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Investigate the metabolic changes in intestinal diseases by employing a ^{1}H-NMR-based metabolomics approach on Caco-2 cells treated with cedrol

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Abstract

Mitochondrial dysfunction may precipitate intestinal dysfunction, while inflammatory bowel disease manifests as a chronic inflammatory ailment affecting the gastrointestinal tract. This condition disrupts the barrier function of the intestinal epithelium and alters metabolic products. Increasing mitochondrial adenosine triphosphate (ATP) synthesis in intestinal epithelial cells presents a promising avenue for colitis treatments. Nevertheless, the impact of cedrol on ATP and the intestinal barrier remains unexplored. Hence, this study is dedicated to examining the cedrol's protective effect on an inflammatory cocktail (IC)–induced intestinal epithelial barrier dysfunction in Caco-2 cells. The finding reveals that cedrol enhances ATP content and the transepithelial electrical resistance value in the intestinal epithelial barrier. Moreover, cedrol mitigates the IC-induced decrease in the messenger ribonucleic acid (mRNA) expression of tight junction proteins (ZO-1, Occludin, and Claudin-1), thereby ameliorating intestinal epithelial barrier dysfunction. Furthermore, nuclear magnetic resonance (NMR)–based metabolomic analysis indicated that IC-exposed Caco-2 cells are restored by cedrol treatments. Notably, cedrol elevates metabolites such as amino acids, thereby enhancing the intestinal barrier. In conclusion, cedrol alleviates IC-induced intestinal epithelial barrier dysfunction by promoting ATP-dependent proliferation of Caco-2 cells and bolstering amino acid levels to sustain tight junction messenger ribonucleic acid expression.

Abbreviations: ATP, adenosine triphosphate; CV, cross-validation; DSS, dextran sulfate sodium; GI, gastrointestinal; IBD, inflammatory bowel disease; IC, inflammatory cocktail; IFN-γ, interferon-gamma; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; qPCR, quantitative reverse transcription PCR; sPLS-DA, sparse partial least squares discriminant analysis; TEER, transepithelial electrical resistance; TNF-α, tumor necrosis factor alpha; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt.

KEYWORDS
^{1}H-NMR, ATP, Caco-2 cells, cedrol, inflammatory bowel disease (IBD), metabolomics

1 | INTRODUCTION

Mitochondria are dual-membraned organelles in most eukaryotic cells. In contemporary medical discourse, mitochondria are often hailed as the cellular powerhouses, primarily responsible for generating adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC).[1] Beyond energy production, mitochondria play crucial roles in cell apoptosis, oxidative phosphorylation, signaling of reactive oxygen species, and maintaining intracellular Ca^{2+} balance, positioning them as pivotal components in cellular metabolism and internal equilibrium.[2] Mitochondria deliver energy through ATP and metabolic signals, providing essential heat for organisms and fortifying cells during stress responses.[3] Under pathological conditions, mitochondria undergo restructuring to uphold the delicate balance between ATP supply and demand, addressing the requirements of cellular biosynthesis, signal transduction in stress responses, and biochemical processes. Severe ATP imbalances and defects can compromise cellular function, leading to diseases such as cardiovascular disorders, neurodegenerative conditions, metabolic disorders, and cancer.[4]

Moreover, mitochondrial dysfunction is intricately linked to a spectrum of diseases and pathologies, encompassing metabolic disorders, neurodegenerative diseases, cardiovascular diseases, and cancer.[2] Disruptions in mitochondrial function can also adversely affect intestinal function, and colitis is recognized as a condition associated with energy deficiency.[5–7] Inflammatory bowel disease (IBD) is a persistent inflammatory ailment affecting the gastrointestinal (GI) tract, characterized by impairment of the intestinal epithelial barrier function. Intestinal epithelial cells (IECs) act as the host's defensive barrier against the intestinal lumen environment, resisting pathogens, regulating metabolism, and maintaining internal and external equilibrium in the intestine. The integrity of this barrier function primarily relies on tight junction (TJ) proteins.[8] IECs undergo continuous renewal every 4–5 days to uphold a consistent turnover state, necessitating a substantial supply of energy.[9] The reliance on energy for maintaining intestinal epithelial integrity implies that mitochondrial function may be pivotal in preserving barrier function.

