou City, Gansu Province, People's Republic of China; [4]Tibetan Medical College, Qinghai University, Xining City, Qinghai Province, People's Republic of China; [5]Research and Experimental Center, Gansu University of Chinese Medicine, Lanzhou City, Gansu Province, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jianzheng He, Email hejianzheng1006@163.com

Background: Ulcerative colitis (UC) is a chronic intestinal inflammation that is prone to relapse and is difficult to fully recover; therefore, there is a need for safer alternative treatments. Caffeic acid (CA) is a natural polyphenolic compound that has antioxidant and anti-inflammatory properties. However, the beneficial effects and mechanisms of action of CA in UC remain unclear.

Purpose: This study evaluated the protective effect of CA against dextran sulfate sodium (DSS)-induced intestinal injury in *Drosophila* melanogaster model.

Results: Oral administration of CA significantly reduced body damage in UC flies, improved their survival rate, restored damaged digestion, and improved locomotion. CA supplementation significantly alleviated intestinal damage in UC flies by restoring excretion balance, repairing intestinal atrophy, improving acid-base balance imbalance, inhibiting intestinal structural destruction, inhibiting intestinal epithelial cell death and intestinal stem cell (ISC) excessive proliferation, and reducing the number of harmful bacteria. Mechanistic studies found that CA significantly reduced the expression of Toll and Imd pathway genes (including *Myd88, Dif, PGRP-LC, Imd, Rel*, and *Dpt*), reduced ROS levels and the expression of apoptosis-related genes (*Debcl, Cyt-c-p, DrlCE, Dronc*, and *Dark*), and increased ATP and MFN2 levels.

Conclusion: CA alleviated intestinal damage mainly by inhibiting the Toll and Imd signaling pathways and inhibiting apoptosis mediated by mitochondrial damage. These findings suggest that CA holds promise as a potential therapeutic for UC treatment.

Keywords: ulcerative colitis, caffeic acid, toll and Imd pathways, apoptosis, *Drosophila melanogaster*

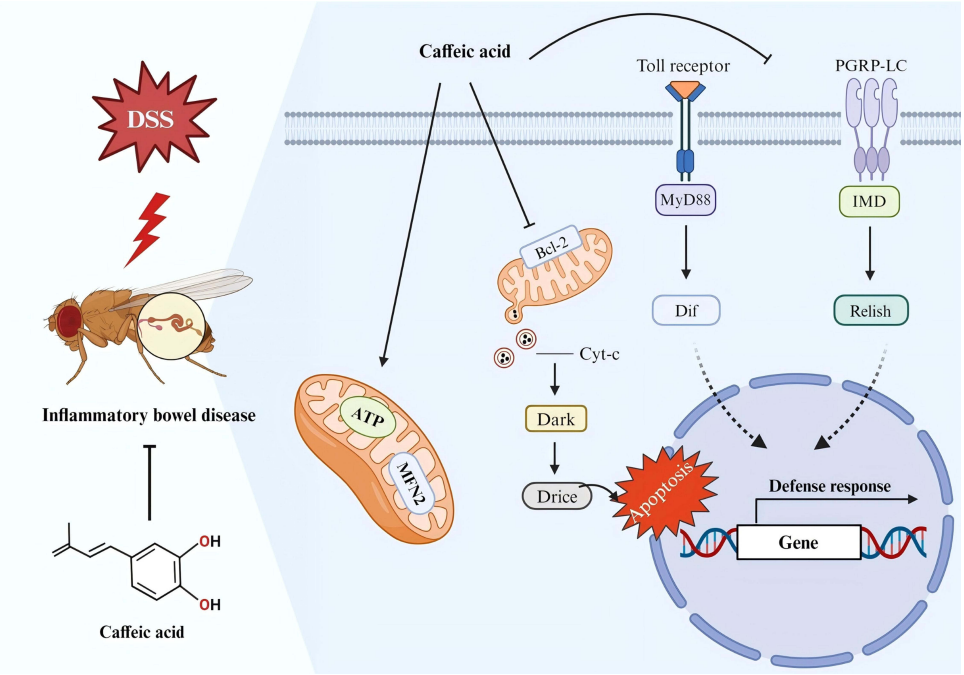
Introduction

Ulcerative Colitis (UC) is a chronic non-specific inflammatory bowel disease characterized by inflammatory lesions of the colorectal mucosa and its underlying mucous purulent and bloody stools, and absorption disorder.[1] Along with the westernization of lifestyle, the incidence of UC in developing countries is increasing.[2,3] Drugs for the treatment of UC usually include aminosalicylic acid, glucocorticoids, and immunosuppressants, but they have disadvantages such as high cost, limited long-term efficacy, and significant side effects.[4,5] Therefore, it is critical to search for safe and effective drugs for UC treatment.

UC involves multiple genetic targets and is primarily associated with intestinal immune dysfunction and mitochondrial apoptosis.[6,7] Some studies have indicated that abnormal immune responses contribute to the pathogenesis of UC.[8,9] This process is regulated by many molecular signaling pathways, including JAK/STAT, PI3K/Akt, NF-κB, Toll, and Imd.[10–12] These related components of cell signaling pathways have the potential to be potential therapeutic targets. In addition, excessive apoptosis and insufficient proliferation of intestinal epithelial cells are important mechanisms of intestinal mucosal

Received: 25 November 2024
Accepted: 12 March 2025
Published: 22 March 2025

Graphical Abstract



injury in UC.[13] Studies have confirmed that Bcl-2 and Bax are common marker proteins for apoptosis and may be involved in regulating apoptosis in the mitochondrial pathway, thereby attenuating UC colon injury.[14,15] Therefore, modulation of host immune homeostasis and blocking the necrotic apoptotic pathway of epithelial cells are essential for the treatment of UC.

Caffeic acid (CA) is a natural polyphenolic compound derived from plants, which possesses various pharmacological activities, such as antioxidant, anti-inflammatory, antibacterial, and antiviral.[16–18] For example, CA supplementation attenuated the age-associated hyperproliferation of intestinal stem cells (ISCs) by suppressing oxidative stress-associated JNK signaling in flies.[19] In Alzheimer's disease rodent models, administration of CA improved cognitive skills and redox state by lowering GSK3β levels and reducing the formation of amyloid-like plaques.[20] Although CA has demonstrated antioxidant effects in aging and neurodegenerative diseases, its role in intestinal inflammation and immune modulation, particularly through the Toll andImd pathways, remains unexplored. *Drosophila melanogaster* has the advantages of excellent genetic tools, short lifespan, easy handling, and remarkable phenotype, and its intestinal function is similar to that of mammals, which makes it a classical in vivo model for studying intestinal physiology and immunity.[21,22] Many studies have shown that *Drosophila* can be used to screen plant extracts for potential applications in many chemical-induced intestinal disorders.[23–25] Moreover, compared to rodent models, *Drosophila* offers a genetically tractable and cost-effective platform for studying intestinal physiology and immune responses, which are highly conserved across species.

In summary, this study aimed to investigate the potential protective effects of CA against dextran sodium sulfate (DSS)-induced intestinal injury by utilizing a *Drosophila* model. Through this exploration, we attempted to elucidate the underlying mechanisms by which CA may help protect against intestinal injury, thereby gaining insight into its potential for application in the treatment of UC.

Methods and Materials

Drosophila Strains and Culture

All fruit flies were reared on common corn agar medium at 25 °C under a relative humidity (65%) and a 12 h light/dark cycle. The fly strains used in this study were as follows: w^{1118} (#5905) that got from the Bloomington *Drosophila* Stock

Center (BDSC); *Esg-Gal4, UAS-GFP* was generously provided by Dr. Lihua Jin, Northeast Forestry University; and *Myo1A-GAL4, UAS-GFP* was kindly given by Dr. Rongwen Xi, NIBS, Beijing, China.

Reagents

CA (#B206220, ≥98%) was purchased from Yuanye Biotechnology Co. (Shanghai, China). DSS was obtained from MP Biomedicals LLC (USA). Sucrose, Erioglaucine disodium salt (SHBN8755), bromophenol blue (SHBN7603), and dihydroethidium (088M2512V) were purchased from Sigma-Aldrich (USA).

Induction of Ulcerative Colitis

2–4d aged adult fruit flies were fed 4% DSS to establish an UC model. The experiment was divided into control, model, 0.5 and 1 mm CA groups. After feeding with culture medium or culture medium containing different concentrations of CA for seven days, flies were transferred to vials containing filter paper soaked in different solutions (control group: 5% sucrose; model group: 5% sucrose and 4% DSS; treatment groups: different concentrations of CA, 5% sucrose, and 4% DSS) for further research (Supplementary Figure 1).The above methods refer to previous studies.[25]

Survival Rate

20 males or females per vial were transferred into vials containing five layers of filter paper infiltrated with 4% DSS and 5% sucrose solution or 0.6% SDS and 5% sucrose solution, with or without CA (0.5 and 1 mm), respectively. Filter papers were changed every three days, and the number of dead flies was recorded until all flies died. 6 vials replicates were used for each treatment group.

Climbing Ability

Different groups of female flies (20/vial) were transferred to plastic tubes (12×2 cm) for acclimatization for 5 min. Afterwards, the bottom of the test tube was gently tapped every 2 min, and crawling time and distance were recorded using a video camera. Each treatment group was repeated 6 vials. Climbing indices were calculated according to a previously described method with minor modifications.[26]

Food Intake

Different groups of female flies (20 flies/vial) were transferred to plastic tubes (12×2 cm) for acclimatization for 5 min. Each treatment group was repeated 6 vials.Food consumption was performed as previously described with minor modifications.[23] Briefly, female flies were starved for 18 h, and then were fed with food containing 5% sucrose and 2% Bromophenol blue for 4 h. Fly abdomen colour was observed under a microscope and scored. Abdomen scores range from 0 to 3, with a colorless abdomen indicated as 0, less than 1/3 of the length of the abdomen as 1, between 1/3 and 1/2 as 2, and greater than 1/2 of the length of the abdomen as 3.

