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7,8-Dihydroxyflavone suppresses ferroptosis to alleviate sleep deprivation-induced gut damage

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ABSTRACT: Sleep is essential for maintaining physiological homeostasis, and sleep deprivation can lead to severe health problems including ferroptosis-driven intestinal damage. Due to the complexity of the intestinal microenvironment, research on intestinal ferroptosis remains in its early stages. Moreover, the relationship between 7,8-dihydroxyflavone and ferroptosis has not yet been clearly defined. This study aims to explore the regulatory role of 7,8-dihydroxyflavone on ferroptosis in sleep-deprived mice. The results revealed that 7,8-dihydroxyflavone alleviates colonic ferroptosis by activating the Nrf2 signaling pathway. 7,8-dihydroxyflavone treatment mitigated weight loss, colon shortening, and the reduction of tight junction proteins in sleep-deprived mice. In colonic epithelial cells, 7,8-dihydroxyflavone suppressed ferroptosis, and this protective effect was dependent on Nrf2 activation. Transcriptomic analysis further demonstrated that 7,8-dihydroxyflavone modulates Nrf2 and its downstream antioxidant targets through the TrkB-Akt signaling cascade. Together, these findings identify 7,8-dihydroxyflavone as a promising therapeutic candidate for preventing intestinal ferroptosis induced by sleep deprivation via the TrkB-Akt-Nrf2 pathway.

Keywords: 7,8-dihydroxyflavone; ferroptosis; sleep deprivation; intestinal damage

1. Introduction

Sleep is a periodic and reversible physiological state for most animals, characterized by reduced motor activity and responsiveness to external stimuli^[1, 2]. Accumulating evidence demonstrates that sleep is intricately linked to cognitive function^[3], immune regulation^[4] and metabolic homeostasis^[5]. Insufficient sleep can cause a range of health problems^[6], and even excessive sleep deprivation (SD) could contribute to premature mortality in different model species, including mice^[7], cockroaches^[8], flies^[9], dogs^[10] and rats^[11]. Emerging evidence reveals a strong association between sleep deprivation and gastrointestinal dysfunction, with sleep loss triggering reactive oxygen species (ROS) accumulation in the gut^[9]. Meanwhile, evidence

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expatiated SD could exacerbate the pathogenesis of inflammatory bowel disease (IBD)^[12]. Intestinal clock dysfunction triggers host immune imbalance and promotes the development and progression of IBD-like colitis^[13, 14]. Nowadays, the impact of sleep deprivation on gut health has received growing attention. Once a study identified d-serine as a crucial regulator of gut ROS during SD. Knockdown of serine racemase (SR), the enzyme responsible for d-serine production, prevents the enhanced ROS buildup during SD in *Drosophila*^[15]. Our previous investigation has consistently shown that SD results in the disruption of the intestinal barrier^[16, 17]. The gut comprises diverse and specialized epithelial cells that exert functions ranging from facilitating nutrient absorption to pathogens sensing and intestinal homeostasis^[18]. Intestinal epithelial homeostasis is intricately associated with the integrity of tight junction proteins such as the claudins (CLDNs) and Zonula occludens protein 1 (ZO1), which connect adjacent intestinal epithelial cells^[19]. Additionally, mucus is key for intestinal barrier, particularly MUC2, which was secreted by goblet cells^[20]. Goblet cells safeguard the intestinal barrier by restricting bacterial penetration and attenuating inflammatory responses^[21]. Disruption of the intestinal epithelium is a defining feature of many diseases. Thus, elucidating the molecular targets involved in SD-induced intestinal injury is of critical importance.

Intestinal dysfunction impairs nutrient absorption and increases susceptibility to infections. Research on intestinal diseases has yielded valuable clues regarding their pathogenesis and the factors contributing to their severe pathology. Aberrant inflammatory responses participate in the process of gut injury^[22]. Previous studies have also linked intestinal damage with intestinal stem cells (ISCs) regeneration^[23]. For example, hyperproliferation of ISCs lead to intestinal tumorigenesis^[24, 25]. Moreover, intestinal injury is linked to excessive ROS production, diminished antioxidant capacity, and heightened oxidative stress^[26]. Correspondingly, these factors may serve as potential targets for alleviating SD-induced gut damage.

SD induced ferroptosis in hippocampal neurons of mice, and led to impairments in learning and memory^[27]. However, our earlier findings indicated that ferroptosis functions as a key pathogenic driver of intestinal injury caused by SD^[16]. Sleep restriction altered gut microbiota composition and intestinal barrier function in rodents in previous study^[28]. This observation suggests that targeting ferroptosis may provide a potential therapeutic strategy for sleep deprivation-induced gut injury. The pathogenesis of ferroptosis involves three primary components: the accumulation of lipid peroxides, disorders of the antioxidant system, and disruption of iron homeostasis^[29]. ALOX12 plays crucial roles in the synthesis of lipid hydroperoxides, it can aggravate the ferroptotic process^[30]. Meanwhile, the by-products of lipid peroxidation including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which can give rise to cellular damage^[31]. The primary proteins of the antioxidant system related to ferroptosis are SLC7A11, and GPX4^[32, 33]. Of note, Nrf2, a master regulator of the antioxidant response, suppresses ferroptosis by enhancing the expressing of GPX4, SLC7A11 and HO-1^[34]. In addition, the regulation of iron is related to ferroptosis. Ferritin, composed of the heavy (FTH) and light (FTL) chains, functions as the major intracellular iron-storage complex, whereas transferrin (TF) and its receptor (TFRC) are the key proteins responsible for iron transport and cellular uptake^[33].

Despite extensive research on ferroptosis inhibitors, their clinical use remains highly restricted owing to pharmacokinetic limitations and toxic side effects^[35]. Flavonoids are (poly)phenolic compounds that are widely consumed as part of the human diet^[36]. Their dietary sources are quite diverse, ranging from fruits and vegetables to nuts and legumes, as well as wines and teas^[37]. 7,8-Dihydroxyflavone (7,8-DHF) is a naturally flavone identified in *Godmania aesculifolia*, *Tridax procumbens*, and the leaves of *Primula* species^[38]. In addition, 7,8-DHF has been detected in the whole herb of *Lepisorus ussuriensis*, a traditional Chinese medicinal plant used for heat-clearing and detoxification^[39]. Some reports further indicate that 7,8-DHF may also be present in fruits, vegetables, and other dietary sources, although typically at very low concentrations^[40]. 7,8-DHF exhibits anti-inflammatory, antibacterial, and antioxidant activities^[41, 42]. Moreover, numerous studies have reported that 7,8-DHF exerts therapeutic effects in various animal models of disease associated with impaired BDNF signaling pathway^[43]. In recent years, 7,8-DHF has gained considerable attention as a bioactive flavone with substantial pharmacological relevance, particularly due to its potent neurotrophic and antioxidant properties. Notably, several commercially available dietary supplements have incorporated 7,8-DHF as a principal active component, each capsule typically containing 25 mg of 7,8-DHF. Previous studies reported 7,8-DHF inhibited BMSC oxidative stress to improve symptoms of postmenopausal osteoporosis^[44]. Du studied 7,8-DHF protected against mitochondrial dysfunction of ischemic cardiomyocytes by rebalancing OPA1 processing^[45]. Accordingly, we postulated that 7,8-DHF could also play an antioxidant role in mitigating SD-induced colonic damage. These observations prompted us to investigate further, ultimately showing that 7,8-DHF protects against SD-induced intestinal damage by suppressing ferroptosis in a Nrf2-dependent manner.

