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Melezitose inhibited glycolytic pathway and enhances anti-Crohn's disease activity via binding to PGK1

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ABSTRACT

Ethnopharmacology relevance: Alhagi honey is a light yellow sugar granule formed by concentrating the liquid secreted by Alhagi branches and leaves. It is a traditional Uygur medicine often used to treat abdominal pain, diarrhea, dysentery, and other conditions. Modern research has indicated that the main active components of Alhagi honey are oligosaccharides and polysaccharides. Our previous research had identified that the extract of Alhagi honey exhibits good anti-inflammatory pharmacological activity, however, its efficacy against Crohn's disease (CD) remains to be elucidated.

Aim of the study: To determine the efficacy of the extract of Alhagi honey in CD and to explore its potential targets and mechanisms.

Materials and methods: Mel (melitriose) is extracted from dried Alhagi honey. In vivo, 2.5% 2,4,6-trinitrobenzenesulfonic acid (TNBS, At a dosage of 100 mg/kg) is used as an enema to induce CD-like changes in the rat colon. Over the subsequent fortnight, the modeled rats were treated with Mel via gavage. The histopathological alterations and repair ability of colonic injury in the colon tissue were evaluated using hematoxylin and eosin (H&E), Masson's trichrome, and immunofluorescence staining. Additionally, the amelioration of inflammatory responses in the colon was assessed using enzyme-linked immunosorbent assay (ELISA). The reparative capacity of Mel on inflammation was evaluated by inducing inflammation in RAW264.7 cells with lipopolysaccharide (LPS). The Drug Affinity Responsive Target Stability (DARTS) experiment was used to explore the relevant targets of action. Furthermore, network pharmacology was used to investigate the mechanism of action of Mel, to further validate its effects at the cellular level.

Results: In the CD rat model, treatment with Mel significantly improved colonic mucosal damage and inflammatory infiltration. It also demonstrated a reduced collagen fiber deposition, thereby ameliorating fibrotic changes in colonic tissue. Furthermore, Mel decreased the expression of pro-inflammatory factors and increased the expression of anti-inflammatory factors in colonic tissue and cell supernatants. Further research confirmed that Mel influences the glycolytic pathway by binding to phosphoglycerate kinase 1 (PGK1) and suppressing its activity, leading to reduced production of adenosine triphosphate (ATP) and its metabolites, 2-phosphoglycerate (2-PG), 3-phosphoglycerate (3-PG); thus, playing a role in anti-inflammation and promotion of repair. This mechanism was further validated using the PGK1 inhibitor NG52, which also demonstrated a reduction in the production of ATP, 2-PG, and 3-PG.

Conclusions: This study revealed that Mel exerts its anti-inflammatory and reparative capabilities *in vitro* and *in vivo* by inhibiting the activity of the key glycolytic enzyme PGK1, leading to reduced production of ATP and its

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products 2-PG and 3-PG, thereby ameliorating the symptoms of CD. It can emerge as a promising candidate for CD treatment.

1. Introduction

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD) that usually affects the terminal ileum and proximal colon, with discontinuous, patchy, segmental, and transmural inflammation (Dolinger et al., 2024). It may lead to complications associated with the condition, potentially increasing the incidence of cancers such as colorectal and breast cancer(Gao et al., 2023). Although CD pathogenesis remains to be further elucidated, substantial evidence suggests that CD results from the interplay of genetic, environmental, immunological, and microbial factors(Cockburn et al., 2023). Relevant reviews have reported novel epidemiological trends in CD globally since the 21st century, with a marked escalation in hospitalization rates for CD in emerging industrialized nations, including those in Asia and Latin America(Buie et al., 2023). By 2023, the incidence and prevalence rates of CD have escalated dramatically, imposing a substantial burden on global healthcare systems(Gorospe et al., 2024).

Currently, CD treatment primarily involves drug therapy to control inflammation and timely surgical intervention to alleviate symptoms of intestinal damage. The European Crohn's and Colitis Organization's updated 2024 guidelines for CD treatment include new targeted drugs, such as risankizumab and upadacitinib, expanding treatment options (Gordon et al., 2024). Mesalazine (Mesa), a first-line drug for IBD treatment, is mainly used for the maintenance treatment of CD during the postoperative remission phase(Hart et al., 2020). It can restore the integrity of the intestinal epithelial barrier by modulating intercellular junctional complexes and inhibiting the synthesis of inflammatory mediators, thereby reducing inflammatory responses(Khare et al., 2019). However, long-term use may lead to side effects, such as dizziness, drug resistance, drug allergy, and a decrease in body weight and appetite(Liu et al., 2024). To circumvent the severe side effects of medications and enhance survival rates, researchers are focusing on developing new anti-CD drugs and improving prognosis.

It is recognized that inflammatory responses involve the activation of various cell types to combat damage and maintain tissue homeostasis. Pro-inflammatory cells such as M1 macrophages, Th1, and Th17 lymphocytes rapidly provided energy through glycolysis and high lactate production to exacerbate inflammation(Soto-Heredero et al., 2020). Glycolytic rate-limiting enzymes or glycolytic metabolites like lactate may regulate inflammatory responses by promoting the activation of inflammasomes in macrophages(Bae et al., 2021). Modern research has demonstrated that clinically applied poly (ADP-ribose) polymerase-1 (PARP-1) inhibitors enhance glycolysis (extracellular acidification rate) under oxidative stress and improve mitochondrial function in epithelial cells, thereby improving experimental CD by preventing energetic collapse and protecting intestinal barrier integrity. Thus, they can be repurposed for the treatment of CD(Kovács et al., 2021). Phosphoglycerate kinase 1 (PGK1) plays an essential role in the glycolytic pathway. As the first key metabolic enzyme producing ATP in the glycolytic process, it is crucial for the sustained generation of cellular energy. Reports indicate that Th17 cells from patients with CD exhibit upregulated PGK1 expression. *In vivo*, silencing PGK1 could enhance the beneficial effects of trinitrobenzene sulfonic acid (TNBS)-induced colitis, achieving control over disease activity and an enhanced immunoregulatory phenotype(Vuerich et al., 2022). Therefore, regulating the glycolytic pathway to control inflammatory responses is a focal point of our current research.