Previous research confirms that elevating mucosal oxidative phosphorylation complex activity and ATP levels in mice can shield them from colitis. It suggests that enhancing mitochondrial ATP synthesis in intestinal epithelial cells may represent a potential therapeutic strategy for colitis.[10] Caco-2 cells, derived from human colon adenocarcinoma, are one of the in vitro models for investigating intestinal epithelial cells. Following cultivation, Caco-2 cells adhere closely to neighboring cells and differentiate into a single-layered epithelial-like structure adorned with microvilli, closely resembling the characteristics of small intestinal epithelial cells. Assessing Caco-2 cells' monolayer barrier function differentiation involves measuring transepithelial electrical resistance (TEER).[11] Van De Walle et al. exposed Caco-2 cells to interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), and lipopolysaccharide (LPS) to establish an in vitro model mimicking impaired barrier function in IBD.[12]

In recent years, metabolomics has provided a more intricate understanding of cellular and organismal metabolism, elucidating the regulatory dynamics between health and disease.[13] Prior research has unveiled metabolic disturbances in individuals with IBD, indicating modifications in the tricarboxylic acid cycle, amino acid, fatty acid metabolism, and oxidative pathways.[14,15] Nuclear magnetic resonance (NMR) stands out as a spectroscopic technique leveraging the energy transitions of nuclear spins in a magnetic field. Recently, NMR has become pivotal in metabolomics analysis, offering advantages such as simplicity in sample preparation, quantifiability, high reproducibility, stability, high resolution, and sensitivity. Moreover, NMR's non-contact with the sample and its capability for real-time or discrete-time point sampling make it particularly suitable for diverse applications. NMR probe technology excels in detecting nanomole-level samples, showcasing exceptional sensitivity.[16] Additionally, NMR can quantify over 65 intracellular and extracellular metabolites and possesses the capability to identify unknown metabolites in complex mixtures.[17,18] Consequently, NMR holds significant promise as a technology for the analysis of colitis metabolomics.

Cunninghamia lanceolata var. *konishii* is an endemic tree species in Taiwan. The primary components of its wood essential oil include cedrol, α-pinene, α-cedrene, and α-terpinol.[19] The main constituent of the essential oil, cedrol, constitutes as much as 78.48% of the total composition.[20] Cedrol, classified as a sesquiterpene compound, is known for its various properties, including anti-inflammatory, analgesic, sedative, anxiolytic, anticancer, antibacterial, and hair growth-promoting effects.[21] It has been reported to ameliorate rheumatoid arthritis,[21] alleviate

neuropathic pain in rats subjected to chronic constriction injury,[22] and effectively promote hair growth in mice.[23] However, the functional impact of cedrol on ATP and intestinal barrier function has not yet been investigated.

Therefore, this study aims to investigate whether an increase in ATP within mitochondria can assist in the restoration of intestinal barrier function and ameliorate metabolic dysfunction. Accordingly, this research involves the treatment of Caco-2 cells with cedrol to examine its potential impact on intracellular ATP levels. On the other hand, an inflammatory cocktail (IC) is used to induce damage in Caco-2 cells, serving as a model to simulate impaired intestinal barrier function. The therapeutic effects of cedrol are then observed to assess its potential to improve the intestinal barrier. Finally, NMR is employed as a diagnostic platform for intestinal diseases, evaluating changes in intracellular metabolites. Moreover, previous studies have demonstrated that quercetin can restore FCCP-induced mitochondrial dysfunction and increase the TEER value and TJ proteins of Caco-2 cells.[24,25] Therefore, quercetin was used as the positive control group in this study.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA), LPSs from *Escherichia coli* O55:B5 (Sigma-Aldrich, Germany, L2880-25MG), recombinant human TNF-α lyophilized (Gibco, USA, PHC3011), recombinant human IFN-γ (Gibco, USA, PHC4031), recombinant human IL-1B lyophilized (Gibco, USA, PHC0815), ATP Detection Assay Kit-Luminescence (Cayman Chemical, USA), total RNA purification kit (GeneMark, Taiwan, TR01-150), DMSO (D2650, Sigma-Aldrich, Germany), methanol-d4 (Sigma-Aldrich, Germany, 151947-10G-GL, >99.8 atom% D), deuterium oxide (Sigma-Aldrich, Germany, 151882-100G, >99.9 atom% D), 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP) (Sigma-Aldrich, Germany, 269913-1G), and Enhanced Cell Counting Kit 8 (Elabscience, China, E-CK-A362).