2. Materials and methods

2.1 Animals and treatment

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The present study was approved by the Animal Use Committees of South China Agricultural University (Guangzhou, China; approval number: 2023b118). Male wild-type C57BL/6J mice were procured from GemPharmatech Co., Ltd. (Nanjing, China). After one week of acclimation, the mice were randomly divided into 3 groups: the non-sleep-deprived control group (Control), the SD group (SD) and the SD group with 7,8-DHF treatment (SD+7,8-DHF). For evaluating ANA-12 function, based on the above experiments, we added another two groups, which were SD+ ANA-12 and SD+7,8-DHF+ANA-12. For each experimental group, we used eight mice, with a total of sixty-four mice every per experiment. To assess protein expression at various time, four mice were assigned to each group, yielding a total of twenty-four mice per experiment. The specific methods of SD were operated according to previous study^[16]. In brief, we used an SD apparatus (KEW-BASIS, Nanjing, China). This device allows mice free access to water and food and is equipped with an adjustable-speed rotating rod at the bottom. During the SD period, the rod was set to rotate at a speed of 10 RPM, alternating between clockwise and counterclockwise directions, for 22 h per day.

2.2 Reagents and antibodies

7,8-DHF was purchased from Yuan ye (Shanghai, China), dissolved in dimethyl sulfoxide (DMSO, MCE, USA), and stored at -20°C for subsequent use. Ferrostatin-1 (Fer-1), RSL3 and Ipatasertib were purchased from Med Chem Express (MCE, State of New Jersey, USA). All primary antibody used in the experiments included GAPDH (1:2000; Proteintech, Wuhan, China), β -actin (1:2000; Proteintech, Wuhan, China), GPX4 (1:1000; Abcam, England), SLC7A11 (1:1000; Abcam, England), MUC2 (1:1000; Abcam, England), HO-1 (1:1000; Abcam, England), Nrf2 (1:1000; Cell Signaling Technology, America), p-Nrf2 (1:2000; ABclonal, China), Akt (1:1000; Cell Signaling Technology, America), p-Akt (1:1000; Cell Signaling Technology, America), ZO1 (1:1000, ABclonal, China), Occludin (1:1000; ABclonal, China). ALOX12 (1:1000; Santa Cruz, America), TrkB (1:1000; Santa Cruz, America), p-TrkB (1:1000, Affinity, China).

2.3 Drug treatment

For the *in vitro* assays, reagents were applied at the following concentrations: 7,8-DHF (Yuan ye, S24970) (50 μ M), RSL3 (MCE, HY-100218A) (5 μ M), Fer-1(MCE, HY-100579) (10 μ M), Ipatasertib (MCE, HY-15186) (10 μ M). For the *in vivo* assays, mice received 7,8-DHF (10 mg/kg) and ANA-12 (1 mg/kg) via intraperitoneal injection. 7,8-DHF was administered once daily for three consecutive days before SD induction, whereas ANA-12 was administered intraperitoneally 30 min prior to each dose of 7,8-DHF.

2.4 Disease Activity Index (DAI)

The DAI score consisted of three indicators: percentage of body weight loss, fecal viscosity and fecal occult blood. A FOB kit was used to evaluate the blood in faeces (BaSo, China). The scoring criteria referred to a previous study^[46].

2.5 Periodic Acid-Schiff's (PAS) staining

PAS staining was performed as described in our previous study (Yuan ye, China)^[16]. Briefly, colon specimens were fixed in 4% paraformaldehyde (Yuan ye, China), embedded in paraffin and sectioned at a thickness of 5 μ m for staining. Goblet cells were quantified using ImageJ software.

2.6 Real-time fluorescence quantitative PCR

Total RNA from colon tissues or MC38 cells was extracted using the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, China) and cDNA was synthesized using the Vazyme HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, China). Quantitative real-time PCR was performed with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China). *Actb* was used as the internal standard. All primers used in this study are provided in Supplementary Table 1.

2.7 Transcriptome sequencing & analysis

RNA-sequencing was conducted following the protocol reported previously^[47]. In a nutshell, total RNA was extracted from colon tissue of different groups, and RNA purity was assessed using the RNA Nano 6000 Assay Kit on the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Library preparation and sequencing were carried out on an Illumina Novaseq platform to generate 150 bp paired-end reads. KEGG

enrichment analysis of differentially expressed genes was performed using the KOBAS database (<http://bioinfo.org/kobas>).

2.8 Western blotting

Cells or tissue samples were lysed in ice-cold RIPA Buffer (R0010, Solarbio) supplemented with 1% PMSF (P0100, Solarbio) on ice for 10 min. The supernatant was collected after centrifugation and the protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher, USA). Equal amounts of protein were separated by 10%SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with the appropriate secondary antibody for 1h. GAPDH or β -actin was selected as an internal loading control. The protein bands were visualized with the ECL (Millipore, USA) and analyzed with ImageJ software.

2.9 Flow Cytometry and Fluorescence Imaging

For ROS and Fe^{2+} measurements, MC38 cells were seeded in 24-well plates and treated with 50 μM 7,8-DHF, 5 μM RSL3 and 10 μM Fer-1 respectively. After 24 h, the cells were rinsed with PBS and detached using trypsin. Then cells were incubated by 10 μM DCFH-DA (S0033S, Beyotime), 10 μM C11-BODIPY (RM02821, ABclonal) and 10 μM FeRhoNox-1 (MX4558, MKBi) working solution at 37°C for 30 min. Regarding to the tissue imaging for ROS and Fe^{2+} detection, it was performed as previous study. In summary, colonic tissues were isolated and processed into single-cell suspensions, ensuring a cell viability of over 80%. Subsequently, the cells were incubated with the respective probes as previously described. Finally, the stained cells were analyzed by flow cytometry (CYTOFLEX, Beckman Coulter, USA) or a Leica TCS SP8 confocal microscope laser scanning microscope (Leica, Germany).

2.10 Measurement of GSH, MDA and Fe^{2+} levels

Total GSH, MDA and Fe^{2+} concentration in serum and colon were determined by GSH Assay Kit (MM-43694M1, Wuhan, China), MDA Assay Kit (BY-JZF0071, Nanjing, China), and Iron Assay Kit (ab83366, USA). All procedures were conducted following the manufacturer's protocol.

2.11 Cell lines and culture

MC38 cells (purchased from the cell bank of the Chinese Academy of Sciences) were cultured in Dulbecco's Modified Eagle Media (DMEM, GIBCO) supplemented with fetal bovine serum (10%FBS, GIBCO) and penicillin–streptomycin (1%) at 37°C in 5% CO_2 . First, MC38 cells were incubated with 0, 10, 20, 30, 40, 50, 100, or 200 μM 7,8-DHF in DMEM for 24 h to evaluate the cytotoxicity of 7,8-DHF. Subsequently, the cells were treated with 5 μM RSL3 for 24 h. Prior to co-treatment with RSL3, 7,8-DHF (50 μM) and Fer-1 (5 μM) were applied as pretreatments for 2 h. Cell viability was assessed using the CCK8 reagent (Vazyme, China).