"*Alhagi honey*" is an important ethnic medicine. It is a solid sugar particle secreted by the leaves of the leguminous plant *Alhagi sparsifolia* Shap. It is widely found in saline-alkali arid lands of Northwest China, Central Asia, and North Africa(Ghosal and Srivastava, 1973). In traditional medicine among the Uyghur people in Xinjiang, China, it is commonly used to treat dysentery, diarrhea, and liver and gallbladder diseases(Song et al., 2024) Our previous research has identified and purified an oligosaccharide extracted from Alhagi honey, which has been structurally characterized as Melezitose (Mel)(Lv, Zhiyuan et al., 2024). Mel is known for its ability to promote the growth of beneficial bacteria such as bifidobacteria and lactobacilli in the gut, which helps in establishing a healthy gut microbiota(Singh et al., 2017). Studies have demonstrated that the metabolism of Mel to fructose is intricately linked with stress-induced gut microbiota remodeling, intestinal stem cell (ISC) renewal, and epithelial homeostasis, forming a positive feedback metabolic loop. Fructose, a product of melitriose metabolism, enhances glycolysis, which in turn fuels ISC proliferation, thereby offering a potential therapeutic approach for alleviating gastrointestinal diseases (Hou et al., 2021). Additionally, research has indicated that Mel targets the inflammatory TLR4-MyD88-NF-κB signaling pathway, mitigating dextran sulfate sodium (DSS)-induced colitis in mice and normalizing the levels of inflammatory cytokines in serum(Zhang, P. et al., 2024). However, the potential preventive and therapeutic effects of Mel on CD have not yet been explored, and further research is urgently needed.

This study investigated the protective effects of Mel on pathological damage, inflammation, and intestinal barrier dysfunction in rats with TNBS-induced colitis by assessing the content of inflammatory factors in colonic tissue, changes in pathological damage, and colonic tissue fibrosis. *In vitro*, we observed the anti-inflammatory effects of Mel on RAW264.7 cells and improved repair capabilities, which were supplemented by Drug affinity responsive target stability (DARTS) experiments to explore relevant targets and further validate them. Collectively, this study assessed the therapeutic effects of Mel on CD and its mechanisms, and explored the potential of Mel as a functional food and natural medicine for CD prevention and treatment.

2. Materials and method

2.1. Cells and reagents

RAW264.7 cells, NCM460 cells and cell medium were provided by Procell (Wuhan, China). The cells were cultured in DMEM and supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and streptomycin at 37 °C in 5% CO_2. The antibodies against phosphoglycerate kinase 1 (PGK1, 380602) was obtained from Zenbio (Chengdu, China), The β-actin (bs-0061R) antibodies was obtained from Bioss (Beijing, China),and the Anti-Tumor Necrosis Factor-alpha, (Anti-TNF-α,GB115701-50) was obtained from Servicebio (Wuhan, China).The test kits Interleukin-12 (IL-12, MM-0105M1), TNF-α (MM-0132M1), IL-1β (MM-0040M1), adenosine triphosphate (ATP, MM-43789M1), 2-phosphoglycerate (2-PG, MM48222M1), 3-phosphoglycerate (3-PG, MM-47241M1), IL-10 (MM-0195R1), IL-1β (MM-0047R1), TNF-α (MM-0180R1), IL-12 (MM-7074R1) were obtained from Jiangsu Meimian industrial Co., Ltd (Jiangsu, China), IL-6 (E-EL-M0044c) and IL-10 (E-EL-M0046c) were obtained from Elabscience Biotechnology (Wuhan, China). Lipopolysaccharide (LPS, L8880), 3-PG (G9030), ATP (C0550), ; Nicotinamide adenine dinucleotide (NADH, N8110), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, G9030) and PronaseE (P8360) was obtained from Beijing Solarbio Science (Beijing China), NG52 (HY-15154), PGK1 Protein, Human (HEK293, His, HY-P71049) was purchased from Chemexpress Inc (Shanghai, China), and ameliorated trinitrobenzene sulfonic acid (TNBS,R41119) was obtained from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). Productsused for staining (hematoxylin and eosin (H&E), Masson's trichrome, and immunofluorescence staining) were from Servicebio Technology

(Wuhan, China).

2.2. Preparation of Mel

The extraction of oligosaccharides from the herbaceous material of Alhagi honey was performed using an aqueous extraction and ethanol precipitation method. After precipitation with 80% ethanol to separate the oligosaccharides and obtain crystalline oligosaccharides, the purified oligosaccharides were structurally characterized using Fourier-transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

2.3. Animals and treatment

Thirty rats were evenly divided into six groups based on body weight. Rats were anesthetized with pentobarbital sodium (30 mg/kg) after fasting for 24 h. The catheter was then lubricated with liquid paraffin and inserted into the colon (approximately 8 cm from the anus), followed by a gradual injection of 2.5% TNBS. To establish a successful acute model of CD, the rats were administered TNBS enema twice. The Control and model groups were given saline daily, while the other four groups were intragastrically administered with different doses of Mel (100, 200, or 400 mg/kg/day) or Mesa (200 mg/kg/day). The fecal characteristics, mental state of rats were observed daily, and body weight was recorded. Finally, the rats were sacrificed, and colon samples were collected for subsequent analysis. All experimental procedures were conducted in accordance with the standards for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, updated 1996). The animal research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xinjiang Medical University (Ürümqi, China, ethical approval number: IACUC-20220802-02).

2.4. Histological evaluation

H&E staining was performed to assess colonic damage. The rats were euthanized, and a distal colon segment (0.5 cm) was excised and fixed in 4% paraformaldehyde (Solarbio Life Sciences, Beijing, China) for 24 h. Tissue was processed through a graded series of ethanol for dehydration and embedded in paraffin. Paraffin sections (0.4 μm) were then stained with H&E. These sections were then examined under a microscope (Nikon Eclipse E100, Tokyo, Japan) and scored (0–4) to determine the degree of tissue damage based on (1) the severity of inflammation, (2) the extent of inflammation, (3) crypt damage, and (4) the proportion of tissue involvement.

2.5. Masson's staining

Paraffin blocks of colon tissue were sectioned to a thickness of 5 μm and stained with Masson's trichrome staining, according to the manufacturer's instructions. The images were acquired using an optical microscope. Under low magnification, collagen fibers were stained blue, while cytoplasm and muscle fibers were stained red. The percentage of the total area occupied by collagen fibers was then calculated.

2.6. Immunofluorescence staining of colon tissue

Paraffin-embedded colon tissue samples were used to examine TNF-α and PGK1 expression. Briefly, the sections were treated with 0.4% Triton X-100 for 30 min at room temperature. Then, 5% goat serum was added for another 30 min. The sections were incubated with anti-TNF-α or anti-PGK1 antibodies at a dilution of 1:200 at 4 °C overnight. After three consecutive washes with PBS, a secondary antibody was added in a dark room for 1 h at 37 °C, and the nuclei were stained with DAPI. Stained sections were then examined under a microscope (Nikon Eclipse C1, Tokyo, Japan).

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to determine the levels of IL-6, IL-12, TNF-α, IL-1β, IL-10, ATP, 2-PG, and 3-PG in colonic tissue or cell supernatant, following the manufacturer's instructions.