Excretion

Different groups of female flies (20/vial) were transferred to plastic tubes (12×2 cm) for acclimatization for 5 min.Each treatment group was repeated 6 vials. Female flies were starved for 6 h and cultured in dyed medium for 12 h. The staining medium was prepared as previously described.[25] Then, the cells were washed with PBS, and the absorbance of the mixed solution was measured at 625 nm.

Intestinal Length

Female flies were transferred to vials containing filter paper moistened with 5% sucrose, 4% DSS, and 1 mm CA, and stressed for 72 h. Randomly select 12 female flies from each group of 20 for dissection in paraformaldehyde. Measure intestinal length using a vernier caliper.

"Smurf" Assay

Different groups of female flies (20/vial) were transferred to plastic tubes (12 × 2 cm) for acclimatization for 5 min.Each treatment group was repeated 6 vials.The "Smurf" assay can be used to analyze the integrity of the intestinal barrier in *Drosophila*.[25] Briefly, adult females were placed in a tube with filter paper saturated in 5% sucrose with or without 4% DSS for 72 h. After starvation for 4 h, the flies were transferred to vials containing food with blue dye (2.5% w/v) for 18 h. The number of "Smurfs" was observed under a microscope.

Intestinal Acid-Base Homeostasis

Different groups of female flies (20/vial) were transferred to plastic tubes (12 × 2 cm) for acclimatization for 5 min.Each treatment group was repeated 6 vials. Intestinal acid-base homeostasis was evaluated by observing the *Drosophila* copper cell region(CCR).[27] Briefly, Female flies were starved for 6h, and cultured on dyed medium for 4 h. The intestines were dissected to observe the CCR and immediately photographed. The staining medium was prepared as previously described.[28] Each set of experiments was repeated three times.

Trypan Blue Assay

The methodology and scoring rules for Trypan blue were performed as previously described.[25] Briefly, randomly select 12 female fruit flies from each group, the intestines of females fed with or without DSS for 72 h were dissected, washed in cold PBS, and stained with trypan blue for 20 min. After washing with PBST, blue staining of the midgut was performed under a microscope.

Ultrastructure of the Epithelial Cells

The ultrastructure of the fly gut was detected using transmission electron microscopy (TEM).[28] Female fly midguts were dissected in pre-cooled PBS, fixed in 2.5% glutaraldehyde at 4 °C overnight, and washed with PBS. The ethanol gradient was then dehydrated and rinsed with acetone. Subsequently, the samples were transferred to embedding plates by adding 100% Spon812 resin and heating at 60 °C for 24 h. Ultrathin sections (100 nm) were cut, transferred to silicon, stained with drops of 1% lead citrate, and rinsed. The samples were then observed using a transmission electron microscope (Hitachi HT7700).

Immunohistochemistry

The dissected intestines of transgenic *Myo1A-GAL4; UAS-mCD8::GFP* and *Esg-GAL4; UAS-mCD8::GFP* females were exposed to 5% sucrose with or without 4% DSS for 48 h, fixed with 4% paraformaldehyde for 30 min, and washed with PBST. Subsequently, the samples were observed under an Olympus FV1000 confocal laser scanning microscope (Olympus, Japan). Randomly select 6 flies from each group of 20.The fluorescence of GFP^{+} cells was measured using the ImageJ software.

Tunnel Staining

To detect DSS-induced apoptosis in intestinal cells, a tunnel assay was performed as previously described with minor modification.[29] Briefly, 5 intestines per group were dissected in cold PBS, the isolated guts were fixed with 4% paraformaldehyde for 20 min, and washed in PBS. The guts were permeabilized with 0.1% Sodium Citrate buffer and 0.1% Triton X-100 in PBS for 30 min and washed briefly with PBS. Afterwards, the guts were incubated in a Tunnel reaction mix at 37 °C for 1 h and rinsed with PBS. Finally, the samples were stained with 4'6-diamidino-2-phenylindole (DAPI) for 10 min and observed under a fluorescence confocal microscope.

DHE Staining

6 flies in each group were dissected for their intestines in cold PBS, incubated with 30 μM DHE for 15 min, washed with PBST, and fixed with paraformaldehyde in a dark environment. Subsequently, the images were visualized using a confocal laser scanning microscope and processed using the ImageJ software.

ATP and MFN2 Analysis

9 vials in each group with 20 flies in each vial were homogenized in the pyrolysis liquid and centrifuged at 3000 rpm for 10 min at 4°C. ATP content and MFN2 (mitofusin 2) content were determined using the ELISA kit (Enzyme-linked Biotechnology Co., Ltd, Shanghai, China). The ATP and MFN2 concentrations were determined using a SpectrARax i3x Microplate reader.[30]

Transcriptome Analysis

According to the manufacturer's instructions, total RNA was extracted from the intestines of 180 flies in each vial using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), with 3 vials replicates per group. RNA purity and quantification were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit, according to the manufacturer's instructions. Transcriptome sequencing and analysis were conducted by OE Biotech Co. Ltd. (Shanghai, China). The samples were further screened and analyzed for differential gene expression, enrichment, and cluster analysis. DEG screening was performed using universal parameter thresholds, specifically q value < 0.05, fold change >1.5, or fold change < 0.5. All functional and enrichment analyses of the DEGs were performed using R (v. 3.2.0) and ChiPlot (http://www.chiplot.online).

RT-qPCR Analysis

Total RNA was extracted from 90 female guts per vial using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with 9 vials replicates per group. Total RNA was reverse-transcribed into cDNAusing Hieff® reverse transcriptase (Shanghai YEASEN, China) and quantified by real-time PCR using SYBR Green (Shanghai YEASEN, China). Rp49 was used as the reference gene, and calculations were performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Molecular Docking

Molecular docking was used to predict the binding affinity and conformation of the CA and its core targets. The sdf format files of CA and receptor protein structures were downloaded from PubChem and RCSB PDB databases, respectively. The CA and protein structures were processed using PyMOL software and AutoDock Tools 1.5.6. Molecular docking of compound targets was performed using Autodock Vina 1.1.2, and visualized using Discovery Studio software. Ligand binding was considered stable if the ligand-receptor binding energy was less than −5.0 kcal/mol.

Table 1 List of Forward and Reverse Primers

Genes	Forward	Reverse
Bcl-2	CTGATCGACAACGGCGGATGG	ACAGCAGCGAATACAGTTGACCTC
Cyt-c-p	CTGAAGTCGGCGACCAAGTA	GCCGGACTGTGACTGAACAC
Caspase-3	GATTCGATGTACGCACCTTCG	ATCCTCCCGAGCAACCTCTT
Dronc	CCTTTATCTCGCTAAACGAACGG	AGCTTGCTAACGCAGGGTC
Apaf-1	GAGCATTCGGGATGGTCTGG	TATGTGGGCTGATGGAGGGA
PGRP-LC	AAACGATCCGTTGACTGGAC	TACGCTTGGATTCCGTTTTC
IMD	TTCGGCTCCGTCTACAACTT	GTGATCGATTATGGCCTGGT
Relish	ACAGCTACAGGAACTGCATCAGGAA	TCATCCTCCTCGAAGAACCTCACT
Myd88	GGCTCGTTCCCTACACGATC	GAATGCTGGGAGTGGTCACC
Dif	ATGTTTGAGGAGGCTTTCGG	GAACCGGCGGTGCGACCCTCGC
PGRP-SC1a	CATCGACGAGGGCTGTAAAT	CCCGACTGCCTAATAAACCA
ND42	CGTTTCGATGTCCCGGAGCT	GTCTGCATTGTAGCCAGGAC
SDHC	TGACCGCCCTCAAGTTTATC	GCCAAGATAGCGGATAGCAC
RP49	CTTCATCCGCCACCAGTC	GCACCAGGAACTTCTTGAATC

https://doi.org/10.2147/DDDT.S499284

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (S.E.M). Statistical analyses were performed using GraphPad Prism, version 9.3.0. The survival rates of the groups were compared using the log-rank (Mantel-Cox) test for significance. The two trial groups were analyzed using unpaired Student's *t*-test. Differences between three or more groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The significance levels were $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

CA Has a Strongly Anti-Inflammative Effect

To determine the protective effect of CA against inflammation, adult female and male mice were treated orally with SDS. SDS can interfere with the normal function of the intestinal barrier, and stimulate local and systemic inflammation.[31] After exposure to SDS, administration of CA at 0.5 and 1 mm significantly improved the survival rate of female or male flies in a dose-dependent manner (Figure 1A and B). To further determine the rescue effect of CA on UC, survival rates of female and male flies under DSS stimulation were measured. DSS can destroy the integrity of the intestinal mucosal barrier, increase the permeability of the colonic epithelium, and aggravate intestinal bleeding.[32] 0.5 and 1 mm CA supplementation remarkably increased the survival rate of male or female flies under DSS stimulation in a dose-dependent manner (Figure 1C and D). These findings suggest that CA plays an important role in flies.

CA Improves Motility and Digestion in UC Flies

To determine the rescue effect of CA on UC flies, 1mM CA was used to assess climbing ability, food intake, and excretion. The climbing ability of adult flies was significantly reduced in the model group, and 1mM CA supplementation restored the reduced locomotion ability of UC flies (Figure 2A). Administration of CA at 1 mm significantly increased food consumption and excretion in UC flies (Figure 2B–D). These results suggest that CA plays a role in restoring the physiological status of flies with UC.