2.12 Lactate dehydrogenase (LDH) cytotoxicity assay

According to the manufacturer's protocol, the cytotoxicity was evaluated by applying the LDH detection kit (C0018S, Beyotime, China). The OD value was measured at 450nm, employing a microplate reader to

determine the relative amount of LDH. The LDH relative release amount was calculated using the following formula: LDH relative release amount (%) = (OD [treated sample] – OD [control sample]) / (OD [maximum enzyme activity of cells] – OD [control sample]) × 100%.

2.13 Separation of cell nuclear and cytoplasmic fractions

The nuclear and cytoplasmic fractions from the cell were separated with a cell nuclear and cytoplasmic isolation Kit (Beyotime). The detection of nuclear proteins were performed using the BCA reagent, followed by their transfer to a 10% SDS-PAGE gel. PCNA served as an internal reference gene.

2.14 Cell transfection

For transfection of siRNA, MC38 cells were seeded in 12-well plates and cultured for 24 h, after which they were transfected with Nfe2l2 siRNA (Tsingke, China), or control siRNA (Tsingke, China) using the jetPRIME transfection reagent (Polyplus, Lot#16Y0304K4) according to the manufacturer's instructions. The Nfe2l2 siRNA sequence are listed in Supplementary Table 1.

2.15 Immunohistochemistry staining

Immunohistochemical (IHC) staining was performed as described in a previous study^[48]. Briefly, colon samples were fixed in paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 µm. Endogenous peroxidase activity was quenched with 3% H₂O₂, followed by blocking with 10% goat serum. The sections were then incubated with an anti-Nrf2 antibody (1:100 in PBS) at 4°C overnight. After washing, the sections were incubated with a goat anti-rabbit IgG secondary antibody at 37°C for 1 h. Immunoreactivity was visualized using a DAB Substrate Kit (SK-4100, Vector, USA) and HRP (434323, Invitrogen, USA). Finally, these slices were examined under a light microscope.

2.16 Statistical analysis

Data are presented as mean ± SEM. Statistical significance between two groups was determined using Student's *t*-test, whereas comparisons among more than two groups were analyzed by one-way ANOVA. *P* < 0.05 was considered statistically significant.

3. Results

3.1 7,8-DHF ameliorates sleep deprivation-induced intestinal damage

Previous study has proved that SD can induce intestinal damage^[9, 17]. In this study, intestinal function was evaluated based on a previously established SD model^[16]. 7,8-DHF has garnered considerable attention owing to its multiple biological activities^[49]. Nevertheless, the potential interaction between 7,8-DHF and gut damage remains unexplored. Here, 7,8-DHF was utilized to evaluate its effects on intestinal damage. The procedure of the animal trial was schematically illustrated as Fig.1A. In this SD model, pre-treatment of 7,8-DHF significantly attenuated SD-induced damage, as reflected by body weight (Fig.1B), colon length (Fig.1C-D) and fecal occult blood scores (Fig.1E). Besides, the DAI also provided evidence that 7,8-DHF could alleviate colonic damage (Fig.1F). To further investigate whether 7,8-DHF supplementation could

affect the colonic mucosal integrity, we detected colonic goblet cells. Sleep deprivation significantly reduced goblet cell numbers, while 7,8-DHF treatment partially restored their abundance (Fig.1G-H). In addition, 7,8-DHF also restored expressions of intestinal barrier-associated molecules like ZO1, MUC2 and Occludin in the colon (Fig 1I and S1). These results collectively suggested that 7,8-DHF could ameliorate colon damage induced by SD to some extent. It is of great value to reveal relevant mechanisms.

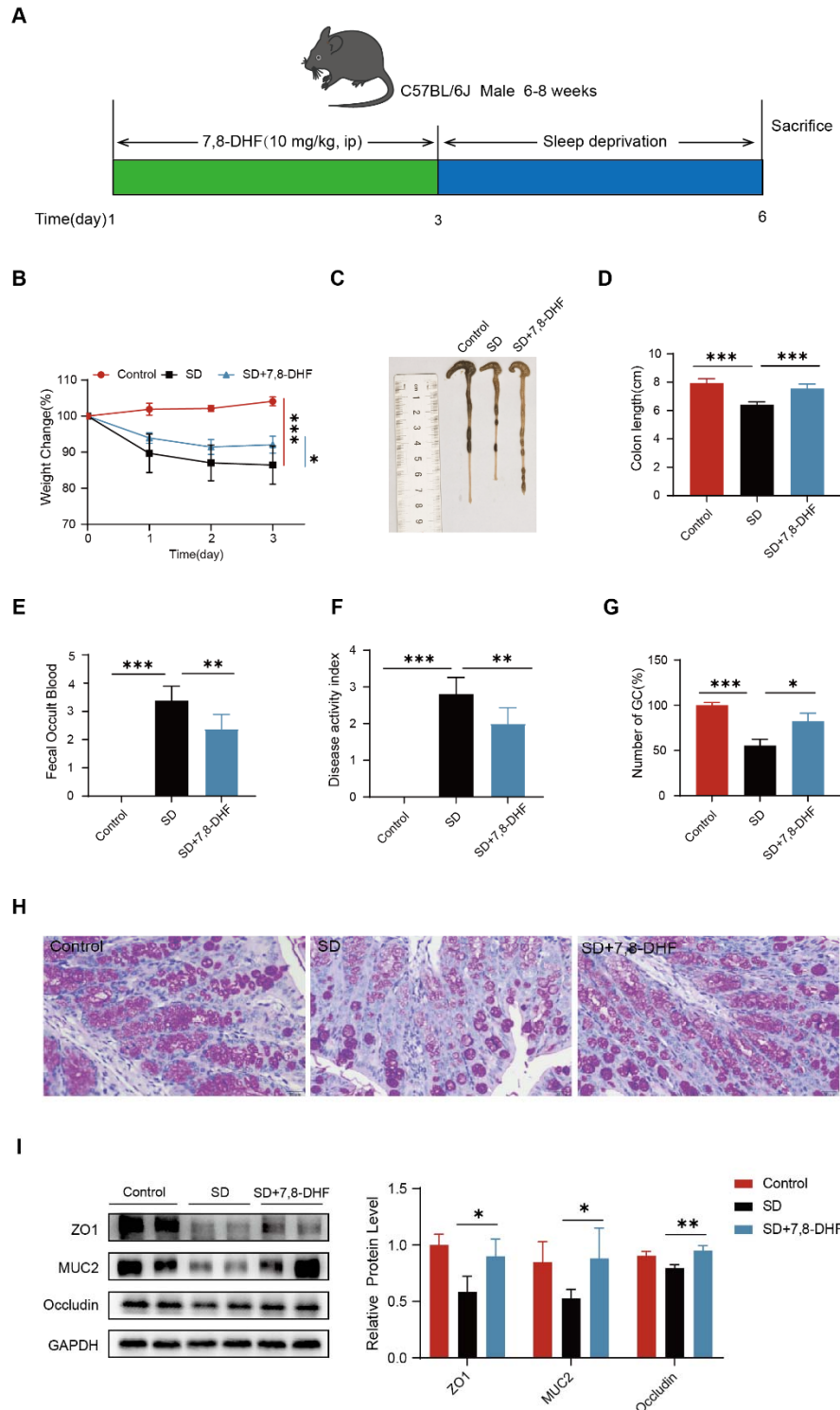
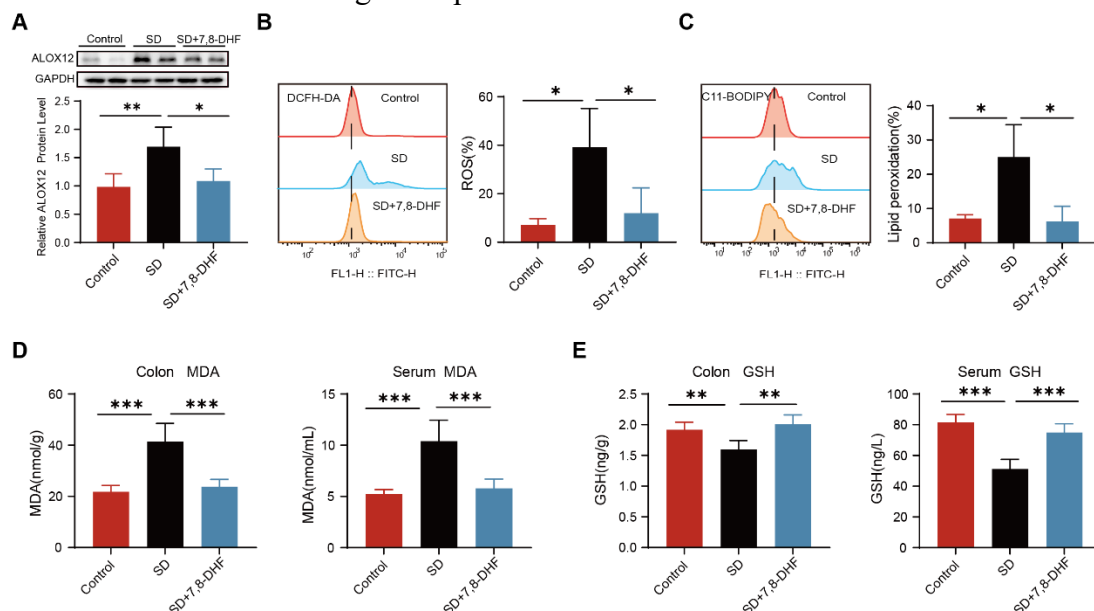