2.8. Cell migration assays

NCM460 cells were cultured in a six-well plate. The mechanical injury was induced using a 200 μL pipette tip by making a fine scratch on the confluent epithelial cell layer when they reached 90% confluence. The bottom of each well was marked to identify the initial position of the wound, and a fresh cell culture medium was added for the cells(Mulati et al., 2022). After cell adhesion, the Ctrl group of NCM460 cells was incubated with the supernatant from untreated RAW264.7 cells, while the model group of NCM460 cells was incubated with the supernatant from RAW264.7 cells that had been treated with LPS (1 μg/mL) for 12 h. PBS was added after 24 h to the Ctrl group, and different concentrations of Mel were added to the model group(Zhang, H. et al., 2024). An inverted bright-field microscope equipped with a camera (Olympus GX41, Tokyo, Japan) was used to capture images of the cell-free area in the marked region at specified times after wound healing.

2.9. Drug affinity responsive target stability (DARTS) experiment

Briefly, approximately 1×10^7 RAW264.7 cells, either treated or untreated with LPS, were collected and lysed with 600 μL of M-PER™ buffer supplemented with a 1% protease inhibitor. After lysing on ice for 15 min, the cell lysate was centrifuged at 4 °C for 15 min at 12000 g. The supernatant was mixed with 10 x TNC buffer (50 mM Tirs-HCl, 50 mM NaCl, 10 mM $CaCl_2$) and divided into two equal samples. Each sample was incubated with Mel (0.4 mM) or PBS at 37 °C for 60 min. After quantification using a BCA assay kit, the incubated samples were digested with 0.05% PronaseE at 37 °C for 30 min(Ren et al., 2021). The samples were then boiled in a loading buffer and analyzed by SDS-PAGE. Subsequently, the samples were subjected to Western blotting.

2.10. Molecular docking

The 3D structure of Mel was retrieved from the PubChem database and subjected to energy minimization under the MMFF94 force field. The crystal structure of PGK1 (PDB ID: 5NP8) was downloaded from the PDB database. Molecular docking was performed using AutoDock Vina 1.2.3 software. Prior to docking, the receptor protein was processed using PyMol 2.5.5, which involved removing water molecules, salt ions, and small molecules. Subsequently, a docking box was defined to encompass the entire protein structure. During the docking process, the global search precision was set to 32, while all other parameters were kept at their default settings. The docking conformation with the highest scoring output was considered the binding conformation, and the results were visualized and analyzed using PyMol 2.5.5.

2.11. Determination the IC_{50} of Mel

The IC_{50} detection of Mel was carried out according to the methods in the references(Liao et al., 2022). In the enzymatic reaction, the generation rate of NADH is directly proportional to the activity of PGK1. Therefore, the rate of PGK1 enzymatic reaction is reflected by detecting the absorbance changes of NADH at 340 nm. The inhibition rate was calculated by formula 1.

Compound inhibition rate (%)=(Initial velocity of DMSO - initial velocity of the compound) / Initial velocity of DMSO × 100 (1)

2.12. Bioinformatics analysis

Genes with significant interactions with PGK1 were downloaded from the String database (https://cn.string-db.org/). All obtained genes were subsequently analyzed for KEGG (Kyoto Encyclopedia of Gene and Genomes) pathway and GO (Gene Ontology) enrichment using the "clusterProfiler" package and the "org.Hs.eg.db" package in the R software. The top 12 pathways were selected for visualization.

2.13. Western blotting

Western blotting was performed as previously described(Jiang et al., 2023; Mulati et al., 2022).

2.14. Cellular thermal shift assay (CETSA)

RAW264.7 cells were suspended in 300 mL of pre-cooled PBS containing PMSF (100 mM) and a mixture of protease inhibitors and lysed on ice for 20 min. The supernatant obtained from the centrifuged lysate was divided into two equal parts and incubated at 37 °C for 30 min in the presence or absence of Mel (0.4 mM). Then, each part was aliquoted into 50 μL portions and heated at 37–98 °C for 3 min. The heated lysates were centrifuged at 12,000 rpm for 15 min, and the PGK1 protein levels in the supernatant were analyzed by Western blotting.

2.15. Statistical analyses

The results are expressed as mean ± standard deviation. A one-way analysis of variance was used to examine all of the results of the experiment. Differences of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mel alleviates intestinal damage and fibrosis in CD model rats

The structural identification of the purified extract was conducted, confirming it to be Mel with a purity of 99.85% (Lv, Z. et al., 2024) (Fig. 1A). The therapeutic results of Mel on CD showed that in the "Mel 0″ group of rats, the colon length was significantly shorter than in the "Ctrl" group, while the "Mel" group exhibited a significant increase in colon length compared to the "Mel 0″ group. Additionally, it was observed that rats in the "Mel 0″ group developed mega colon, intestinal stenosis, and even obstruction; besides this, the colonic wall appeared to be markedly thickened upon longitudinal incision along the mesentery, with the presence of extensive or characteristic longitudinal ulcers, which appeared grayish-white or grayish-black and were associated with fecal matter that was challenging to remove (Fig. 1B). Furthermore, the body weight changes in rats showed a dose-dependent decrease (Fig. 1C), and the relative weight of the spleen in treated rats was reduced (Fig. 1D). HE staining and Masson staining results indicated that Mel effectively improved colonic mucosal injury and inflammatory infiltration, and reduced the deposition of collagen fibers, improving the fibrosis of colonic tissue (Fig. 1E–G). The results suggest that Mel treatment can reduce pathological damage.

3.2. Mel alleviates intestinal inflammation in CD rat models

The expression of inflammatory factors was assessed in colonic tissue. As illustrated in Fig. 2A–D, the levels of IL-1β, IL-12, and TNF-α were decreased, whereas the IL-10 level was increased (Fig. 2A–D). Subsequently, immunofluorescence detection of TNF-α in the colonic tissue yielded the same results (Fig. 2E–F). These findings suggested that treatment with Mel can reduce colonic inflammatory shortening and alleviate the inflammatory response in the colon.

3.3. Mel reduces the production of inflammatory factors in vitro and promotes repair

To verify the anti-inflammatory effect of Mel at the cellular level, RAW264.7 cells in LPS-induced inflammatory models were incubated with Mel at different concentrations, and the production of relevant pro- and anti-inflammatory factors in the cell supernatant was measured. The results revealed that Mel inhibits the secretion of IL-6, IL-1β, IL-12, and TNF-α (Fig. 3A–D), promoting IL-10 release (Fig. 3E). These results indicate that Mel can effectively alleviate the inflammatory response of RAW264.7 cells induced by LPS.