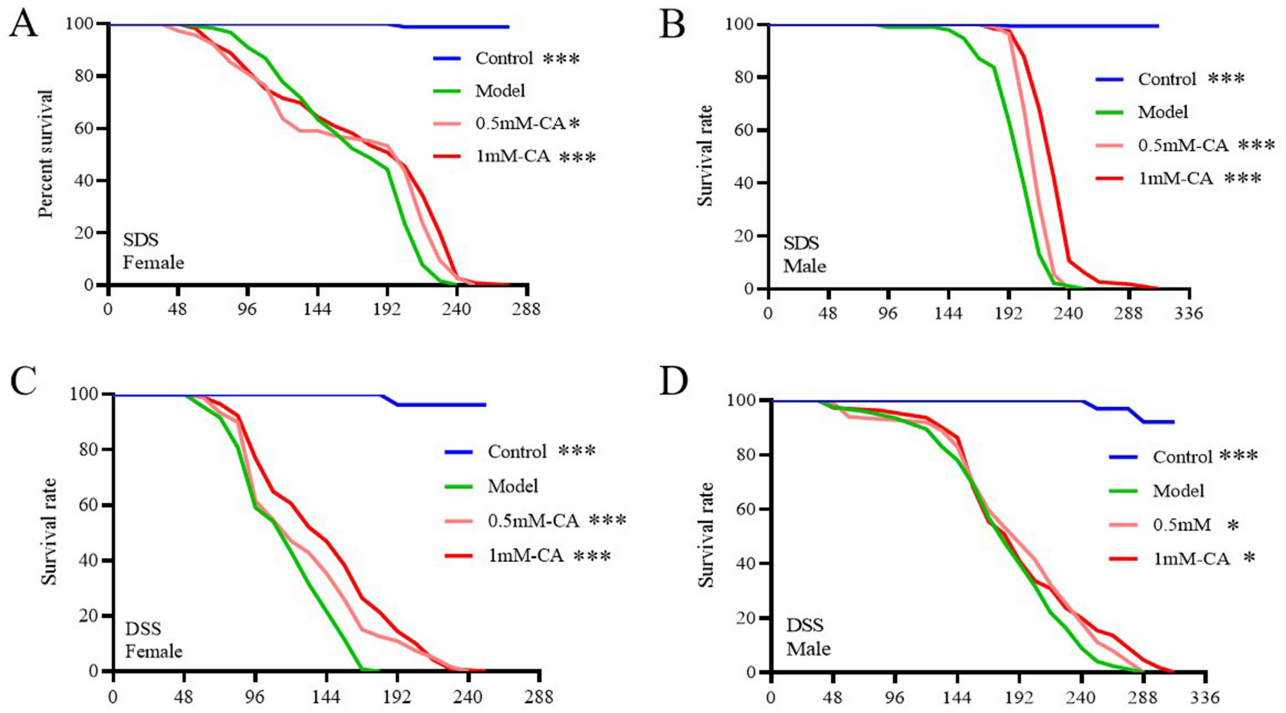


Figure 1 Effect of CA on the survival rate of adult flies under SDS or DSS stimulation. Oral administration of CA at 0.5 mm and 1 mm significantly elevates the decreased survival rate of female flies (**A**) (n =6 vials) and male flies (**B**) (n =6 vials) exposed to 0.6% SDS. Both 0.5 mm and 1 mm CA supplementation elevates the decreased survival rate of female flies (**C**) (n =6 vials) and male flies (**D**) (n =6 vials) flies exposed to 4% DSS. The *p*-value represents the significance of each group compared to the model group. $*p < 0.05$ and $***p < 0.001$.

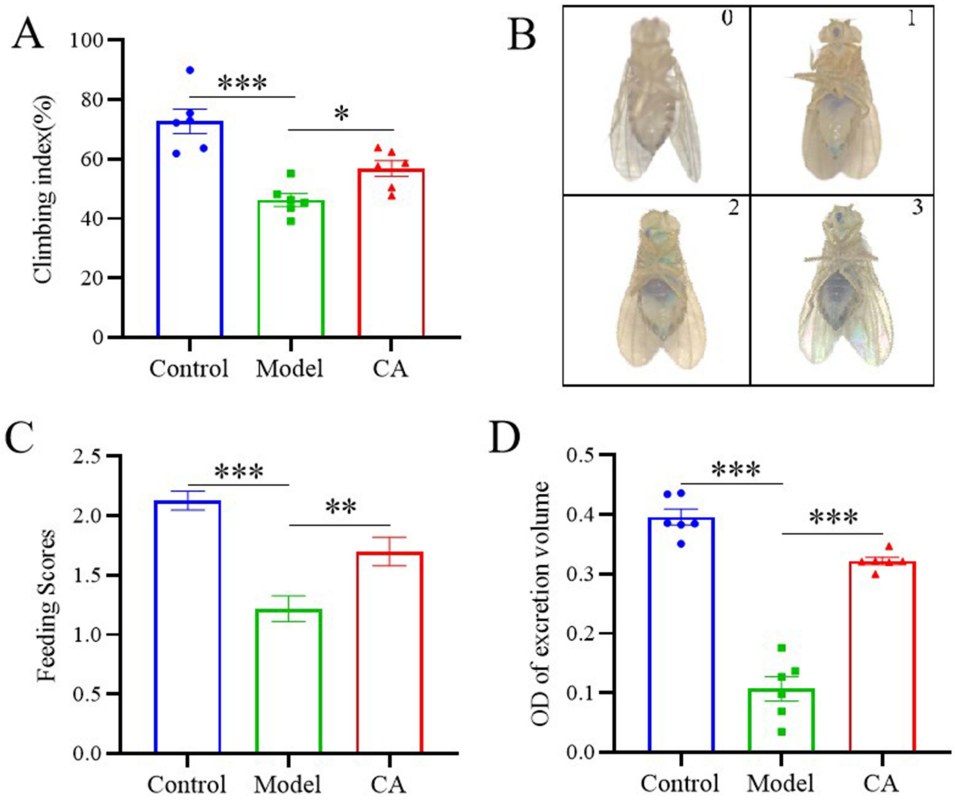


Figure 2 Effect of CA on climbing ability, feeding score and excretion in flies treated with DSS. (**A**) 1 mm CA supplementation improves the locomotor activities of female flies fed with DSS (n=6 vials). (**B**) Food intake is quantified by uptake of the blue dye. CA supplementation increases the food intake (**C**) (n=6 vials) and the excretion (**D**) (n=6 vials) of female flies under DSS stimulation. The *p*-value represents the significance of each group compared to the model group. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

CA Protects Intestinal Morphological Integrity and Intestinal Homeostasis in UC Flies

To investigate whether CA has a protective effect against morphological damage in UC flies, intestinal permeability and length were measured. Intestinal permeability was assessed using the "Smurf" test (Figure 3A).[33] The results showed that the proportion of "Smurf" in flies treated with DSS was significantly higher than control flies, and 1mM CA supplementation could significantly reduce the proportion of "Smurfs" in UC flies (Figure 3B). Administration of 1 mm can markedly increased the intestinal length of UC flies (Figure 3C). These results suggest that CA has a protective effect against damage to the intestinal morphology in UC flies. In addition, intestinal homeostasis was determined by measuring the intestinal acid-base balance (Figure 3D).[19] In UC flies, administration of CA at 1 mm dramatically increased the percentage of the female homeostatic phenotype and decreased the percentage of perturbed A (Figure 3E). These results suggest that CA plays an important role in alleviating intestinal damage.

CA Rescues the Intestinal Cell Damage in UC Flies

To determine whether CA supplementation could alleviate intestinal cell death in UC flies, the trypan blue staining scoring method was used to assess intestinal cell death.[25] Specifically, the intestinal color was quantitatively scored as follows: 1 point for similarity to Figure 4A, 2 points for similarity to Figure 4B, and 3 points for similarity to Figure 4C. The results showed that DSS treatment could increase the intestinal staining score in adult flies, while administration of CA significantly reduced the intestinal staining score (Figure 4D). In addition, the fluorescence intensity of intestinal epithelial cells was measured using the transgenic fly strain Myo1A>GFP.[34] Compared with the control group, DSS-treated flies had significantly weakened fluorescence intensity of intestinal epithelial cells (Figure 4E and F), while 1 mm CA supplementation caused an increase in epithelial cell fluorescence intensity (Figure 4G and H). These results indicated that CA significantly reduced intestinal cell death. Next, ISC proliferation was determined by using the transgenic fly strain Esg-GAL4; UAS-mCD8::GFP.[23] The number of GFP-positive cells was significantly increased in