2.2 | Extraction of cedrol

The *C. lanceolata* var. *konishii* wood used in this study was provided by Dr. Min Jay Chung (Experimental Forest Management Office of National Taiwan University, Nantou County, Taiwan). Initially, 200 g of *C. lanceolata* var. *konishii* wood was placed in a round-bottomed flask with 1 L of distilled water to extract essential oil via steam distillation. After a 6-h period, the essential oil was gathered, subsequently undergoing recrystallization to obtain high-purity cedrol.[26] The purity and structure of cedrol were determined by GC–mass spectrometry (MS) and NMR spectroscopy. NMR experiments (^{1}H and ^{13}C) were carried out on an NMR spectrometer (Advance III-400, Bruker, USA) at 300 K using $CDCl_3$ as solvents (Figure S1A,B).[27] The GC–MS data showed that the purity of cedrol (96.4%) was higher than 95% by normalizing the area (Figure S1C).

2.3 | Cell culture

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC HTB-37™, USA) and was cultured at 37°C and 5% (v/v) CO_2 in high glucose DMEM. The medium included 3.7 g $NaHCO_3$, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin, 1% (v/v) of 100× MEM nonessential amino acids, and 1% (v/v) of 100 mM sodium pyruvate and 1% (v/v) of 100× Glutamax™. Cells were used between 43 and 60 passages. The medium was replaced every 2–3 days during cell growth and differentiation, and cells were differentiated for 21 days before drug treatment.

2.4 | Cell viability assay

The cell viability assay was conducted using the Cell Counting Kit-8 (CCK-8) assay, which contains WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). WST-8 is a pink molecule that can be reduced in living cells by dehydrogenase enzyme activity and generates an orange product (formazan). The number of viable cells can be determined by dehydrogenase activity, and the number of viable cells was measured at an absorbance of 450 nm. The CCK8 assay was performed according to the previously described procedures with minor modifications.[28] Caco-2 cells were cultured in 12-well plates at a density of 2×10^5 cells per well, with the culture medium replaced every 2–3 days. Following 21 days of differentiation, the cells were exposed to various concentrations of cedrol (containing 0.2% DMSO). After 12, 24, and 48 h of incubation, the culture medium was replaced with medium containing 1% Enhanced Cell Counting Kit 8 (WST-8/CCK8) and incubated at 37°C for 1 h. Cell viability was determined by measuring absorbance at 450 nm using a Microplate Spectrophotometer (BioTek, USA, μQuant).

$$\text{Cell viability}\,(\%) = \left(\frac{\text{Treatment OD}_{450}}{\text{Control OD}_{450}}\right) \times 100.$$

2.5 | ATP assay

The ATP content of Caco-2 cells was assessed using an ATP assay, which was performed according to previously described procedures with minor modifications.[29] Caco-2 cells were cultured at a density of 2×10^5 cells per well in 12-well plates, with the culture medium replaced every 2–3 days over a 21-day period. The treatment included 0.2% DMSO (control), various concentrations of cedrol, and 150 μM quercetin (positive control) administered for a 12-h duration. ATP measurements were conducted utilizing the ATP Detection Assay Kit-Luminescence (Cayman Chemical, USA). Absorbance values at 535 nm were measured using a Multilabel Microplate Reader (Hidex, Finland). Subsequently, ATP concentrations were calculated for the samples, with the ATP concentration of the control set as 100% to normalize all values.

2.6 | Establishment of intestinal inflammation model of Caco-2 cells

Caco-2 cells were utilized as an in-vitro model to simulate intestinal epithelial barrier function in IBD, as described Van De Walle et al.[12] Before treatment with the IC, Caco-2 cells were differentiated for 21 days. The IC consisted of 50 ng/mL TNF-α, 50 ng/mL IFN-γ, 25 ng/mL IL-1β, and 1 μg/mL LPS, administered for a duration of 12 h to induce increased permeability of the cell monolayer.