Figure 1. 7,8-DHF significantly alleviates SD-induced intestinal damage *in vivo*

A Schematic illustration of experimental protocol for the animal trial. B The weight change of mice (n=8 mice per group). C-D The colon length and quantitative analyses (n=8 mice per group). E The mice fecal occult blood score (n=8 mice per group). F The DAI score (n=8 mice per group). G-H PAS staining of colonic tissue sections (n=3 mice per group). The scale bar is 20 μ m. I Western blot of ZO1, MUC2, Occludin in SD model (n=4 mice per group). All the quantitative data are presented as mean $\pm$ SD. * P < 0.05; ** P < 0.01; *** P < 0.001.

3.2 7,8-DHF attenuates intestinal damage via regulating ferroptosis.

In view of the promising protection effects of 7,8-DHF, we endeavored to explore the underlying mechanisms. Then we utilized the Swiss Target Prediction database to predict the related pathway (<http://swisstargetprediction.ch/>). The results indicated a potential association with ALOX12 (Fig.S2). Notably, ALOX12 is essential for mediating ferroptosis^[50]. We previously identified ferroptosis as a major pathogenic target underlying SD-induced intestinal damage^[16]. Here, we broadened our investigation to determine whether 7,8-DHF exerts a regulatory effect on ferroptosis. Quantitative analysis of the ferroptosis-associated protein ALOX12 showed a significant reduction after treatment with 7,8-DHF (Fig.2A). Accordingly, we hypothesized that 7,8-DHF has the potential to regulate ferroptosis. To validate this assumption, we further assessed the intracellular ROS and lipid peroxides in SD mice. The results demonstrated that, comparing with SD group, 7,8-DHF treatment partially reduced lipid ROS accumulation in colonic cells (Fig.2B-C). Furthermore, 7,8-DHF restored GSH and MDA in serum and colon (Fig.2D-E). Subsequently, 7,8-DHF could increase the expression of ferroptosis-related proteins, including GPX4, SLC7A11 and HO-1 (Fig.2F). Meanwhile, iron homeostasis is a crucial indicator for ferroptosis. By detecting iron homeostasis-related proteins, 7,8-DHF administration decreased the protein expression of TFRC (transferrin receptor) and increased FTH (ferritin heavy chain) in colon tissues (Fig.2G). Besides, 7,8-DHF significantly prevented SD-induced iron accumulation in colon and serum (Fig.2H-I). Considering the findings observed *in vivo*, it is essential to assess the efficacy of 7,8-DHF *in vitro*. To this end, MC38 cells were selected as a representative cell line to be employed. In order to ascertain the appropriate concentration of 7,8-DHF for subsequent studies, the cell viability of 7,8-DHF was performed by cell counting Kit-8 assay (Fig.S3). A final concentration of 50 μ M of 7,8-DHF was applied to MC38 cells for the following research. Intriguingly, 7,8-DHF abolished cell death induced by RSL3 in MC38 cells (Fig.2J-K). In addition, the flow cytometry and immunofluorescence staining indicated that RSL3-induced lipid peroxides increasing was significantly inhibited by 7,8-DHF (Fig.2L-M). Collectively, 7,8-DHF suppresses ferroptosis through dual mechanisms: activation of antioxidant gene expression and restoration of iron homeostasis.