https://doi.org/10.2147/DDDT.S499284

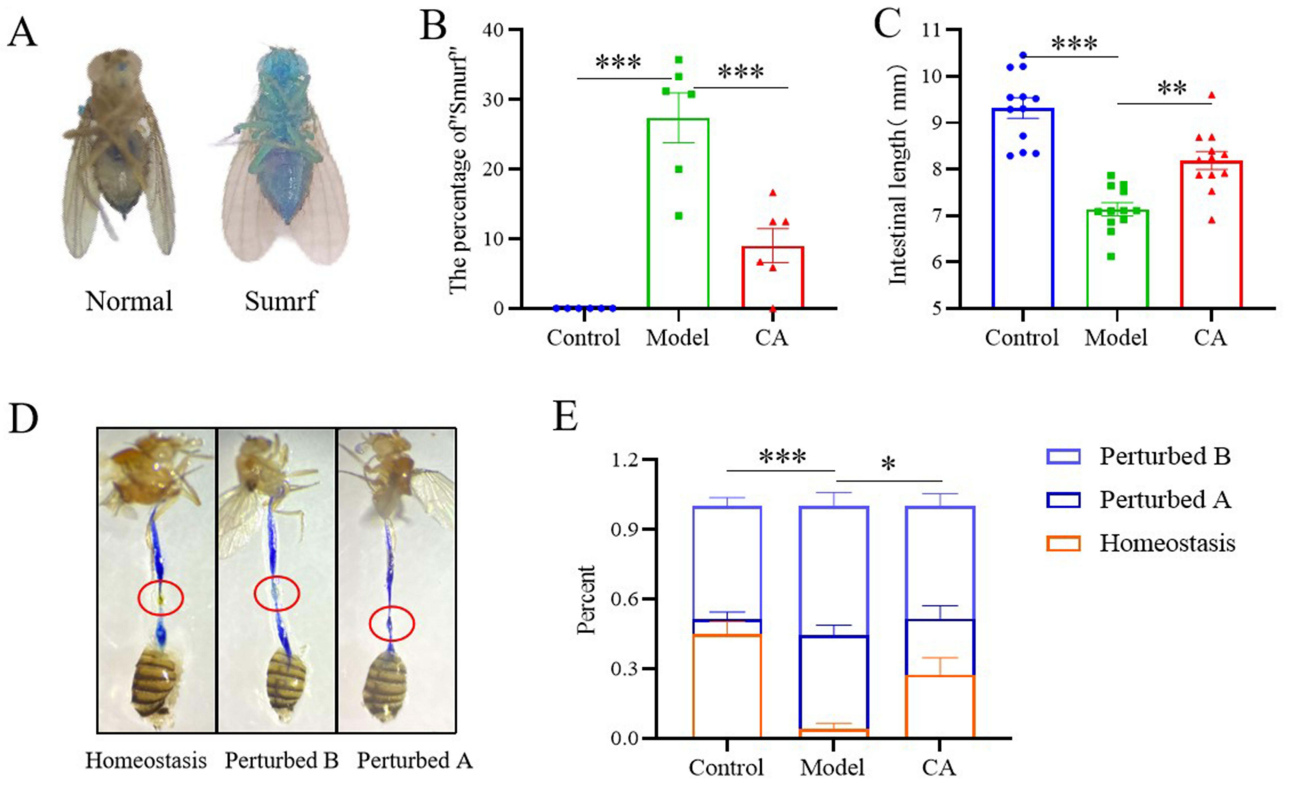


Figure 3 CA ameliorates the intestinal morphological disruption in female flies under DSS stimulation. (**A**) Intestinal barrier function by using "Smurfs" experiments; Left panel, the intestines were normal; right panel, the intestines were damaged and become "Smurf". (**B**) 1 mm CA supplementation significantly reduces the percentage of Smurf in female flies (n=6 vials). (**C**) oral administration of CA at 1 mm relieves the intestinal shortening (n=12 flies). (**D**) Homeostasis represents intestinal acid-base homeostasis, while Perturbed A and B represent intestinal acid-base disorders. (**E**) CA supplementation significantly improves intestinal acid-base disorders (n=6 vials). The *p*-value represents the significance of each group compared to the model group. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

the intestine of the flies treated with DSS, and CA supplementation could restore the number of GFP-positive cells in UC flies (Figure 4I–L). Taken together, these results indicate that CA has a protective effect against intestinal epithelial cell death and ISC over-proliferation in flies.

CA Alleviates the Damage of Intestinal Microstructure in UC Flies

To explore whether CA has a protective effect on the intestinal contents and walls of UC flies, transmission electron microscopy (TEM) at 0.3 a magnification of used. The results showed that the intestinal wall of flies treated with DSS was narrowed and cell apoptosis occurred compared with the control flies (Figure 5A), whereas no apoptosis was observed after the administration of 1 mm CA (Figure 5A). These results suggested that CA alleviated apoptosis. In addition, a large number of microorganisms were present in the intestinal contents of flies treated with DSS (Figure 5A). According to the results of further magnification at 1.0 k (Figure 5B), cocci and bacilli were identified, whereas the contents of the CA group were consistent with the results of the control group (Figure 5B). This result indicates that CA can effectively inhibit the increase in harmful bacteria in flies with UC. TEM at 8.0 k magnification allowed the ultrastructure of the mid-hindgut to be dissected. As shown in Figure 5C, the microvilli of UC flies were sparse or even fell off and were damaged. However, 1mM CA supplementation remarkably restored the sparse microvilli in UC flies without causing shedding damage (Figure 5C). In addition, the mitochondrial and endoplasmic reticulum morphology in UC flies changed significantly, manifested by mitochondrial vacuolization and mitochondrial ridge fragmentation, as well as atrophy and reduction of the endoplasmic reticulum (Figure 5D). In contrast, CA supplementation improved mitochondrial and ER-related damage (Figure 5D). In summary, CA reduced harmful bacteria in the intestines of UC flies and alleviated damage to the intestinal microstructure.

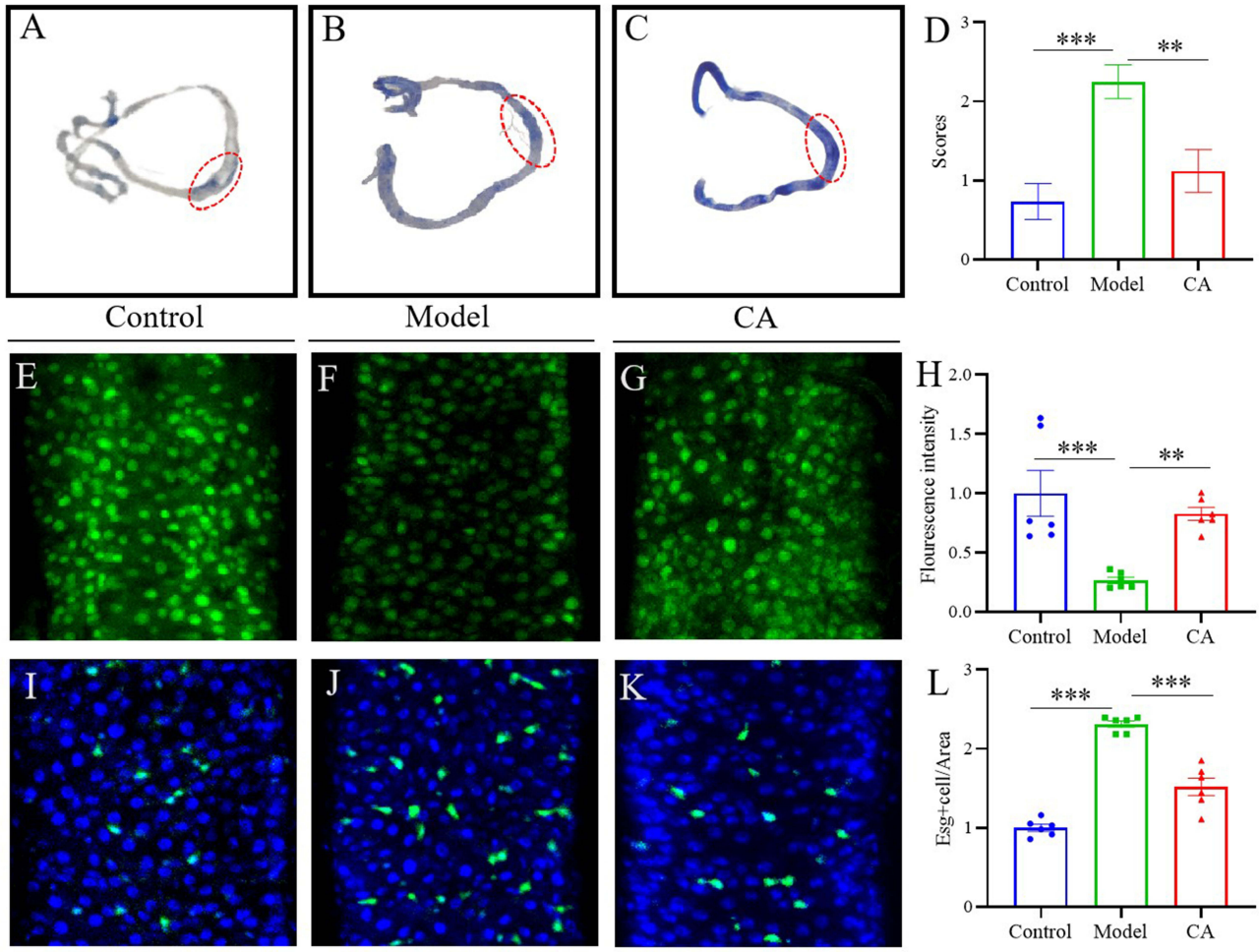


Figure 4 CA protected DSS-induced intestinal epithelial cell damage and prevents the intestinal stem cell (ISC) over-proliferation. (**A-C**) The intestinal cells are stained with Trypan blue, and higher scores indicate the higher number of intestinal cell deaths. (**D**) CA supplementation significantly protects intestinal epithelial cell damage (n=12 flies); (**E-G**) Intestinal Myo1A-GFP staining. (**H**) CA supplementation significantly increases the fluorescence intensity of Myo1A levels in the intestine (n=6 flies). (**I-K**) Intestinal Esg-GFP staining, Esg^{+} cells are labeled with GFP (green) and DAPI (blue). (**L**) CA supplementation decreases the proportions of GFP positive cells per area and number (n=6 flies). The *p*-value represents the significance of each group compared to the model group. $^{**}p < 0.01$, and $^{***}p < 0.001$.