2.7 | Effect of cedrol on intestinal permeability in differentiated Caco-2 cells

The intestinal permeability of differentiated Caco-2 cell monolayers was assessed using TEER, which was performed according to previously described procedures with minor modifications.[30] Caco-2 cells were cultured in the apical chamber of 12 mm transwells (Corning, USA, 3401) at a density of 1.7×10^5 cells per well, with the apical and basolateral chambers containing 0.5 and 1.5 mL of medium, respectively. Medium was replaced every 2–3 days during the 21-day differentiation period. To investigate the impact of cedrol on TEER, 0.2% DMSO (control), varying cedrol concentrations, and 150 μM quercetin (positive control) were added to the apical chamber and incubated for 6, 12, 24, 36, and 48 h. In the IC-induction group, IC was added to the basolateral chamber for 12 h to enhance cell monolayer permeability. Subsequently, the culture medium in the apical chamber was replaced with 0.2% DMSO, cedrol at different concentrations, and 150 μM quercetin for 6, 12, 24, and 36 h. The transwells were then placed on a heating plate at 37°C at specified time points, and TEER values were measured using TEER measurements (World Precision Instruments, Germany, EVOM2). TEER values ranging between 400 and 500 $\Omega \cdot cm^2$ confirmed the successful differentiation of the Caco-2 monolayer cells. Here, "*A*" represents the measured resistance value, while "Blank" indicates the resistance value of cell-free culture. Finally, the resistance values of each group were normalized based on the TEER value of the control.

$$\text{TEER value}\left(\Omega \cdot \text{cm}^2\right) = (A - \text{Blank}) \times 1.12.$$

2.8 | RNA isolation and quantitative reverse transcription PCR

The expression of TJ genes in a monolayer of differentiated Caco-2 cells was assessed via quantitative reverse transcription PCR (qPCR), which was performed according to previously described procedures with minor modifications.[31] Caco-2 cells were cultured in a 12-well plate at a density of 2×10^5 cells per well, with the culture medium replaced every 2–3 days for 21 days to induce differentiation. Following this, an IC was added to the 12-well plate for 12 h, succeeded by treatment with 0.2% DMSO, varying concentrations of cedrol, and 150 μM quercetin for 24 h. RNA extraction was performed following the Total RNA Purification kit (GeneMark, Taiwan, TR01-150), with the resultant RNA stored at −80°C. Subsequently, RNA concentration was determined using the NanoVue™ Plus Spectrophotometer (GE HealthCare, USA) at 260/280 nm.

For cDNA synthesis, 200 ng of total RNA was reverse transcribed using SuperSAMScript IV Reverse Transcriptase (GeneMark, Taiwan, GRT004L). The reverse transcription process was performed by a PCR Thermal Cycler (Astec, Japan, PC-818A). Finally, the resulting cDNA was stored at −20°C.

The expression of target junction genes in differentiated Caco-2 cell monolayers was evaluated via qPCR, with primer sequences detailed in Table S1. First, 200 ng of cDNA sample used PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) for the PCR reactions. Subsequently, PCR amplification was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, USA). Data analysis was conducted using StepOne Software v2.3 (Thermo Fisher Scientific, USA). β-actin served as the endogenous control gene, and the relative expression of the target gene was calculated as

$2^{-\Delta\Delta Ct}$. Finally, the relative expression values of each group were normalized based on the $2^{-\Delta\Delta Ct}$ value of the control.

2.9 | Metabolite extraction of Caco-2 cells

Caco-2 cells were cultured at a density of 1×10^6 cells per well in a 10-cm dish, with the culture medium replaced every 2–3 days over a 21-day differentiation period. According to previous literature, Caco-2 cells enter the stationary phase and reach a stable number (20×10^6 cells) after 14 days[32]; thus, we can ensure the consistency of metabolite concentrations in all cell samples. The experimental groups consisted of control, cedrol, IC, and IC + cedrol. In the inflammatory groups, the IC was added to the cells in the 10 cm dish for 12 h, followed by treatment with either 0.2% DMSO (IC group) or 300 μM cedrol (IC + cedrol group) for an additional 12 h. For the non-inflammatory groups, cells were exposed to either 0.2% DMSO (control group) or 300 μM cedrol (cedrol group) for 12 h. After treatment, cell samples were harvested from the culture medium and washed twice with cold PBS. Subsequently, cells were collected into 2-mL Eppendorf tubes and centrifuged at $1 \times 1000g$ for 5 min at 4°C to remove PBS. The resulting cell pellet was either stored at −80°C or subjected to immediate metabolite extraction.

Metabolite extraction was carried out by adding 800 μL of colded 70% MeOH to the cell pellet, followed by sonication using an ultrasonic processor (ChromTech, USA, UP-800) at 30% intensity for 10 s, repeated five times. The sample was then vortexed on ice for 10 min to ensure homogenization of cellular metabolites. After centrifugation at $10 \times 1000g$ for 15 min at 4°C, the supernatant containing cell metabolites was transferred to a new 1.5-mL Eppendorf tube. Finally, the cell pellet was subjected to MeOH evaporation in a Napco Vacuum Oven (Napco National, Saudi Arabia, 5831) to collect the cell metabolites.