It has been reported that macrophages of different phenotypes play different roles in epithelial cells, with M1 macrophages being able to disrupt the function of the epithelial barrier(Han et al., 2021; Tao et al.,

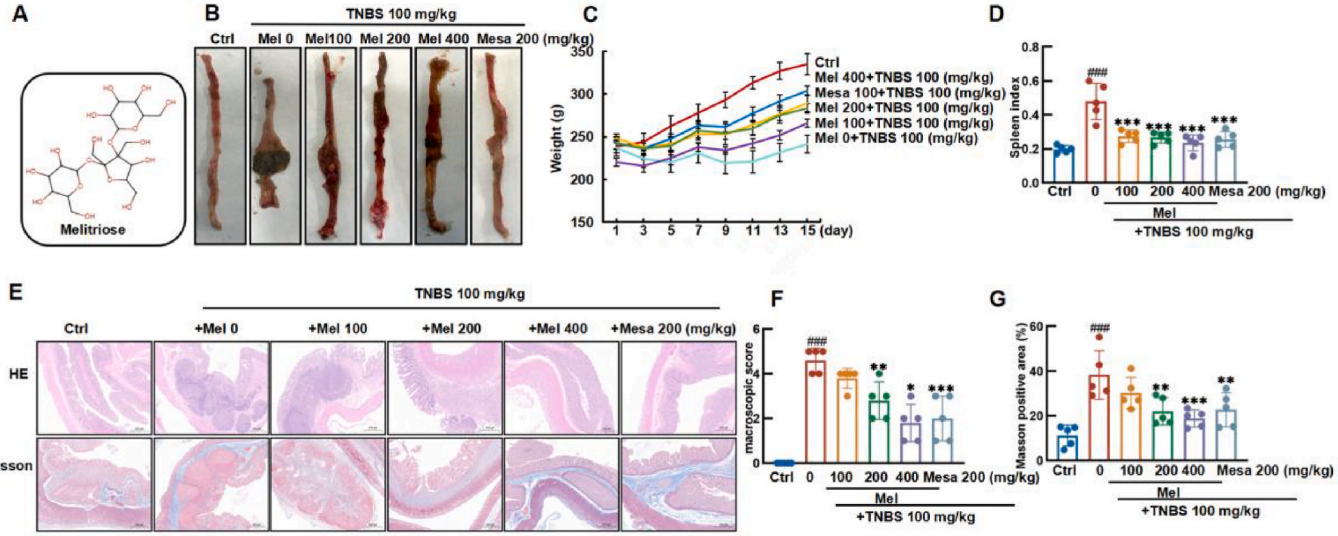

Fig. 1. Mel improved the pathological damage of TNBS induced CD rats. (A) Chemical structure of Mel. (B) Pathological lesion of rat colon. (C) Daily body weight changes of the rats. (D) Changes in the relative weight of the rat spleens. (E–G) Representative H&E and A1Masson staining images of colon tissue sections from each group (n = 5, scale bar, 500 μm). * indicates compared with the "Mel 0" group, and # indicates compared with the Ctrl group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

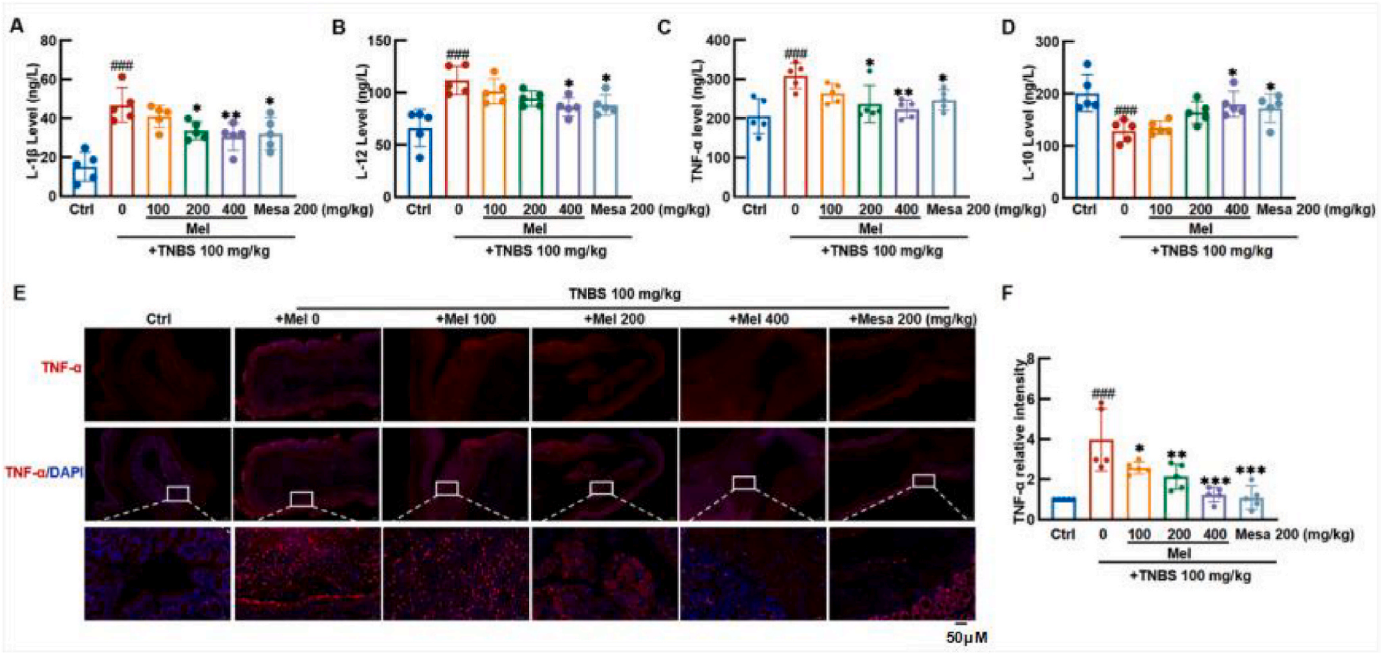

Fig. 2. Mel improves inflammation in rats. (A–D) Levels of pro-inflammatory cytokines IL-1β, TNF-α, IL-12, and anti-inflammatory factor IL-10 in colon tissue were measured using an ELISA kit. (E–F) Representative immunofluorescence images of colon tissue sections from each group (n = 5, scale bar, 500 μm and 50 μm). * indicates compared with the "Mel 0" group, and # indicates compared with the Ctrl group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