CA Inhibits Mitochondrial Damage-Mediated Apoptosis in UC Flies

To further determine whether CA could alleviate intestinal damage by inhibiting cell apoptosis, TUNEL staining was performed. The results showed that 1 mm CA supplementation significantly reduced the expression of TUNEL fluorescence in UC flies, which was similar to the results in control flies (Figure 6A–D). In addition, ROS intensity was remarkably increased in UC flies (Figure 6E–H), whereas administration of CA at 1 mm significantly reduced the content of ROS in UC flies. Mitochondria are necessary for cellular energy metabolism and the production of ROS, and they control the life and death of cells and produce ATP to drive the life activities of cells.[35] To further verify whether CA could treat UC by inhibiting apoptosis mediated by mitochondrial dysfunction, the levels of mitochondrial function indicators ATP and MFN2 were detected. The results showed that the gene expression levels of ATP and MFN2 were significantly downregulated in UC flies and rescued after treated with 1mM CA (Figure 6I). Mitochondrial dysfunction may be related to the *Bcl-2* family (*Debcl* and *Buffy*) on the outer membrane of the mitochondria, which regulates cell apoptosis. *Debcl* In flies, Debcl can promote the release of cytochrome C (*cyt-c-p*), activate the caspase pathway, and lead to cell apoptosis, whereas Buffy can directly inhibit this process. To verify whether CA regulates apoptosis through *Bcl-2*, the mRNA levels of *Buffy, Debcl, Cyt-c-p, DrlCE, Dronc*, and *Dark* were measured. UC flies had a higher expression of these genes than control flies, and CA supplementation rescued the mRNA levels of these genes (Figure 6J). Taken together, CA alleviated intestinal damage mainly by inhibiting cell apoptosis mediated by mitochondrial damage.

https://doi.org/10.2147/DDDT.S499284

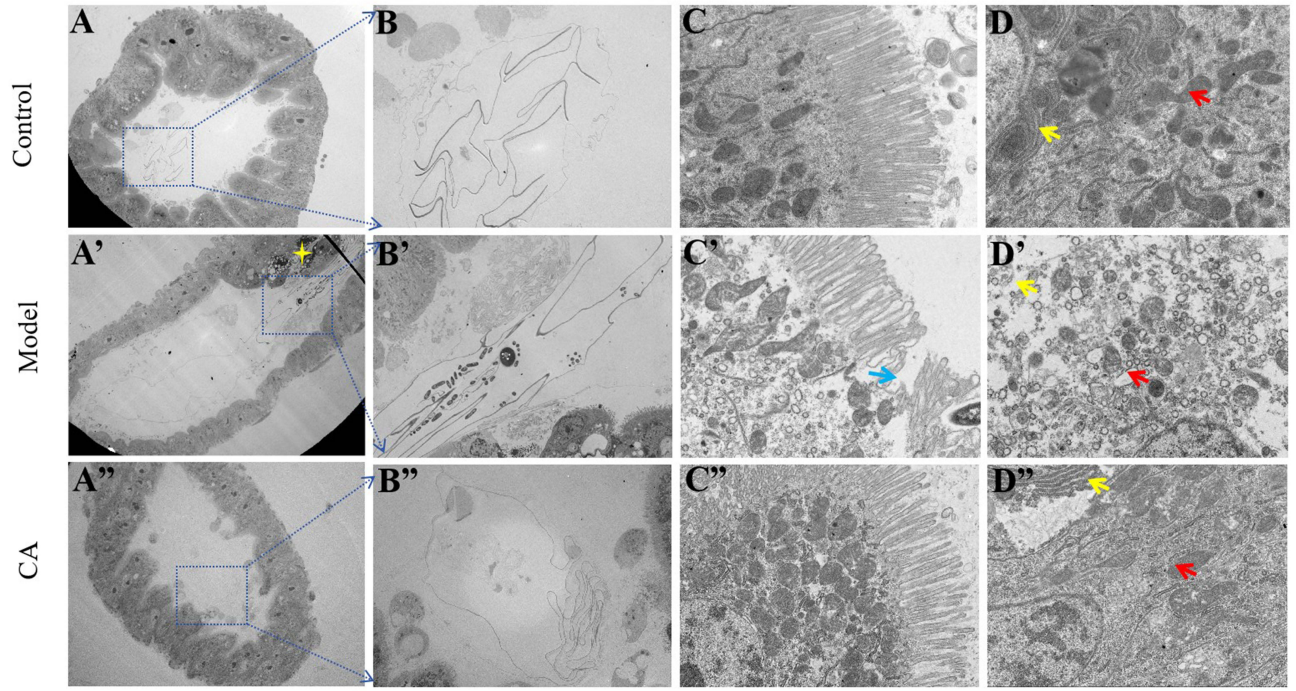


Figure 5 CA protects against DSS-induced intestinal morphological damage. (**A**) Electron microscopic images of representative intestinal cross-sections from each group are analyzed (1.0k). (**B**) Both control and CA are shown to have normal intestinal contents, while Model's intestinal contents are shown to have microflora (3.0k). (**C**) CA supplementation significantly improved intestinal villus length and intestinal barrier function (8.0k), Blue arrows indicate intestinal villus breakage. (**D**) CA supplementation is shown to significantly ameliorate mitochondrial and endoplasmic reticulum apoptosis in intestinal cells (8.0k). Red arrows indicate mitochondria, while yellow arrows indicate endoplasmic reticulum.

CA Restores Excessive Immune Response in UC Flies

To further explore the potential mechanism of CA in UC treatment, transcriptome analysis was performed to analyze the gene expression profile in the fly intestine (Figure 7A and B). There were 1581 differentially expressed genes between control flies and UC flies, including 732 upregulated and 849 downregulated genes. There were 106 differentially expressed genes between UC flies and UC flies fed 1 mm CA, which included 92 upregulated genes and 14 down-regulated genes (Figure 7A). Among these, 15 genes with significantly differential co-expression were screened using Venn diagram analysis and visualized using a heat map (Figure 7B). To investigate the signaling pathways that were enriched in DEGs, KEGG enrichment analysis was performed. The results showed that three pathways were significantly enriched: immune-related, rhythmic, and metabolic (Figure 7B). Among them, the immune regulatory pathways (Toll and Imd signaling pathways) showed significant changes. To verify whether CA could also alleviate UC by regulating the immune response, RT-qPCR was used to detect the gene expression of the Toll signaling pathway (*Myd88, Dif*) and the Imd signaling pathway (*PGRP-LC, Imd, Rel, Dpt*). The mRNA levels of *Myd88, Dif, PGRP-LC, Imd, Rel*, and *Dpt* in the intestines of UC flies were significantly enhanced compared to those in control flies, while 1mM CA supplementation significantly restored the expression of these genes (Figure 7C). Therefore, CA might alleviate UC fly associated immune overactivation in the intestine.

Molecular Docking Confirms the Mechanism of Action of CA in Treating UC Flies

Figure 8A shows the chemical structure of CA.To further confirm the potential therapeutic mechanism of CA, molecular docking was performed using Bcl-2 (PDB ID: 4LXD) (Figure 8B), Myd88 (PDB ID: 4DOM) (Figure 8C), and RIPK1 (PDB ID: 4ITJ) (Figure 8D). The results showed that the binding affinity between CA and the core targets was less than −5.0 kcal/mol, and all were hydrogen-bonded. This indicates that a stable structure can be formed between caffeic acid and its targets. These results confirm that CA alleviates intestinal injury by intervening in apoptosis and the Toll and Imd pathways.