2.10 | Cell culture samples preparation for ^{1}H NMR metabolomics analysis

The metabolic profile of differentiated Caco-2 cell monolayers was analyzed using a NMR spectrometer. The solvent utilized was a 1:1 mixture of deuterium oxide and methanol-d4, with 0.0002% TSP serving as the internal standard. For the NMR analysis, the dried cellular metabolites were dissolved in 650 μL of the deuterium oxide + methanol-d4 solvent mixture containing 0.0002% TSP. Subsequently, 600 μL of this mixture was transferred into a 5-mm NMR tube for ^{1}H spectroscopy.

2.11 | ^{1}H NMR measurements

All spectra were acquired on an NMR spectrometer (Advance III-400, Bruker, USA), operating at a proton frequency of 400 MHz at 300 K. For cell extract samples, methanol-d4 was used as an internal lock to maintain gradient shimming. The basic parameters of ^{1}H spectra were as follows: 90° pulse, with 1000 scans, a spectral width of 20 ppm in the ^{1}H dimension, and a pre-scan delay of 6.5 s. All the spectra were analyzed in TopSpin (version 3.5pl7, Bruker, Germany), and the TSP was used as the standard for zero chemical shift and the internal standard for integration and normalization. The accumulated integrate and chemical shift data were exported to an Excel file, then converted to a csv file and compressed, and finally imported into MetaboAnalyst 6.0 for data analysis. Afterward, the data were normalized by the sum, log-transformed, and Pareto scaling. The data were first performed in an unsupervised multivariate analysis with principal component analysis (PCA) to assess metabolic differences in Caco-2 cells. Finally, the supervised analysis methods of partial least squares discriminant analysis (PLS-DA) and sparse partial least squares discriminant analysis (sPLS-DA) were used to analyze the data to eliminate environmental and systematic errors, thereby obtaining more accurate grouping results. While PLS-DA compares two groups, sPLS-DA analysis compares four groups (control, 300 μM cedrol, IC, and IC + 300 μM cedrol). The variety of metabolites between groups was identified based on variable importance in prediction and loading plot of major predictors. Metabolites were identified with reference to chemical shifts published in the Human Metabolome Database (http://www.hmdb.ca) and Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu) databases and compared with previous literature.[17,33–35]

2.12 | Statistical analysis

The values are expressed as mean ± SD, and all experiments were performed at least three independent times. Statistical analysis was performed using GraphPad Prism 8.0 software (Dotmatics, USA). All data were tested for normality using the Shapiro–Wilk test. The data were analyzed with *t*-tests or one-way ANOVA followed by Tukey's HSD post hoc test. The values of $p < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Cell viability of Caco-2 cells

To investigate the impact of cedrol on differentiated Caco-2 monolayer cells, a CCK8 assay was employed to assess cell viability at different concentrations of cedrol. As shown in Figure 1, cell viability was evaluated after treating Caco-2 cells with cedrol at concentrations ranging from 75 to 400 μM for 12, 24, and 48 h. The results indicated that the cell viability of Caco-2 cells treated with high concentrations of cedrol are significantly different from the control group and can promote Caco-2 cell proliferation (Figure 1A–C). The result demonstrates that 75–400 μM concentration of cedrol does not exhibit cytotoxicity toward Caco-2 cells, making it suitable for subsequent experimental analyses. Additionally, quercetin at a concentration of 150 μM showed no cytotoxicity toward Caco-2 cells and served as a positive control for this study.

3.2 | Effect of cedrol on ATP production

An ATP assay was employed to assess the ATP content within the cells to investigate the impact of cedrol on ATP levels in differentiated Caco-2 cells. As depicted in Figure 2, cells were treated with cedrol at concentrations ranging from 50 to 300 μM, and then, the intracellular ATP levels were measured. The results revealed that the ATP content within Caco-2 cells increased dose-dependently with cedrol concentrations. The ATP content treated with 150–300 μM cedrol was significantly different from the control group at 12 h, indicating that cedrol can increase the ATP level in Caco-2 cells.