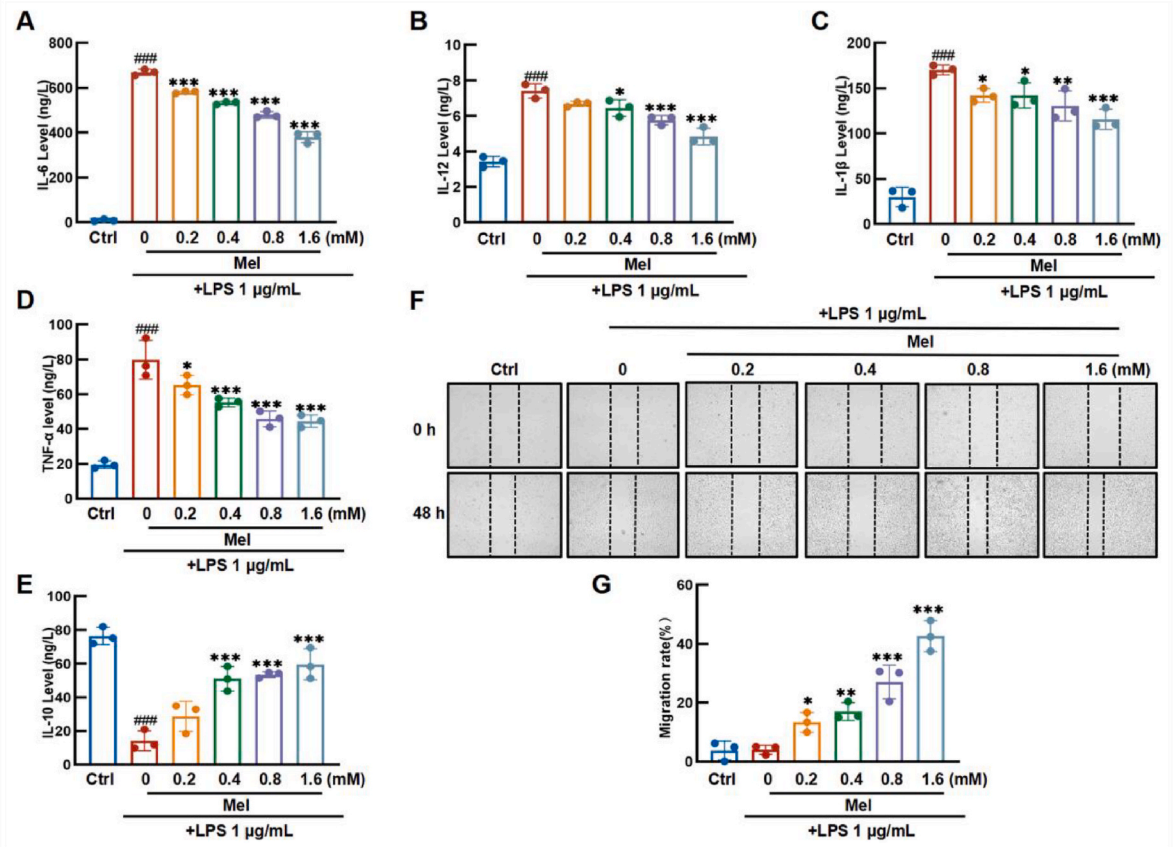

Fig. 3. Mel inhibits the production of inflammatory factors and promotes repair. (A–E) The effect of Mel on the production of inflammatory factors induced by LPS in macrophages. (F–G) The scratch repair of NCM460 cells was photographed, and the percentage of wound closure was calculated using Image J software at designated times, and the histogram displays the migration rate (n = 3, scale bar, 100 μm). * indicates compared with the LPS group, # indicates compared with the Ctrl group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2023). Consequently, the regulatory effect of the supernatant from different groups of RAW264.7 cells was studied on scratch healing in NCM460 cells. There was no significant difference between “M0” and "Ctrl" groups. However, compared with the “M0” group, the migration rate of the Mel treatment group significantly increased, indicating that Mel promotes repair (Fig. 3F–G). These results indicated that Mel can improve the expression of inflammatory factors in the supernatant of macrophages stimulated by LPS and promote the scratch repair of NCM460 cells.

3.4. DARTS assay reveals Mel targeting PGK1 and reduces its protein stability

After determining that Mel can alleviate inflammation and promote repair both *in vivo* and *in vitro*, the target proteins of Mel were further explored using DARTS experiments. Silver staining analysis of pronase-digested protein bands revealed a noticeable change in the degree of enzymatic digestion following the Mel intervention, with a molecular weight of approximately 45 kDa (Fig. 4A). HPLC-MS/MS analysis identified PGK1 as the most likely candidate gene, with a coverage rate of 89% (Fig. 4C). Based on the existing research results, we focused on PGK1 and further validated its interaction with Mel by Western blotting (Fig. 4B). CETSA revealed that the binding changed the protein stability of PGK1 in a temperature-dependent manner (Fig. 4D). In the crystal structure, the ligand binds to the PGK1 protein through various interactions, including hydrophobic interactions, pi-pi interactions, and salt bridge formation. Notably, hydrophobic residues such as PHE-292 and LEU-257 participate in van der Waals and pi-pi interactions with the co-crystal ligand, while charged residues like LYS-216 engage in salt bridge interactions, contributing to the stability of the complex. However, in the docking model, Mel binds to PGK1 solely through hydrogen bonding interactions. The binding sites in the docking model differ from those observed in the crystal structure, likely due to the considerable differences in the two molecules’ nature. Mel, with its multiple polar groups, readily forms hydrogen bonds with both hydrogen bond donors and acceptors, whereas the co-crystal ligand lacks such features, indicating that melezitose binds to the protein in a completely novel manner, and the binding energy of the PGK1 domain to Mel being −5.621 kcal/mol (Fig. 4E–F). The above research results indicated that PGK1 is a potential target of Mel to alleviate macrophage inflammation.

3.5. Bioinformatics analysis in Mel against CD

Subsequently, the bioinformatics analysis was performed to detect the pathways and associated genes implicated in PGK1. Utilizing the STRING analysis, the gene interaction network was delineated, highlighting significant alterations in genes interacting with PGK1 (Fig. 5A–B). The interaction networks, which exhibited substantial changes, were derived from the KEGG pathway and GO enrichment analyses of the 45 intersecting genes. It became evident that Mel is significantly associated with glycolytic pathways (Fig. 5C), and is engaged in various biological processes, predominantly glycolysis (Fig. 5D).

3.6. Mel reduces ATP production by inhibiting PGK1 expression

To further investigate whether Mel treatment affects PGK1 expression *in vivo*, immunofluorescence was used to assess PGK1 levels within colonic tissues. The findings demonstrated significant upregulation of PGK1 in the model group. Notably, the administration of Mel led to a reversal of this elevation (Fig. 6A–B). Consistent results were obtained through Western blot analysis, suggesting that Mel potentially attenuates inflammatory responses and promotes tissue repair by suppressing PGK1 expression in colonic tissues (Fig. 6C–D).