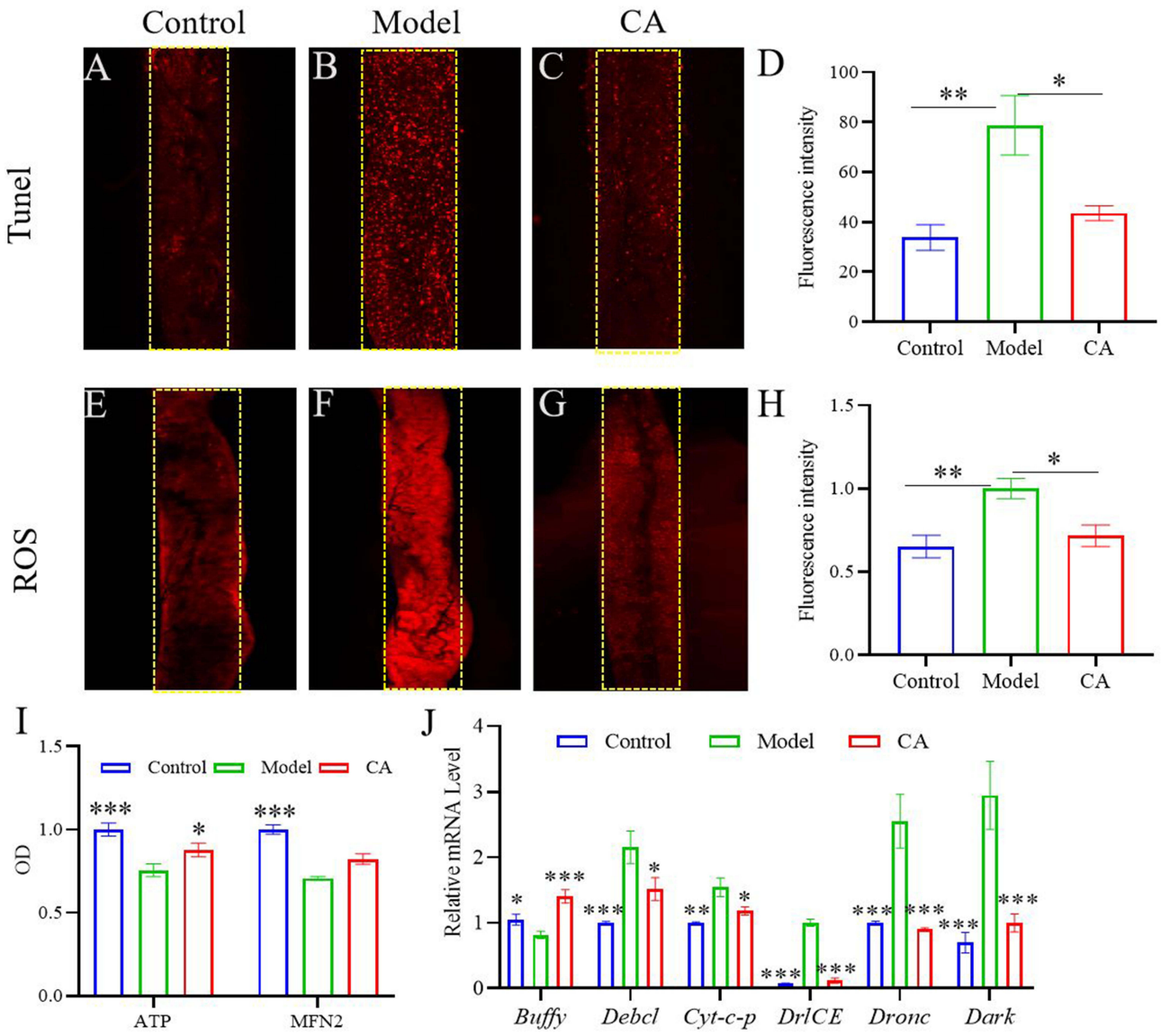


Figure 6 CA improves DSS-induced mitochondrial dysfunction in intestine of adult flies. (**A-C**) Tunel staining of flies intestines. (**E-G**) DHE staining of flies intestines. Oral administration of CA at 1 mm significantly declines the fluorescence intensity of Tunel (**D**) (n=5 flies) and ROS levels in the intestine (**H**) (n=6 flies). (**I**) CA supplementation significantly increases the relative ATP and MFN2 levels (n=9 vials). (**J**) CA supplementation reduces the relative mRNA level regarding apoptosis (n=9 vials). The *p*-value represents the significance of each group compared to the model group. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Discussion

UC is a chronic intestinal inflammation that is difficult to cure and is prone to relapse.[36] Natural molecular compounds show great potential in treating UC.[16,37] CA is a hydroxycinnamic acid found in many plants.[38] Fly has been reported as a well-established UC model in many previous studies.[25,37] Therefore, this study investigated the efficacy and potential mechanism of CA in the treatment of UC using a fly model. The results showed that CA supplementation improved the survival rate of UC flies, restored their motility and digestive abilities, protected their intestinal morphology and homeostasis, and reduced intestinal cell damage in UC flies. In particular, oral administration of CA reduced harmful bacteria in the intestine and alleviated the damage to the intestinal microstructure of UC flies. In addition, CA alleviated intestinal disruption in UC flies by inhibiting apoptosis mediated by intestinal epithelial mitochondrial damage and by alleviating UC-related immune overactivation in the intestine.

The survival rate of flies intuitively reflects the effectiveness of the drug. SDS can cause local and systemic inflammation in model animals, while DSS stimulation is usually used to construct US models in rodents and flies.[32]

https://doi.org/10.2147/DDDT.S499284

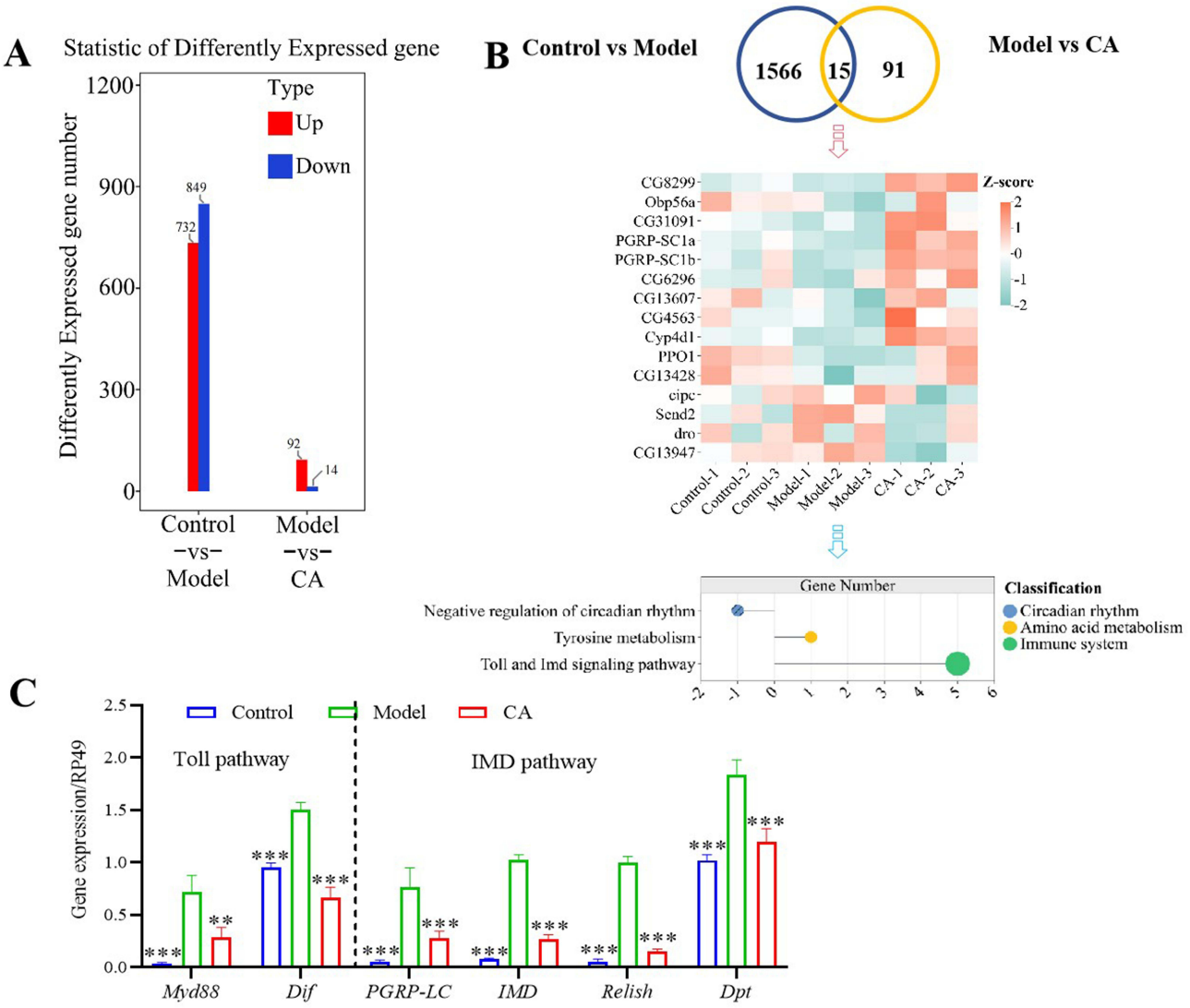


Figure 7 CA alleviates DSS-induced intestinal injury by inhibiting Toll and IMD signaling pathways. (**A**) Bar graphs showing the number of differential expressed genes between control and model, as well as between model and CA groups (n=3 vials). (**B**) Venn plot and heatmap indicated the screening of differentially regulated genes in DSS model after CA intervention. Enriched relevant pathways after CA intervention based on GO and KEGG databases (n=3 vials); (**C**) CA supplementation decreases the expression of genes related to Toll and IMD pathways in the intestine (n=9 vials). The *p*-value represents the significance of each group compared to the model group. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Here, we found CA supplementation could remarkably extend the survival rate in flies under SDS stimulation or DSS stimulation, which suggests that CA has great potential in alleviating UC. In addition, a fly's climbing ability, food intake, and excretion volume can indicate its athletic ability and digestive status. Previous studies have shown that climbing ability, food intake, and excretion of flies were significantly reduced after DSS treatment,[25] which is consistent with our results. Administration of CA significantly rescued the climbing ability, food intake, and excretion in UC flies, indicating that CA alleviates the weakening of the climbing and digestive abilities of UC flies.

Integrity of the intestinal barrier and intestinal acid-base homeostasis play an important role in maintaining intestinal homeostasis.[28,39] Here, we found CA supplementation dramatically reduced the "Smurf" ratio, increased the intestinal length, and alleviated the intestinal acid-base homeostasis imbalance in UC flies, indicating that CA can protect against intestinal damage in UC flies. In addition, Studies have confirmed that excessive death of intestinal epithelial cells and excessive proliferation of intestinal stem cells are closely related to the occurrence of intestinal inflammation.[40] This study found that the number of intestinal epithelial cells was decreased and the number of intestinal stem cells increased