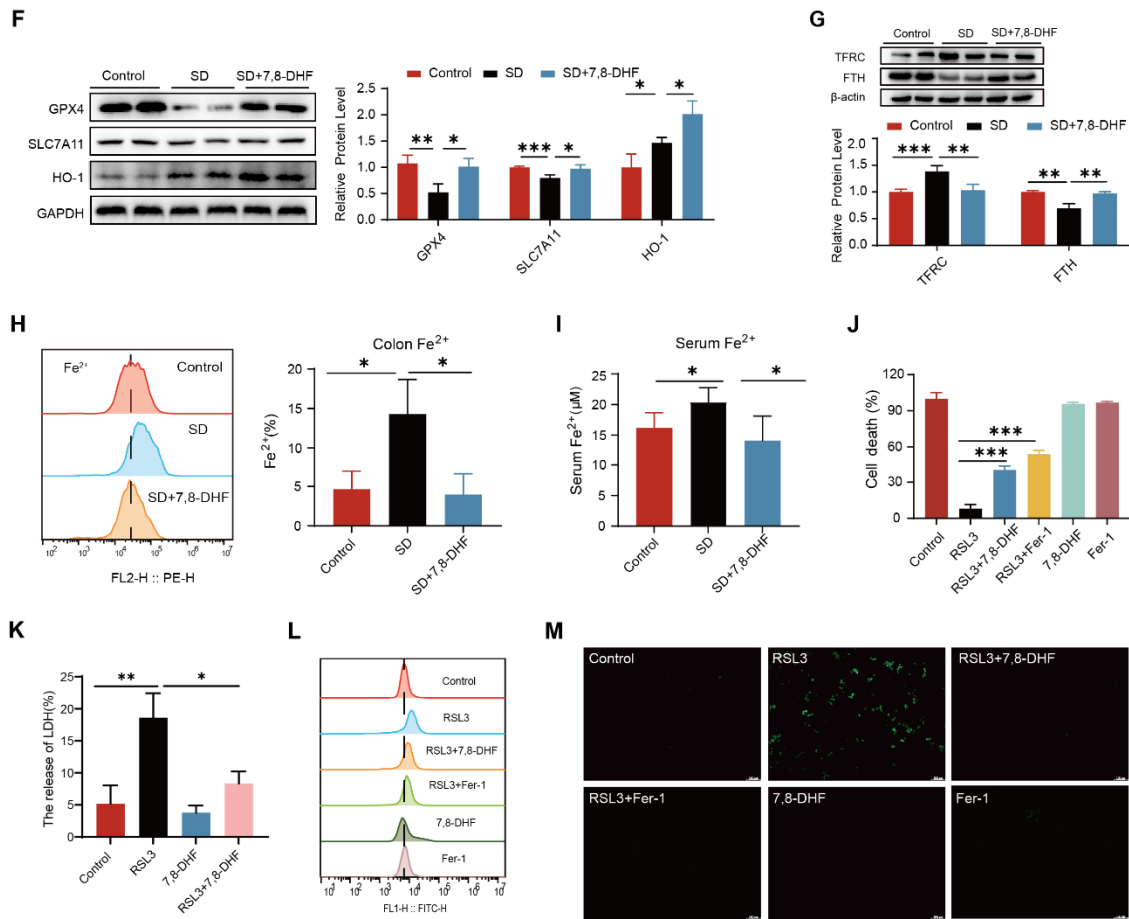


Figure 2. 7,8-DHF inhibits ferroptosis in mice during SD-induced intestinal damage

A The protein level of ALOX12 was examined in colon. (n=4 mice per group) B ROS level was assessed by flow cytometry using DCFH-DA. (n=3 mice per group) C Lipid peroxidation level was assessed by flow cytometry using C11-BODIPY. (n=3 mice per group). D-E MDA and GSH levels were quantified in the colonic tissue and serum of mice (n=6 mice per group). F The protein levels of GPX4, SLC7A11 and HO-1 were examined in colon. (n=4 mice per group). G The protein levels of TFRC and FTH were examined in colon. (n=4 mice per group). H The accumulation of Fe²⁺ was detected in colon. (n=3 mice per group). I The accumulation of Fe²⁺ was detected in serum. (n=6 mice per group). J Cell viability assayed by CCK-8. (n=6 per group).

K The LDH assay in MC38 cells. (n=4 per group). L-M Lipid ROS levels were measured by flow cytometry and immunofluorescence staining with C11-BODIPY in MC38 cells. (n=3 per group). The scale bar is 100 μ m. * P < 0.05; ** P < 0.01; *** P < 0.001.

3.3 Nrf2 plays a critical role for 7,8-DHF in mitigating ferroptosis

Considering that GPX4, HO-1 and FTH are downstream targets of Nrf2, meanwhile Nrf2 is critically for ferroptosis^[51], it is possible that 7,8-DHF protected intestine against ferroptosis through activating and increasing Nrf2 signaling pathways (Fig.3A). Therefore, we conducted western blot analysis to assess whether 7,8-DHF enhanced Nrf2 expression. Consistently, 7,8-DHF activated Nrf2 and downstream proteins including SLC7A11, HO-1 and GPX4 in a time-dependent manner in mice (Fig.3B). This was consistent with the results of cell experiments (Fig.S4). Nuclear entry of Nrf2 serves as a critical indicator for the activation of this pathway. According to the results of immunofluorescence and western blot, 7,8-DHF facilitated the translocation of Nrf2 into the nucleus in MC38 cells (Fig.3C-D). Consistently, we observed the same phenomenon in the colonic tissues (Fig.3E). To reinforce the notion of 7,8-DHF inhibits ferroptosis by increasing Nrf2, a direct cell assay was conducted in MC38 cells, the result demonstrated that 7,8-DHF enabled the mRNA expression of *Hmox1*, *Gpx4*, *Slc7a11* and *Gclm*, which were related to *Nfe2l2*(Fig.3F). In

contrast, the protection of 7,8-DHF for increasing the expression of *Hmox1*, *Gpx4*, *Slc7a11* and *Gclm* were abolished after silencing *Nfe2l2* (Fig.3G and S5). Hence, it was plausible that 7,8-DHF inhibited ferroptosis by activating Nrf2.