As a key metabolic enzyme in the glycolytic pathway, PGK1 facilitates the conversion of 1,3-bisphosphoglycerate (1,3-BPG) to 3-PG, producing the inaugural ATP of the glycolytic pathway. Moreover, 2-PG is sequentially derived from 3-PG, with both metabolites playing pivotal roles in glycolysis(He et al., 2024; Liu et al., 2022). To further validate this result, ATP, 2-PG, and 3-PG were quantified in the cell supernatant. The assay outcomes demonstrated marked elevations of 3-PG, 2-PG, and ATP levels in the supernatant following LPS stimulation. Conversely, the application of Mel significantly abrogated this increase in a concentration-dependent fashion (Fig. 6E–G). Subsequently, NG52,

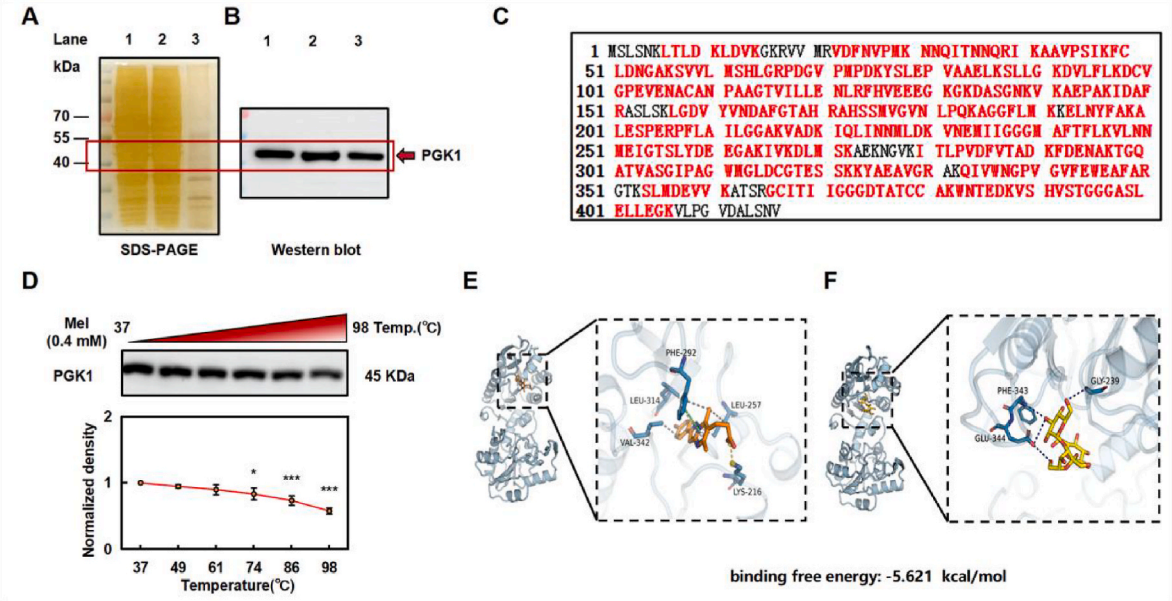

Fig. 4. Mel inhibits the production of inflammatory factors *in vitro* and promotes repair. (A–C) Target identification using DARTS experiments (Lane 1. Ctrl, Lane 2. LPS, Lane 3. LPS + Mel). (D) Mel altered the thermal stability of PGK1 protein. * indicates compared with the 37 °C group. *$p < 0.05$, ***$p < 0.001$ (E) The binding mode of the crystal ligand with the PGK1 protein in the crystal structure. (F) Molecular docking analysis of PGK1 with Mel (yellow sticks: Mel; cyan: PGK1; blue lines: hydrogen bonding interactions; gray dashed lines: hydrophobic interactions; yellow dashed lines: salt bridge interactions; green dashed lines: pi-pi interactions). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

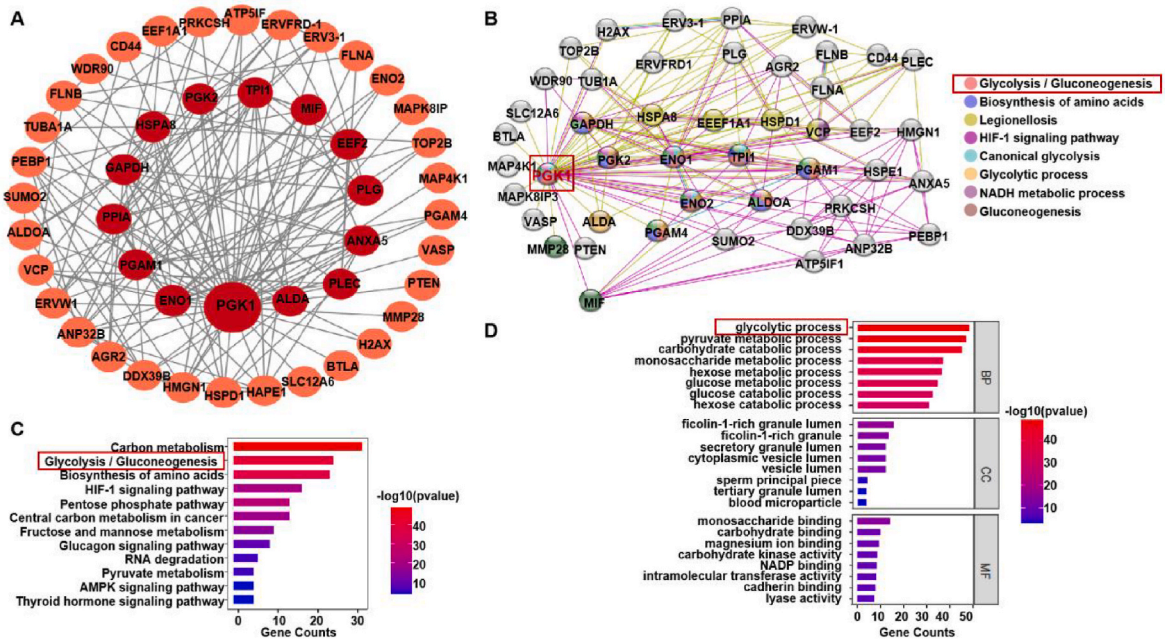

Fig. 5. The PGK1 involved pathways and associated genes. (A,B) Search tool for recurring instances of neighboring genes (STRING) network visualization of the genes in differentially expressed genes related to PGK1. Edges represent protein-protein associations. (C) KEGG analysis. (D) GO analysis.