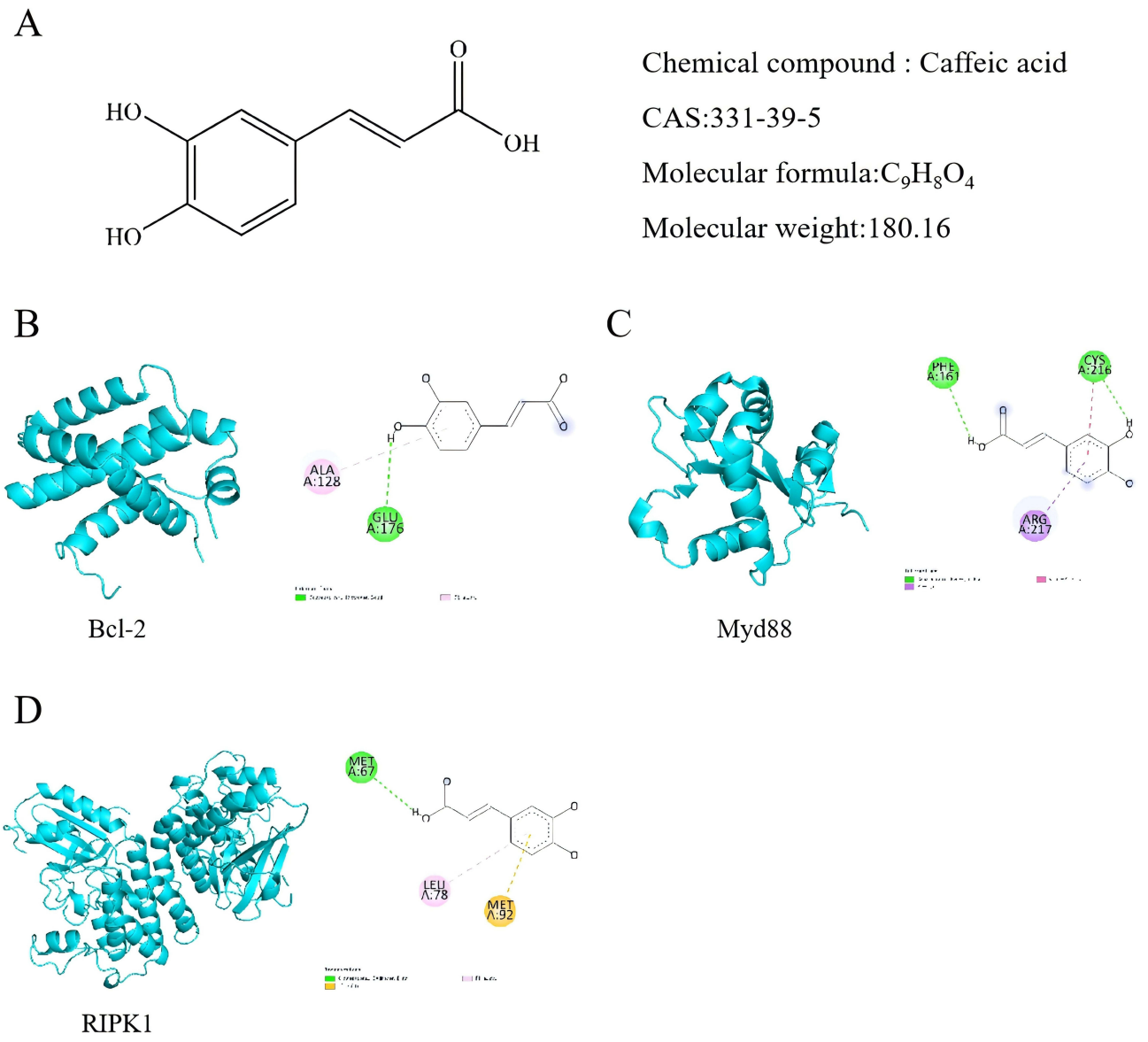


Figure 8 CA forms a stable binding structure with the core targets. (**A**) Basic information of CA; (**B**) CA -Bcl-2 complex, binding energy: −5.8 kcal/mol; (**C**) CA -Myd88 complex, binding energy: −5.4 kcal/mol; (**D**) CA -RIPK1 complex, binding energy: −6.6 kcal/mol.

in UC flies, which is consistent with the previous studies.[25] CA supplementation could significantly enhance the number of intestinal epithelial cells and inhibit the excessive proliferation of intestinal stem cells, indicating that CA plays an important role in maintaining the intestinal epithelial barrier.

Imaging results showed that UC flies supplemented with CA showed alleviated intestinal cell apoptosis and improved damage to the mitochondria and endoplasmic reticulum in intestinal cells. Mitochondrial damage leads to cell apoptosis.[41] Mitochondria are the main site of ROS generation in cells,[42] and ROS production increases significantly when mitochondria are damaged.[43] Here, CA supplementation restored the intestinal ROS levels in UC flies. Mitochondria are also the site of ATP production.[44] The significant decrease in ATP in the model group indicates that the intestinal mitochondria of UC flies were damaged, thus limiting the production of ATP. CA supplementation restored ATP levels. MFN2 is essential for maintaining mitochondrial morphology and function.[45] Under stress conditions, MFN2 function may be inhibited, leading to mitochondrial fragmentation and makes cells more susceptible to apoptosis.[46] In this study, the expression of MFN2 was inhibited in the model group but restored in the CA group. *Bcl-2* is a gene family that regulates apoptosis and is located in the outer membrane of the mitochondria. In flies, *Buffy* gene is an anti-apoptotic

https://doi.org/10.2147/DDDT.S499284

gene homologous to *Bcl-2* in mammals and can prevent the release of *cyt-c-p* during cell apoptosis; *Debcl* is a pro-apoptotic gene homologous to the mammalian *Bcl-2* family that induces the release of *cyt-c-p*.[47] This release triggers an apoptotic cascade involving *Apaf-1, caspase-9*, and *caspase-3*, ultimately leading to cell death. Here, CA supplementation rescued the downregulated expression of *Buffy* and upregulated the expression of *Debcl, Cyt-c-p* (a cytochrome c homolog), *Dark* (an Apaf-1 homolog), *Dronc* (a caspase-9 homolog), and *DrlCE* (a caspase-3 homolog), suggesting that the specific mechanism by which CA inhibits mitochondrial damage-mediated cell apoptosis is to promote the expression of the Buffy gene while inhibiting the expression of pro-apoptotic genes (*Debcl*) and proteins (*Cyt-c-p, Dark, Dronc, DrlCE*). Taken together, our results suggest that CA may alleviate intestinal damage mainly by repairing mitochondrial damage and inhibiting cell apoptosis.

CA effectively inhibited the increase in *bacilli* and *cocci* in the UC fly model, suggesting that CA can repair the imbalance in intestinal microorganisms. Previous study has found that CA supplementation changed the composition of the intestinal microbiome of UC mice and reduced the relative abundance of *Bacteroides* and *Turicibacter* in the intestines of UC mice.[16] *Bacteroides* belongs to *bacilli* and *Turicibacter* belongs to cocci, which is consistent with the results of this study. Although this study demonstrated a reduction in harmful bacteria, further characterization of the microbiota composition (eg, by sequencing) will enhance the understanding of the role of CA in regulating the gut microbiota. In addition, transcriptome analysis and RT-PCR results showed that CA supplementation could downregulate the innate immune pathways (Toll and IMD pathways) in UC flies. These two signaling pathways are activated under intestinal stress.[48,49] Intestinal stress may cause intestinal hyperimmunity, and an excessive immune response can aggravate intestinal inflammation.[50,51] This suggests that CA can alleviate intestinal damage by regulating Toll and IMD signaling pathways. Furthermore, the Toll and Imd pathways are highly conserved, and their modulation by CA suggests potential therapeutic implications beyond UC, such as in other inflammatory or immune diseases.

As a molecular compound with anti-inflammatory activity, CA was confirmed to be effective in alleviating UC using a UC fly model. First, CA can alleviate UC by inhibiting apoptosis mediated by mitochondrial damage in the intestinal cells. Second, CA can alleviate UC by alleviating excessive intestinal immunity caused by UC. However, although the results found in *Drosophila* are reliable and mechanistically insightful, preclinical validation in mammalian models is necessary to evaluate the therapeutic potential of CA in humans. This study effectively evaluated the short-term results of CA treatment for UC. Future studies evaluating the long-term effects of CA on intestinal damage, mitochondrial function, and immune regulation will provide more comprehensive insights into the specific mechanisms of CA treatment for UC. In conclusion, the data of this study provide sufficient guidance for future preclinical studies of CA in the treatment of UC and provide ideas for studying the role of other bioactive compounds in the treatment of human diseases.

Acknowledgment

We are grateful to the members of Prof. Liu and the He Laboratory for their useful comments on the manuscript.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; Minghui Xiu and Botong Li took part in drafting, revising or critically reviewing the article; all other authors took part in revising the article; all authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China (No. 82360896), Gansu Provincial Department of Education Innovation Ability Enhancement Project (No. 2023A-086), Lanzhou Science and Technology Plan Project (No. 2023-4-53), and the Foundation of Key Laboratory of Dunhuang Medicine (No. DHYX21-05).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