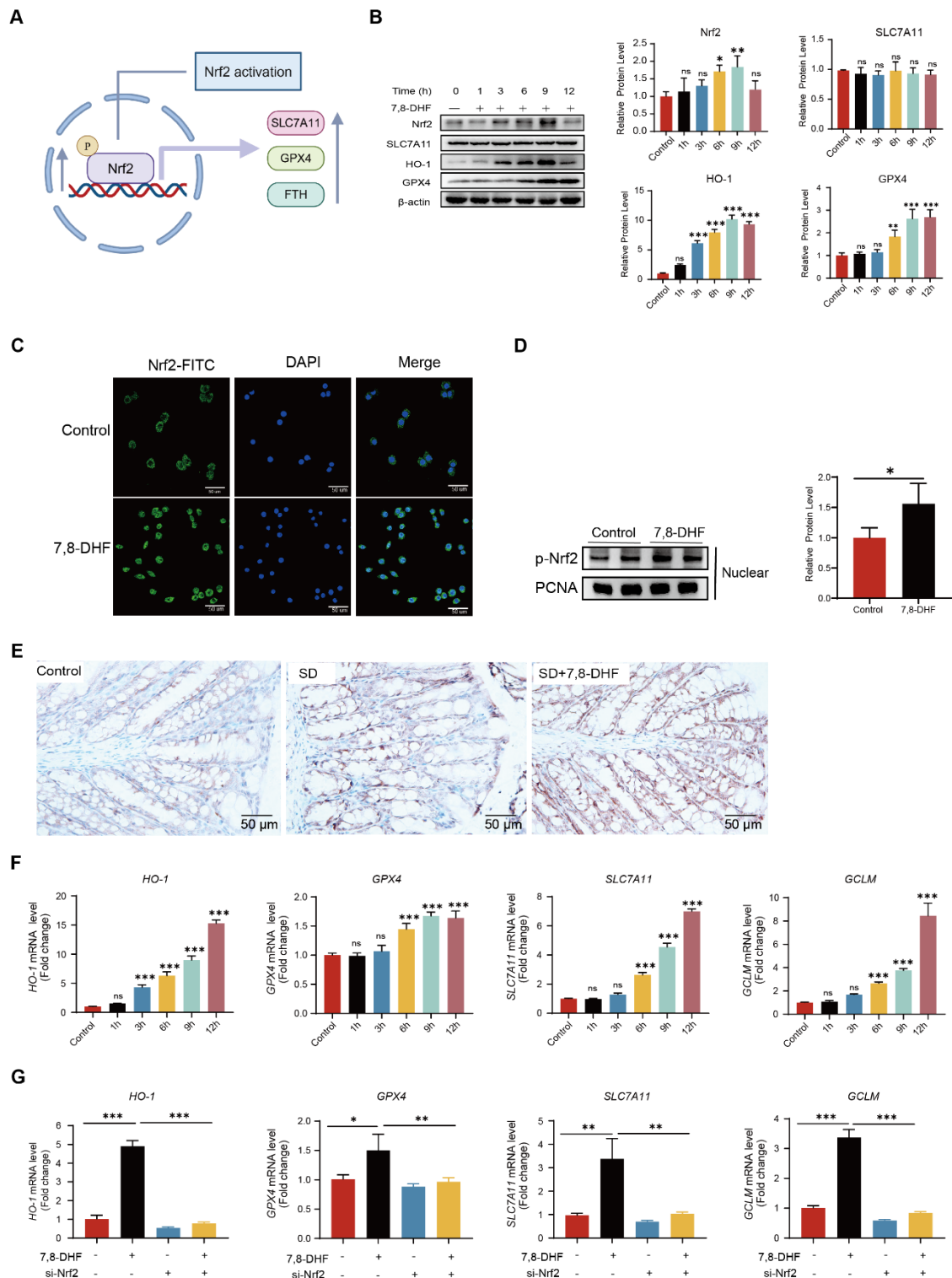
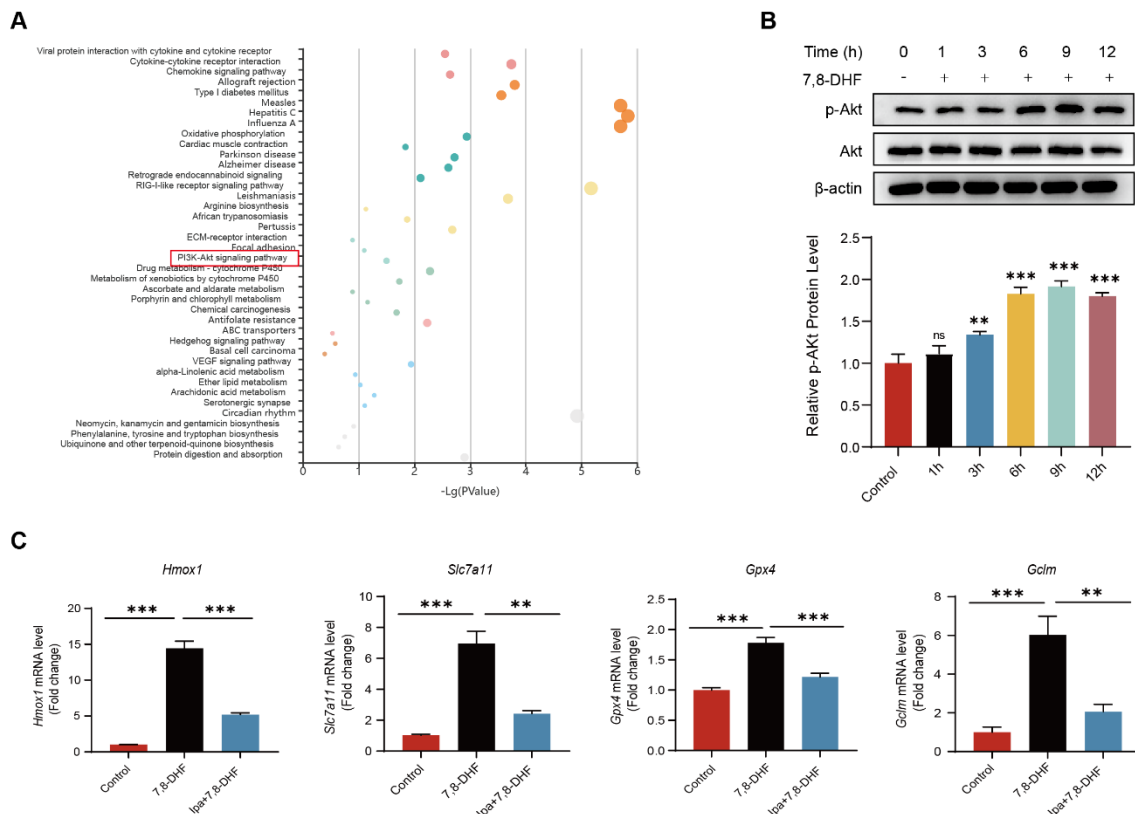


Figure 3. 7,8-DHF activates Nrf2 in colon tissues and MC38 cells

A Schematic diagram of Nrf2 regulation on antioxidative pathway. B The protein levels of Nrf2, SLC7A11, HO-1, and GPX4 were examined in colon tissue. (n=4 mice per group). C Representative images of immunofluorescence staining for Nrf2 in MC38 cells. (n=3 per group). The scale bar is 50 μ m. D Assessment of Nrf2 nuclear translocation in MC38 cells. (n=4 per group) E Representative images of immunohistochemistry staining for Nrf2 in colon tissue. (n=3 mice per group). The scale bar is 50 μ m. F Relative mRNA levels of *Hmox1*, *Gpx4*, *Slc7a11* and *Gclm* in MC38 cells. (n=6 per group). G Relative mRNA levels of *Hmox1*, *Gpx4*, *Slc7a11* and *Gclm* in MC38 cells by Si-Nfe2l2. (n=6 per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.4 7,8-DHF increases Akt phosphorylation to regulate Nrf2

With the role of Nrf2 in 7,8-DHF-mediated ferroptosis confirmed, we subsequently aimed to uncovering the upstream regulatory mechanisms to restrain ferroptosis. Transcriptomic analysis revealed enrichment of PI3K-Akt signaling pathway (Figure.4A). Interestingly, western blot confirmed time-dependent phosphorylation of Akt after 7,8-DHF treatment (Fig.4B). Meanwhile, Ipatasertib (an Akt inhibitor) was employed to conduct the experiment, verified whether 7,8-DHF worked by activating Akt. Accordingly, 7,8-DHF could boost the mRNA level of *Hmox1*, *Slc7a11*, *Gpx4*, *Gclm*, while these promotion effects were partially antagonized by Ipatasertib (Fig.4C). Consistently, 7,8-DHF also activated Nrf2, HO-1, GPX4 protein levels, while these activities of 7,8-DHF were suppressed through Ipatasertib implement (Fig.4D). The String database discovered Pi3k/Akt signal pathway was associated with EGFR (a kind of tyrosine kinase) (Fig.4E). Meanwhile, receptor tyrosine kinases (RTKs) are known to regulate various downstream signaling pathways such as MAPK, PI3K/Akt and JAK/STAT^[52]. Subsequently, the western blot analysis demonstrated the protein level of p-EGFR were significantly increased after 7,8-DHF treatment (Fig.4F). However, its phosphorylation was synchronized with the phosphorylation of Akt, there was no obvious signaling cascade. We hypothesized other tyrosine kinases to regulate Akt phosphorylation. In this regard, we profiled the expression of TrkB and interestingly found that it was upgraded in response to 7,8-DHF (Fig.4G). Meanwhile, the temporal pattern of TrkB phosphorylation preceding EGFR and Akt activation. It is reasonable to assume that TrkB served as a crucial upstream regulator. Thus we supposed TrkB may be more indispensable in intestinal diseases. As a consequence, TrkB and Akt phosphorylation were promoted in response to 7,8-DHF supplement, ultimately exerting a protective effect in SD model.