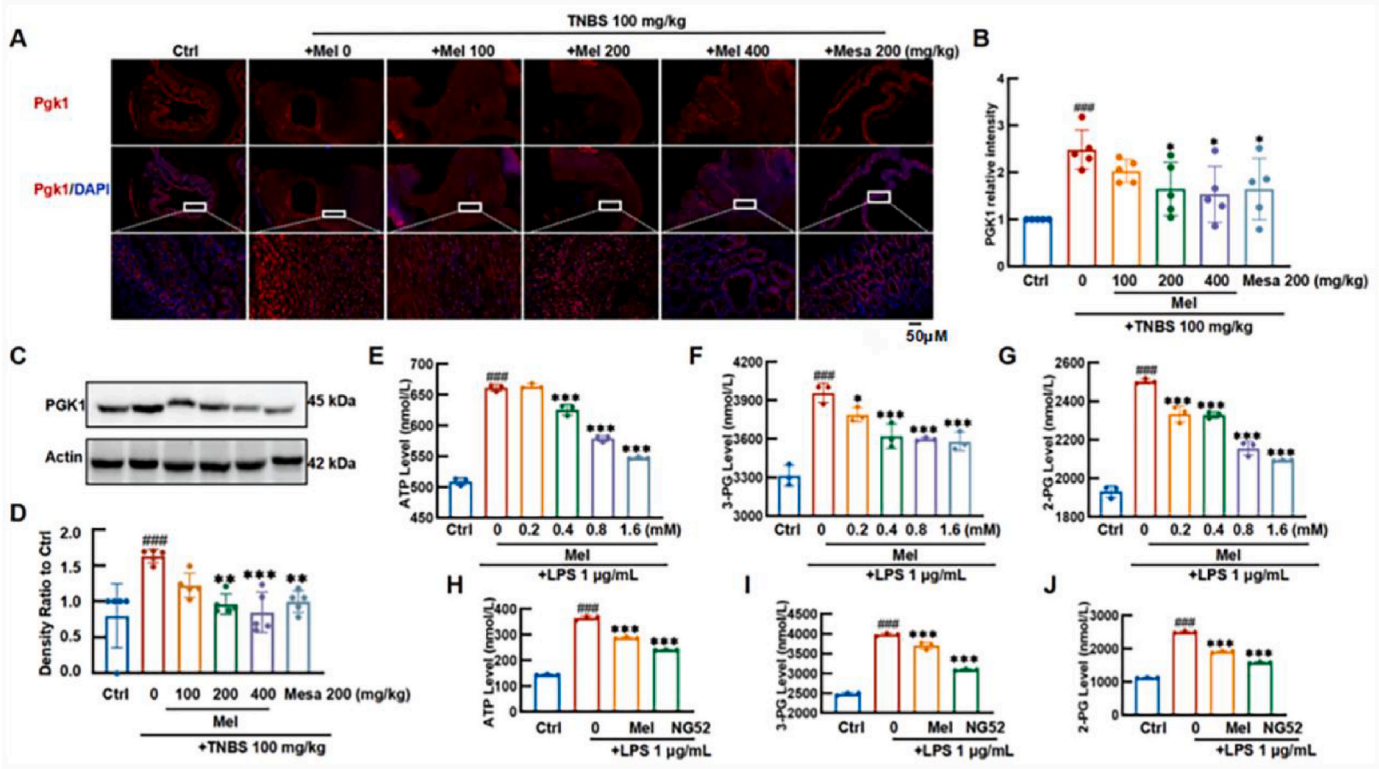

Fig. 6. Mel reduces ATP production by inhibiting the expression of PGK1, thereby easing inflammation and promoting repair. (A–B) Expression of PGK1 in rat colonic tissue with a scale bar of 500 μm and 50 μm (n = 5). (C–D) Protein expression of PGK1 in rat colonic tissue. (E–I) Expression data of ATP, 2-PG, and 3-PG in the cell supernatant detected by ELISA kits (n = 3). * indicates compared with the "Mel 0"group or LPS group, # indicates compared with the Ctrl group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

a competitive inhibitor of PGK1(Wang et al., 2021), was added to the cell supernatant at a concentration of 5 μM, and the levels of ATP, 2-PG, and 3-PG were quantified in the supernatant. The results revealed that treatment with NG52 inhibited the LPS-induced increase. This result further demonstrated that Mel can suppress the PGK1 activity, thereby decreasing the production of ATP and 3-PG via glycolysis and

subsequently attenuating the biosynthesis of 2-PG (Fig. 6H–J).

3.7. Mel-induced PGK1 suppression diminishes ATP production and attenuates inflammatory responses

We further explored the potential of PGK1 in regulating inflammatory responses. By detecting the inhibitory effect of Mel on PGK1 activity, we measured the IC_{50} of Mel (0.38 mM), while the IC_{50} of NG52 has been reported as 2.5 μM (Wang et al., 2021). Additionally, we detecting the effect of Mel on the catalytic activity of PGK1 in cells, results showed that Mel significantly reduced the catalytic activity of PGK1, and this reduction was exacerbated in the presence of NG52 (Fig. 7A–B). Furthermore, "Mel" group and "NG52" group attenuated the production of pro-inflammatory cytokines, and increased the levels of anti-inflammatory cytokines, meanwhile, the "Mel + NG52" group has a synergistic effect (Fig. 7C–G). Collectively, these results proved that Mel could suppress PGK1 activity, consequently mitigating inflammatory responses.

4. Discussion

Chronic idiopathic IBD, known as CD, is characterized by skip lesions and transmural inflammation, which can affect the entire gastrointestinal tract. The clinical symptoms mainly include abdominal pain, diarrhea, and gastrointestinal bleeding, among others(Roda et al., 2020). Among them, 21%–47% of patients will also have systemic extraintestinal manifestations, such as arthritis, oral mucosal ulcers, chronic hepatitis, sclerosing cholangitis, and others(Shen et al., 2022). Currently, pharmacotherapy remains the primary method of clinical treatment for CD. Studies have revealed that the main feature of CD is the excessive infiltration of white blood cells into the inflamed mucosa and the massive secretion of pro-inflammatory cytokines(Clough et al., 2020; Ghiboub et al., 2022). Consequently, clinical drugs mainly regulate the activity of the immune system through 5-aminolevulinic acid, immunosuppressants, glucocorticoids, and others(Clough et al., 2020). However, these drugs are prone to adverse reactions, such as affecting the metabolism of sugar and protein, damaging liver and kidney function, and increasing the incidence of malignant tumors. Besides, due to individual differences and inherent limitations of the drugs, the primary non-response rate and loss of response rate of patients to immunosuppressants are relatively high(Clough et al., 2020). As a result, there is an urgent need to address the lack of novel and effective therapies for CD.

Mel, extracted from Alhagi honey, is derived from natural sources. Compared with chemically synthesized drugs, it typically has fewer side effects and is more suitable for long-term consumption. In Uyghur folklore, Alhagi honey is commonly used to treat various ailments, including digestive issues, such as abdominal pain, diarrhea, and dysentery(Wei et al., 2021). Therapeutic properties are attributed to its rich composition of oligosaccharides and polysaccharides, which have been identified to possess anti-inflammatory effects(Cai et al., 2021, 2022; Wusiman et al., 2022). Functional oligosaccharides, a class of compounds not digested or absorbed by the host, selectively promote the growth and activity of beneficial gut bacteria, conferring health benefits to the host(Yang et al., 2023). In CD treatment, oligosaccharides can improve inflammation and repair intestinal damage(Hu et al., 2022; Leibovitzh et al., 2022). The current research on Alhagi honey primarily focused on polysaccharides, and this study fills the gap in CD research on oligosaccharides.