References

1. Wattacheril JJ, Abdelmalek MF, Lim JK, Sanyal AJ. Aga clinical practice update on the role of noninvasive biomarkers in the evaluation and management of nonalcoholic fatty liver disease: expert review. *Gastroenterology*. 2023;165(4):1080–1088. doi:10.1053/j.gastro.2023.06.013
2. Ng SC, Shi HY, Hamidi N, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. 2017;390(10114):2769–2778. doi:10.1016/S0140-6736(17)32448-0
3. Kaplan GG, Windsor JW. The four epidemiological stages in the global evolution of inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol*. 2021;18(1):56–66. doi:10.1038/s41575-020-00360-x
4. Lertnimitphun P, Jiang Y, Kim N, et al. Safranal alleviates dextran sulfate sodium-induced colitis and suppresses macrophage-mediated inflammation. *Front Pharmacol*. 2019;10:1281. doi:10.3389/fphar.2019.01281
5. Sun X, Zhang Y, Cheng G, et al. Berberine improves dss-induced colitis in mice by modulating the fecal-bacteria-related bile acid metabolism. *Biomed Pharmacother*. 2023;167:115430. doi:10.1016/j.biopha.2023.115430
6. Porter RJ, Kalla R, Ho GT. Ulcerative colitis: recent advances in the understanding of disease pathogenesis. *F1000Res*. 2020;9. doi:10.12688/f1000research.20805.1.
7. Slomski A. Filgotinib induces and maintains ulcerative colitis remission. *JAMA*. 2021;326(4):299. doi:10.1001/jama.2021.11750
8. Roberts-Thomson IC, Bryant RV, Costello SP. Uncovering the cause of ulcerative colitis. *JGH Open*. 2019;3(4):274–276. doi:10.1002/jgh3.12216
9. Lu Q, Yang MF, Liang YJ, et al. Immunology of inflammatory bowel disease: molecular mechanisms and therapeutics. *J Inflamm Res*. 2022;15:1825–1844. doi:10.2147/JIR.S353038
10. Zhou M, Xu W, Wang J, et al. Boosting mTOR-dependent autophagy via upstream TLR4-MyD88-MAPK signalling and downstream NF-kappaB pathway quenches intestinal inflammation and oxidative stress injury. *EBioMedicine*. 2018;35:345–360. doi:10.1016/j.ebiom.2018.08.035
11. Kleino A, Silverman N. Regulation of the drosophila imd pathway by signaling amyloids. *Insect Biochem mol Biol*. 2019;108:16–23. doi:10.1016/j.ibmb.2019.03.003
12. Zheng S, Xue T, Wang B, Guo H, Liu Q. Chinese medicine in the treatment of ulcerative colitis: the mechanisms of signaling pathway regulations. *Am J Chin Med*. 2022;50(7):1781–1798. doi:10.1142/S0192415X22500756
13. Jia Z, Xu C, Shen J, Xia T, Yang J, He Y. The natural compound celastrol inhibits necroptosis and alleviates ulcerative colitis in mice. *Int Immunopharmacol*. 2015;29(2):552–559. doi:10.1016/j.intimp.2015.09.029
14. Wang Q, Zhang L, Yuan X, et al. The relationship between the bcl-2/bax proteins and the mitochondria-mediated apoptosis pathway in the differentiation of adipose-derived stromal cells into neurons. *PLoS One*. 2016;11(10):e0163327. doi:10.1371/journal.pone.0163327
15. Yin Q, Pi X, Jiang Y, et al. An immuno-blocking agent targeting il-1beta and il-17a reduces the lesion of dss-induced ulcerative colitis in mice. *Inflammation*. 2021;44(5):1724–1736. doi:10.1007/s10753-021-01449-4
16. Wan F, Zhong R, Wang M, et al. Caffeic acid supplement alleviates colonic inflammation and oxidative stress potentially through improved gut microbiota community in mice. *Front Microbiol*. 2021;12:784211. doi:10.3389/fmicb.2021.784211
17. Pavlikova N. Caffeic acid and diseases-mechanisms of action. *Int J mol Sci*. 2022;24(1). doi:10.3390/ijms24010588
18. Wen X, Wan F, Wu Y, et al. Caffeic acid supplementation ameliorates intestinal injury by modulating intestinal microbiota in lps-challenged piglets. *Food Funct*. 2023;14(16):7705–7717. doi:10.1039/d3fo02286b
19. Sheng X, Zhu Y, Zhou J, et al. Antioxidant effects of caffeic acid lead to protection of drosophila intestinal stem cell aging. *Front Cell Dev Biol*. 2021;9:735483. doi:10.3389/fcell.2021.735483
20. Chang W, Huang D, Lo YM, et al. Protective effect of caffeic acid against Alzheimer's disease pathogenesis via modulating cerebral insulin signaling, beta-amyloid accumulation, and synaptic plasticity in hyperinsulinemic rats. *J Agric Food Chem*. 2019;67(27):7684–7693. doi:10.1021/acs.jafc.9b02078
21. Guo X, Lv J, Xi R. The specification and function of enteroendocrine cells in drosophila and mammals: a comparative review. *FEBS J*. 2022;289(16):4773–4796. doi:10.1111/febs.16067
22. Xiu M, Wang Y, Yang D, et al. Using drosophila melanogaster as a suitable platform for drug discovery from natural products in inflammatory bowel disease. *Front Pharmacol*. 2022;13:1072715. doi:10.3389/fphar.2022.1072715
23. Yang S, Li X, Xiu M, et al. Flos puerariae ameliorates the intestinal inflammation of drosophila via modulating the nrf2/keap1, jak-stat and wnt signaling. *Front Pharmacol*. 2022;13:893758. doi:10.3389/fphar.2022.893758
24. He J, Li X, Yang S, et al. Protective effect of astragalus membranaceus and its bioactive compounds against the intestinal inflammation in drosophila. *Front Pharmacol*. 2022;13:1019594. doi:10.3389/fphar.2022.1019594
25. Li B, Xiu M, He L, et al. Protective effect of san huang pill and its bioactive compounds against ulcerative colitis in drosophila via modulation of jak/stat, apoptosis, toll, and nrf2/keap1 pathways. *J Ethnopharmacol*. 2024;322:117578. doi:10.1016/j.jep.2023.117578
26. Han S, Xiu M, Li S, et al. Exposure to cytarabine causes side effects on adult development and physiology and induces intestinal damage via apoptosis in drosophila. *Biomed Pharmacother*. 2023;159:114265. doi:10.1016/j.biopha.2023.114265
27. Abu F, Ohlstein B. Monitoring gut acidification in the adult drosophila intestine. *J Vis Exp*. 2021;(176). doi:10.3791/63141
28. He J, Han S, Wang Y, et al. Irinotecan cause the side effects on development and adult physiology, and induces intestinal damage via innate immune response and oxidative damage in drosophila. *Biomed Pharmacother*. 2023;169:115906. doi:10.1016/j.biopha.2023.115906
29. Lee SH, Goo TW, Yun EY. Allomyrina dichotoma larval extract has protective effects against gut permeability of dextran sulfate sodium-fed drosophila by e-cadherin and armadillo. *J Ethnopharmacol*. 2021;279:113786. doi:10.1016/j.jep.2021.113786
30. Dai Y, Wang Y, Kang Q, et al. The protective effect and bioactive compounds of astragalus membranaceus against neurodegenerative disorders via alleviating oxidative stress in drosophila. *FASEB J*. 2024;38(13):e23727. doi:10.1096/fj.202400390R
31. Song G, Chen F, Chen S, Ye S. Polysaccharides from premna microphylla turcz ameliorate inflammation via the enhancement of intestinal resistance in host. *J Ethnopharmacol*. 2021;276:114208. doi:10.1016/j.jep.2021.114208

32. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (dss)-induced colitis in mice. *Curr Protoc Immunol*. 2014;104:15–25. doi:10.1002/0471142735.im1525s104
33. Rera M, Clark RI, Walker DW. Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in drosophila. *Proc Natl Acad Sci USA*. 2012;109(52):21528–21533. doi:10.1073/pnas.1215849110
34. Guo X, Wang C, Zhang Y, Wei R, Xi R. Cell-fate conversion of intestinal cells in adult drosophila midgut by depleting a single transcription factor. *Nat Commun*. 2024;15(1):2656. doi:10.1038/s41467-024-46956-8
35. Figge MT, Osiewacz HD, Reichert AS. Quality control of mitochondria during aging: is there a good and a bad side of mitochondrial dynamics? *Bioessays*. 2013;35(4):314–322. doi:10.1002/bies.201200125
36. Ordas I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet*. 2012;380(9853):1606–1619. doi:10.1016/S0140-6736(12)60150-0
37. Wei T, Wu L, Ji X, Gao Y, Xiao G. Ursolic acid protects sodium dodecyl sulfate-induced drosophila ulcerative colitis model by inhibiting the jnk signaling. *Antioxidants*. 2022;11(2). doi:10.3390/antiox11020426
38. El-Seedi HR, El-Said AM, Khalifa SA, et al. Biosynthesis, natural sources, dietary intake, pharmacokinetic properties, and biological activities of hydroxycinnamic acids. *J Agric Food Chem*. 2012;60(44):10877–10895. doi:10.1021/jf301807g
39. Camilleri M, Madsen K, Spiller R, Greenwood-Van MB, Verne GN. Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterol Motil*. 2012;24(6):503–512. doi:10.1111/j.1365-2982.2012.01921.x
40. Zheng L, Duan SL. Molecular regulation mechanism of intestinal stem cells in mucosal injury and repair in ulcerative colitis. *World J Gastroenterol*. 2023;29(16):2380–2396. doi:10.3748/wjg.v29.i16.2380
41. Jeong SY, Seol DW. The role of mitochondria in apoptosis. *BMB Rep*. 2008;41(1):11–22. doi:10.5483/bmbrep.2008.41.1.011
42. Andrieux P, Chevillard C, Cunha-Neto E, Nunes J. Mitochondria as a cellular hub in infection and inflammation. *Int J mol Sci*. 2021;22(21). doi:10.3390/ijms222111338
43. Yang Y, Karakhanova S, Hartwig W, et al. Mitochondria and mitochondrial ros in cancer: novel targets for anticancer therapy. *J Cell Physiol*. 2016;231(12):2570–2581. doi:10.1002/jcp.25349
44. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, atp, and ros: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol*. 2004;287(4):C817–C833. doi:10.1152/ajpcell.00139.2004
45. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins mfn1 and mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol*. 2003;160(2):189–200. doi:10.1083/jcb.200211046
46. Jiang Y, Krantz S, Qin X, et al. Caveolin-1 controls mitochondrial damage and ros production by regulating fission - fusion dynamics and mitophagy. *Redox Biol*. 2022;52:102304. doi:10.1016/j.redox.2022.102304
47. M'Angale PG, Staveley BE. Bcl-2 homologue debcl enhances alpha-synuclein-induced phenotypes in drosophila. *PeerJ*. 2016;4:e2461. doi:10.7717/peerj.2461
48. Valanne S, Wang JH, Ramet M. The drosophila toll signaling pathway. *J Immunol*. 2011;186(2):649–656. doi:10.4049/jimmunol.1002302
49. Myllymaki H, Valanne S, Ramet M. The drosophila imd signaling pathway. *J Immunol*. 2014;192(8):3455–3462. doi:10.4049/jimmunol.1303309
50. Yue B, Ren J, Yu Z, et al. Pinocembrin alleviates ulcerative colitis in mice via regulating gut microbiota, suppressing tlr4/md2/nf-kappab pathway and promoting intestinal barrier. *Biosci Rep*. 2020;40(7). doi:10.1042/BSR20200986
51. Liu Y, Yang M, Tang L, et al. Tlr4 regulates rorgammat(+) regulatory t-cell responses and susceptibility to colon inflammation through interaction with akkermansia muciniphila. *Microbiome*. 2022;10(1):98. doi:10.1186/s40168-022-01296-x