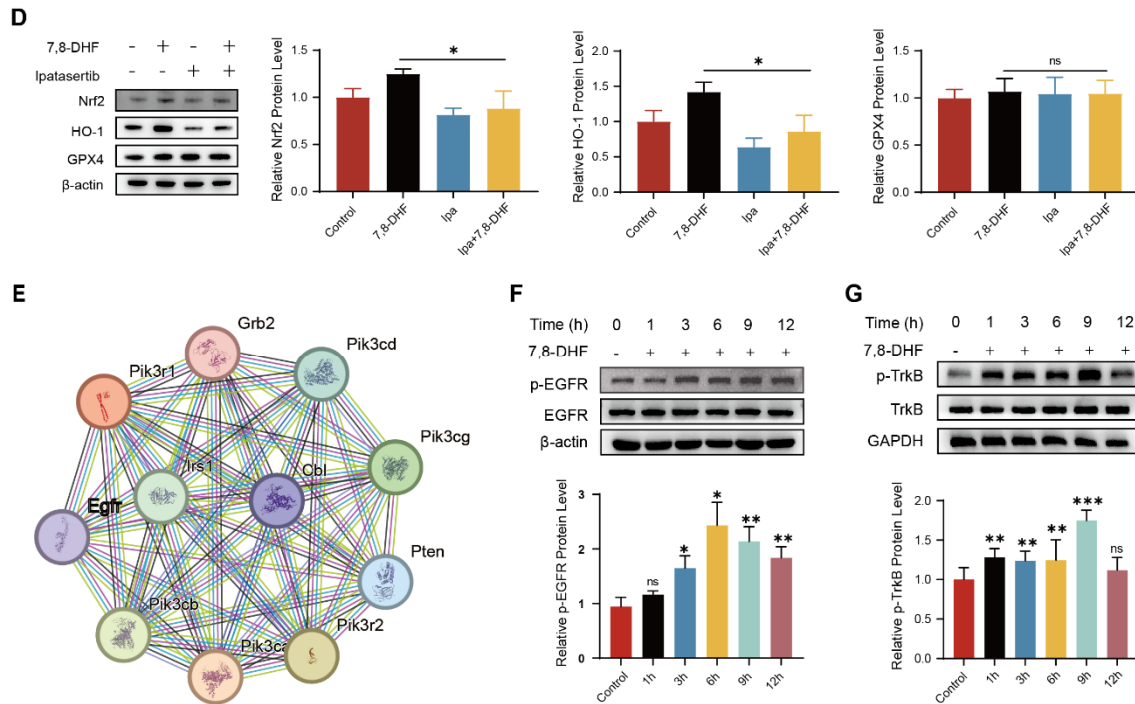
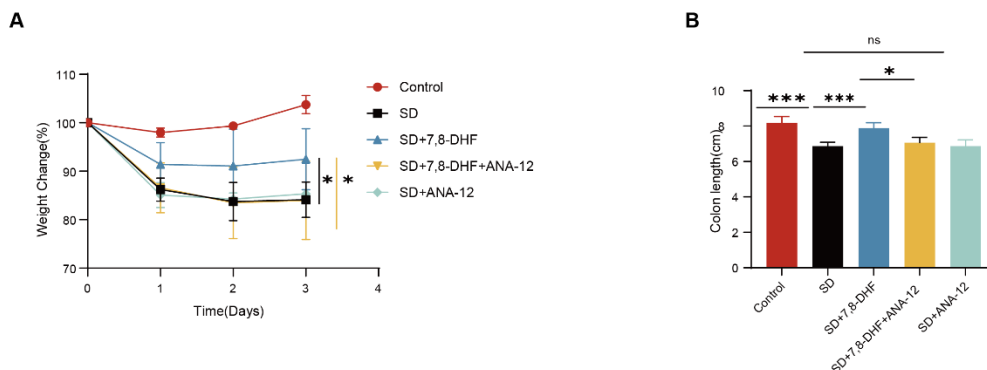


Figure 4. Akt phosphorylation promotes the expression of Nrf2 pathway

A KEGG-enriched pathways of differentially expressed genes in colon of SD mice (n=4 mice per group). B The protein expression of Akt was analyzed by Western blotting in colon tissues. (n=4 mice per group). C Relative mRNA levels of *Hmox1*, *Slc7a11*, *Gpx4*, and *Gclm* in MC38 cells. (n=6 per group) D The protein levels of Nrf2, HO-1, and GPX4 were examined in MC38 cells. (n=4 per group). E Regulatory network of the PI3K-Akt pathway. F-G The protein levels of EGFR and TrkB were examined in colon tissue for different time (n=4 mice per group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.5 TrkB inhibitor abolishes the protection of 7,8-DHF against ferroptosis in SD

To further validate that the protection of 7,8-DHF on ferroptosis was in a TrkB dependent manner, ANA-12 was used to construct mice experiment. ANA-12 is a small-molecule, selective TrkB antagonist that effectively inhibits BDNF-TrkB signaling pathway. Interestingly, ANA-12 abolished the ability of 7,8-DHF to alleviate intestinal damage and restore body weight. (Fig.5A-B). Co-injection of 7,8-DHF and ANA-12 showed no obvious improvement in the expression of Occludin, ZO-1, and MUC2 when compared with the SD group (Fig.5C). The effects of 7,8-DHF on GSH and MDA in the serum and colon were also inhibited by ANA-12. (Fig.5D-E). Besides, the protein expression of Nrf2, HO-1, GPX4 had no significant difference between co-injection ANA-12 of SD group and SD accompany with 7,8-DHF group (Fig.5F). In summary, we confirmed that the protective effects of 7,8-DHF were accompanied by activation of the TrkB pathway.