3.3 | Effect of cedrol on intestinal permeability in differentiated Caco-2 cells

To evaluate the intestinal permeability of differentiated Caco-2 cell monolayers, the TEER was measured. As illustrated in Figure 3, treatment with different concentrations of cedrol (75, 150, and 300 μM) for 6–48 h increased TEER values compared to the control group. Notably, 300 μM cedrol and 150 μM quercetin significantly elevated TEER values at 6 and 12 h, indicating that cedrol alone can enhance TEER in Caco-2 cells (Figure 3A). After pre-treatment with the IC for 12 h, the TEER value of the Caco-2 cell monolayer was reduced to 88.50 ± 3.31% (data not shown), significantly lower than the control group, indicating a decrease in monolayer permeability following IC pre-treatment. However, treatment with different concentrations of cedrol (75, 150, and 300 μM) for 6–36 h increased TEER values compared to the IC group. It significantly improved the decreased TEER values induced by IC. Specifically, 150 and 300 μM cedrol and 150 μM quercetin significantly increased TEER values at 6–24 h after IC induction (Figure 3B). These results suggest that cedrol can restore the decreased TEER values induced by IC, reducing the intestinal permeability of Caco-2 cell monolayers.

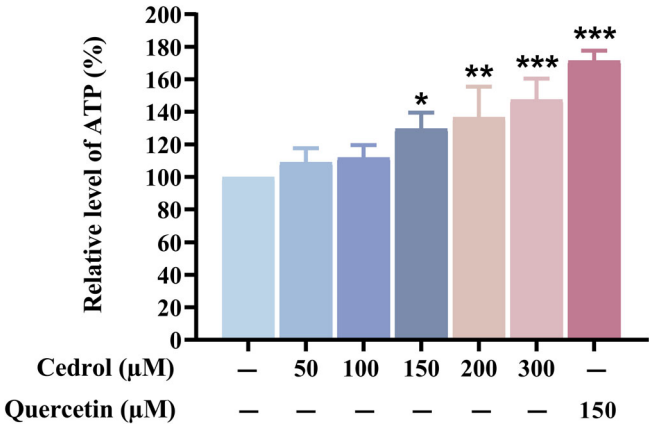

FIGURE 2 Effect of different cedrol concentrations on adenosine triphosphate (ATP) levels in Caco-2 cells. The values are expressed as mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

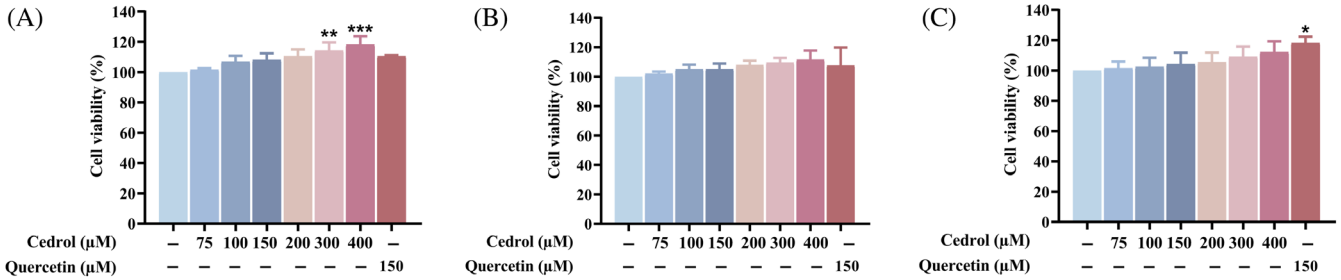

FIGURE 1 Effect of different cedrol concentrations of cell viability in Caco-2 cell. Viability of Caco-2 cells treated with cedrol for (A) 12, (B) 24, and (C) 48 h. The values are expressed as mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