Fibrosis is a key complication of CD, often resulting in the formation of scar tissue within the intestines. As the condition deteriorates, this can lead to intestinal stenosis. The study revealed that Mel treatment significantly decreased intestinal scar tissue in rats when compared to the model group. Masson's findings also demonstrated a reduction in collagen fiber deposition, suggesting that Mel can reduce intestinal fibrosis. Meanwhile, we evaluated the inflammatory factors in the colon tissue, and the results suggested that Mel has notable anti-inflammatory activity. These results indicate that Mel could be developed into a medication for CD treatment.

Glycolysis is a vital metabolic mechanism in living organisms, and PGK1, a key enzyme in the glycolytic pathway, catalyzes the production of ATP and plays a significant role in the pathogenesis and progression of IBD. Research indicates that glycolysis affects the gut microbiota, mucosal barrier function, and immune system and is associated with various signaling pathways and transcription factors, which collectively participate in the pathogenic processes of CD and colorectal cancer(Xia et al., 2023). In CD, increased glycolysis can affect multiple intestinal cells, including macrophages, dendritic cells, T cells, and neutrophils (Xia et al., 2023). Studies have found that the metabolic state of colonic epithelial cells is closely linked to the structure of the gut microbiota, and modulating ATP levels in the gut can suppress the exacerbation of colitis(Kumar and Dikshit, 2019). In our study, it was confirmed by DARTS and other experimental techniques that Mel influences the glycolytic pathway. Further experiments revealed that Mel administration suppressed PGK1 expression, leading to a reduction in ATP levels and its products, 2-PG and 3-PG, thereby downregulating the glycolytic signaling pathway. These results were further confirmed by the addition

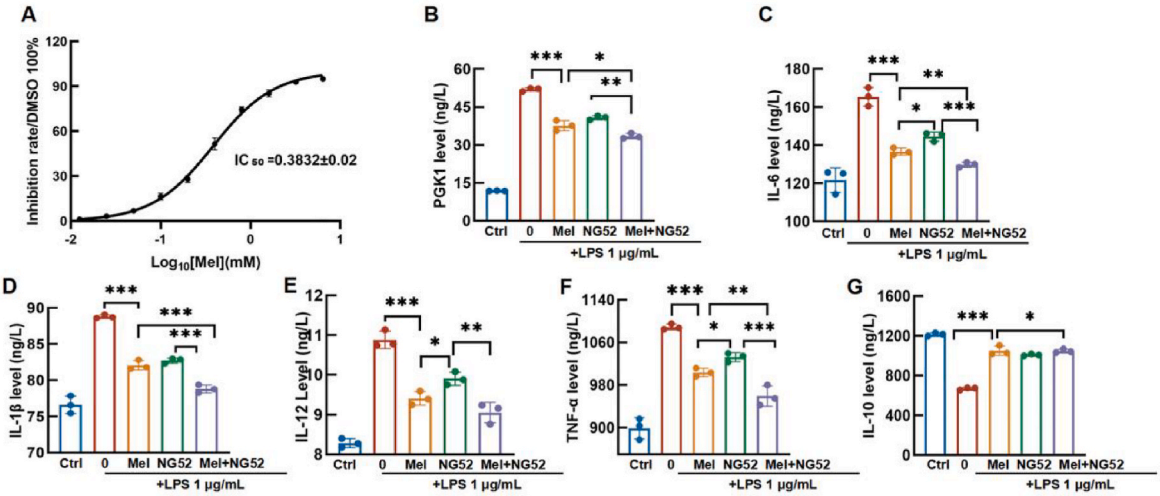

Fig. 7. Mel inhibits the activity of PGK1 and suppresses the production of inflammatory factors. (A)The IC_{50} of Mel was determined. (B) Mel suppresses the generation of PGK1. (C–F) Mel exerts anti-inflammatory effect by inhibiting the activity of PGK1, thereby suppressing the production of inflammatory cytokines. (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of the PGK1 inhibitor NG52. Meanwhile, the combination of Mel with NG52 has a synergistic effect. Furthermore, immunofluorescence was employed to analyze PGK1 expression in colonic tissues. Results indicated that PGK1 expression was significantly upregulated in the model group, while Mel treatment decreased its expression. Although our our research indicates that Mel modulates glucose metabolism to exert anti-inflammatory and reparative effects, the current investigation did not delve deeper into the *in vivo* mechanisms by which Mel affects glucose metabolism.The association between Mel and glucose metabolism will be examined more comprehensively in our upcoming research, potentially providing novel insights for CD therapy.

5. Conclusion

In summary, this study demonstrated that Mel exerts its anti-inflammatory and reparative capabilities *in vitro* and *in vivo* by inhibiting the activity of the key glycolytic enzyme PGK1, leading to reduced production of ATP and its products 2-PG and 3-PG, thereby ameliorating the symptoms of CD. These findings provide new insights into the potential of Mel for CD treatment.

CRediT authorship contribution statement

Miaomiao Zhang: Writing – original draft, Software, Methodology, Formal analysis. **Jianing Ma:** Methodology, Investigation, Formal analysis. **Shulipan Mulati:** Methodology, Investigation, Formal analysis. **Junmin Chang:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization. **Weiyi Zhang:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Institutional Review Board statement

The animal study protocol was approved by the Institutional Review Board (IACUC-20220802-02).

Data availability statement

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

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Declaration of competing interest

We have reviewed the final version of the manuscript and approve it for publication. To the best of our knowledge and belief, this manuscript has not been published neither in whole nor in part or considered to published in other places. The authors declare that there are no conflicts of interest.

Abbreviations

Mel	Melezitose
CD	Crohn's disease
IBD	inflammatory bowel disease
H&E	hematoxylin and eosin
ELIS	Aenzyme-linked immunosorbent assay
DARTS	Drug Affinity Responsive Target Stability
CETSA	Cellular thermal shift assay
PGK1	phosphoglycerate kinase 1
ATP	adenosine triphosphate
2-PG	2-phosphoglycerate 2-PG
1,3-BPG	1,3-bisphosphoglycerate
3-PG	3-phosphoglycerate
Mesa	Mesalazine
TNF-α	Tumor Necrosis Factor-alpha
IL-12	Interleukin-12
LPS	Lipopolysaccharide
TNBS	trinitrobenzene sulfonic acid
PDB	Protein Data Bank
KEGG	Kyoto Encyclopedia of Gene and Genomes
GO	Gene Ontology

Data availability

Data will be made available on request.

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