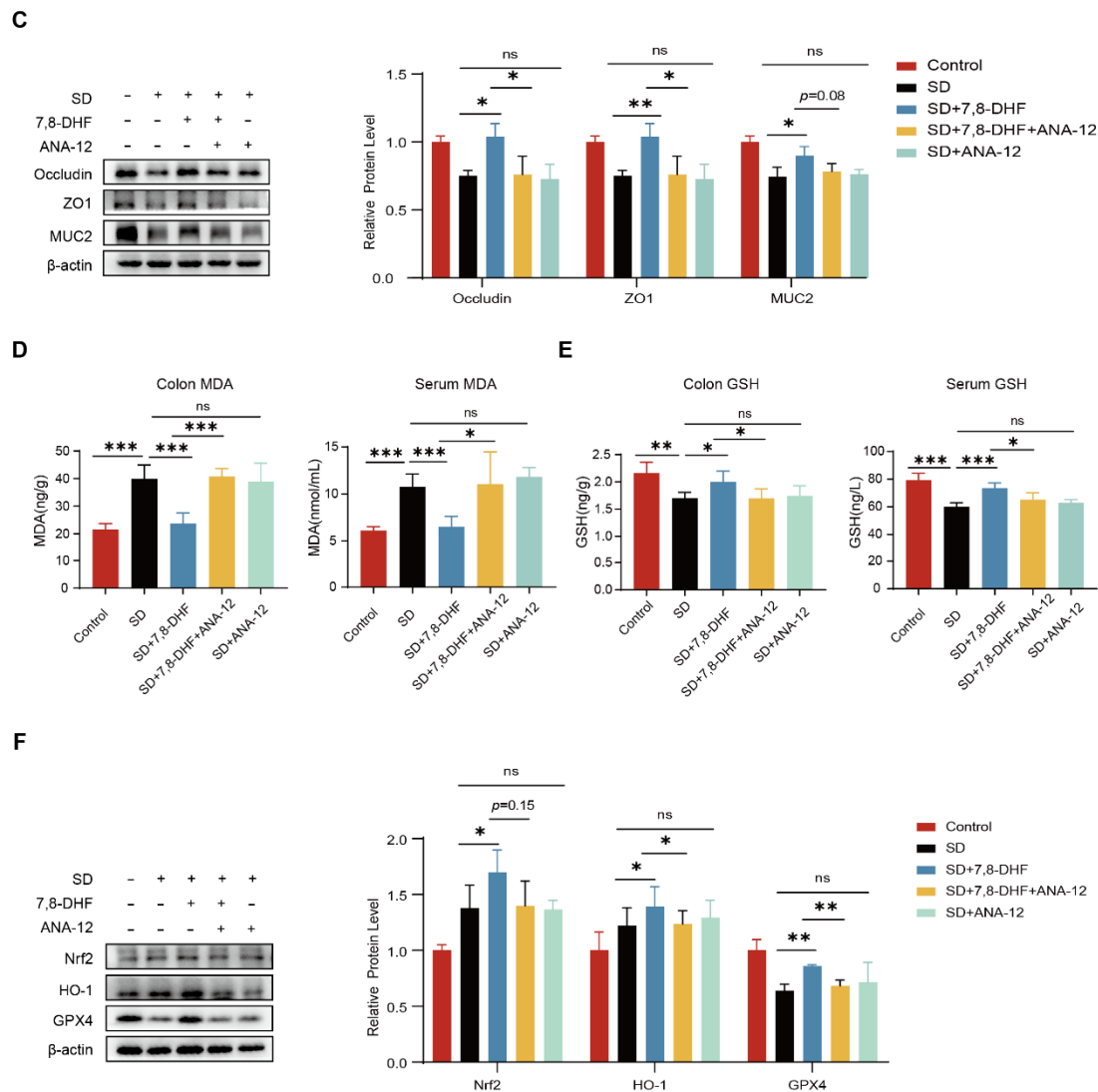


Figure 5. The inhibition of TrkB-Akt pathway abolished the effect of 7,8-DHF on ferroptosis

A Body weight variation in mice treated with ANA-12 (n=6 mice per group). B The colon length of the mice after ANA-12 treatment (n=6 mice per group). C The protein levels of Occludin, ZO1, and MUC2 were examined in colon tissue with ANA-12 treatment. (n=4 mice per group). D-E The levels of MDA and GSH were measured in colon tissue and serum with ANA-12 treatment (n=6 mice per group). F The protein levels of Nrf2, HO-1, and GPX4 were examined in colon tissue with ANA-12 treatment. (n=4 mice per group) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4. Discussion

Accumulating evidence highlights the indispensable role of sleep in animal physiology^[53, 54]. SD can trigger severe diseases of different organs, even cause premature mortality in most animals^[55]. Sufficient sleep is critical for maintaining neurocognitive function and supporting memory consolidation, whereas SD has been linked to a heightened risk of multiple neurocognitive deficits^[56]. Interestingly, administration of serotonin precursor has been proved to ameliorate SD-induced cognitive impairments^[57]. Although these studies highlight the profound damage that SD can inflict on the brain, our work focuses more specifically on its detrimental effects on the gastrointestinal tract. In addition, SD could induce the accumulation of ROS in the intestines of mice and *Drosophila*, ultimately leading to animal death^[9]. Our previous study showed SD has been associated with ferroptosis, it can lead to colonic damage via regulating ferroptosis^[16]. Even worse, SD causes multiple adverse effects on intestinal health, such as impaired intestinal mucosal integrity,

perturbed intestinal microbiota, and imbalanced microbiota metabolism^[58]. Thus, targeting intestinal ferroptosis represents an important therapeutic strategy for intestinal diseases.

To date, ferroptosis has been recognized as a multifaceted process influenced by a combination of endogenous and exogenous determinants. Although a wide range of ferroptosis antagonists has been explored, their unfavorable pharmacokinetic properties—including rapid metabolic clearance, poor solubility, limited cell permeability, and systemic toxicity—substantially constrain their clinical advancement^[59]. Phytochemicals have been proved to have protective effects on hosts via their antioxidative, anti-inflammatory, and antiviral properties. The fact that 7,8-DHF can regulate metabolism, inflammation and exert antioxidant efficacy has been extensively discussed in publications^[60–62], but very few of them mentioned whether 7,8-DHF could suppress ferroptosis to ameliorate intestinal damage. This paper gives evidence of benefit for the application of 7,8-DHF in protecting against SD-induced gut damage.

Mechanistically, 7,8-DHF inhibits lipid peroxidation in a GPX4-dependent manner. Furthermore, its anti-ferroptotic activity relies on Nrf2 activation, which subsequently upregulates the transcription of downstream targets, including *Gpx4*, *Hmox1*, *Gclm*, and *Slc7a11*. Consistent with prior findings, Nrf2 serves as a master regulator of antioxidant defense, as many of its downstream target genes are critical in maintaining redox homeostasis and regulating ferroptosis^[63, 64]. Intriguingly, we further identified Akt as an upstream regulator of Nrf2. In a previous study, curcumin exerted anti-inflammation and antioxidant stress efficacy mediated by anti-inflammatory microglia polarization through Akt/Nrf2 axis^[65]. While the regulatory interplay between Akt and Nrf2 was not explored in this study, it may involve Akt-mediated inactivation of Keap1 and suppression of GSK3 β . Li also reported that 7,8-DHF alleviates oxidative stress via activation of the Nrf2 pathway, thereby improving symptoms of postmenopausal osteoporosis^[44]. Collectively, these results imply that 7,8-DHF has potential utility in other models.

According to the STRING database, Akt activation is linked to EGFR, which is critically involved in mitigating intestinal mucosal damage under multiple stress conditions^[66]. Our findings demonstrate that 7,8-DHF not only activates TrkB, consistent with prior reports, but also upregulates additional tyrosine kinases^[43]. Notably, 7,8-DHF initiated TrkB activation earlier than EGFR, suggesting a temporal hierarchy in its signaling effects. Thus, we hypothesized that TrkB is likely to be the key mediator of intestinal protection. A prior work has shown that ANA-12, a selective TrkB inhibitor, can induce neuroinflammation and mechanical allodynia through BDNF-TrkB-p38/JNK signaling pathway^[67]. In line with these observations, ANA-12 blocked the protective effect of 7,8-DHF against intestinal damage. These findings underscore the crucial role of 7,8-DHF in mitigating intestinal pathologies linked to ferroptosis.

While this study elucidates the mechanism by which 7,8-DHF alleviates intestinal damage through ferroptosis inhibition, several caveats in the study should be taken into account. Firstly, although we demonstrated that 7,8-DHF suppresses lipid peroxidation, its interaction with specific phospholipid substrates remains to be fully characterized. Secondly, apart from its impact on intestinal epithelial cells, 7,8-DHF may also influence immune response and associated cells. We did not identify the potential role of TrkB-Akt-Nrf2

in intestinal immune cells on intestinal function. Thirdly, many kinds of stress in aquaculture production, we only use SD model. Lastly, although many types of stress exist in aquaculture production, our study employs only the sleep deprivation model, and we did not investigate FFA, serotonin, GABA, or other pathways linking insomnia to the intestine. We plan to answer remaining questions in future studies.

5. Conclusion

In summary, 7,8-DHF protected SD-induced colon damage through inhibiting ferroptosis. Mechanistic investigations revealed that 7,8-DHF suppressed ferroptosis via the TrkB-Akt-Nrf2 signaling pathway. This study provides a new perspective on the 7,8-DHF for intestinal damage and highlights the significant role of 7,8-DHF on ferroptosis.

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Ethics Statement

This study protocol was approved by the Ethics Committee of South China Agricultural University (Approval: 2023b118).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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