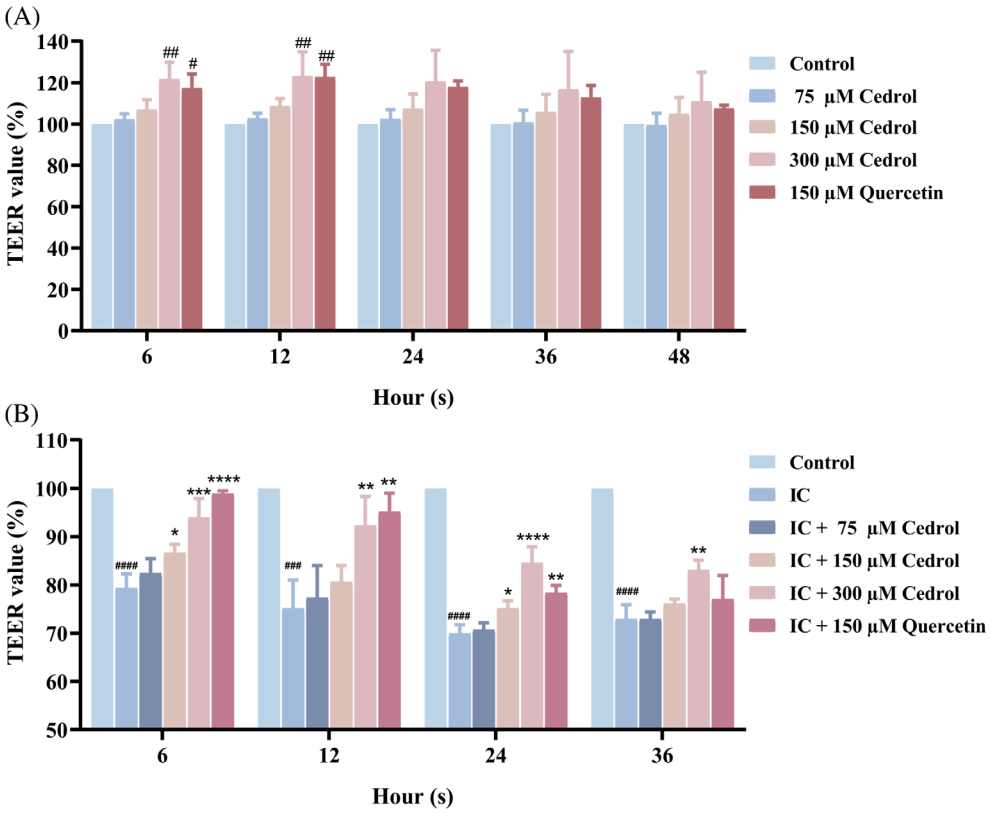

FIGURE 3 Effects of different cedrol concentrations on transepithelial electrical resistance (TEER) value in Caco-2 cell monolayers. (A) Effect of different cedrol concentrations on TEER value of Caco-2 monolayer cells at different times. (B) Different concentrations of cedrol on TEER values of Caco-2 monolayers after induced with inflammatory cocktail (IC) for 12 h. The values are expressed as mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, compared with control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with IC group. And 150 μM quercetin was used as the positive control.

3.4 | qPCR of the expression of Tight junction gene

The expression of TJ genes in differentiated Caco-2 cell monolayers was evaluated using qPCR. As depicted in Figure 4, IC induction reduced the expression of ZO-1, Occludin, Claudin-1, and Claudin-4. Compared to the control group, IC induction significantly decreased the expression of ZO-1, Occludin, and Claudin-1 (Figure 4A–C). Treatment with different concentrations of cedrol (75, 150, and 300 μM) and 150 μM quercetin (positive control) for 24 h restored the expression of TJ genes. Specifically, 300 μM cedrol significantly restored the expression of ZO-1, Occludin, and Claudin-1 compared to the IC group (Figure 4A–C), while 150 μM quercetin significantly increased the expression of Claudin-4 (Figure 4D). These results indicate that cedrol can restore the TJ genes induced by IC, alleviating the intestinal barrier dysfunction caused by IC through the increased expression of ZO-1, Occludin, and Claudin-1.

3.5 | ^{1}H NMR analysis of the metabolites in Caco-2 cell

The metabolic changes in differentiated Caco-2 cell monolayers were assessed using an NMR spectrometer (Advance III-400, Bruker, USA). The ^{1}H NMR spectra of the four treatment groups (control, 300 μM cedrol, IC, and IC + 300 μM cedrol) are shown in Figure 5. Following previous literature[17] and chemical shift information published in the Human Metabolome Database (http://www.hmdb.ca) and the Biological Magnetic Resonance Bank (http://www.bmrb.wisc.edu), a total of 25 metabolites were identified (Table S2). Differences among metabolites across different groups can be observed in the spectra. To understand the variations between metabolites among the groups, multivariate analysis was performed on the ^{1}H NMR spectra, reducing the dimensionality of the data for a more straightforward interpretation of the spectral results.

NMR-based metabolomic analysis was used to evaluate metabolite changes after Caco-2 cell treatment.

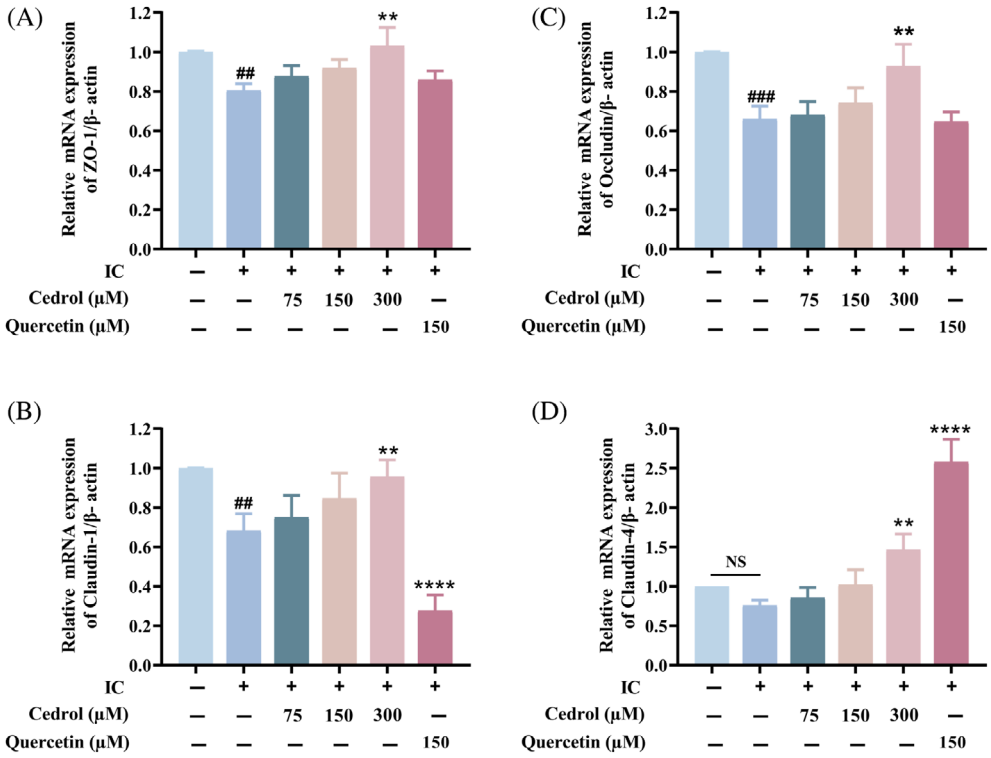

FIGURE 4 The messenger ribonucleic acid (mRNA) levels of tight junction genes in cedrol treated with Caco-2 cell monolayers. The effects of cedrol on the mRNA expression levels of (A) ZO1, (B) Claudin-1, (C) Occludin, and (D) Claudin-4. Caco-2 cells were induced by inflammatory cocktail (IC) for 12 h and subsequently incubated with cedrol for 24 h. Data were normalized to control values. The values are expressed as mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with control group. $^{**}p < 0.01$, $^{****}p < 0.0001$ compared with IC group. And 150 μM quercetin was used as the positive control.

Unsupervised multivariate PCA was initially applied to assess metabolite differences in Caco-2 cells. The results showed that the PCA multivariate model could differentiate metabolites in the two groups (data not shown). After that, the supervised analysis method PLS-DA is used to analyze the data to eliminate environmental and systematic errors, thereby obtaining more accurate grouping results. The PLS-DA model can express the fit of the model through cross-validation (CV), and the study found that the R2 and Q2 values of the PLS-DA model are both higher than 0.7, which means that the model is reliable and not overfitting (data not shown). PLS-DA analysis showed a clear separation of cellular metabolite samples between the control group and the IC group, as well as between the IC group and the IC + 300 μM cedrol group (Figure S2). Compared to the control group, the IC group significantly reduced acetate, creatine, choline, and betaine levels and increased glucose levels. Conversely, compared to the IC group, the IC + 300 μM cedrol group shows a significant increase in leucine, isoleucine, alanine, acetate, choline, glycine, and tyrosine (Table 1). Observations from Table 1 also suggest that the trend of metabolites can be restored through cedrol treatment, indicating that cedrol can recover the metabolic disruptions induced by IC.

The ^{1}H NMR spectra of the four treatment groups (control, 300 μM cedrol, IC, and IC + 300 μM cedrol) were analyzed using PCA to evaluate metabolic differences in Caco-2 cells initially. The results showed that the PCA model could not distinguish the four groups of metabolites well (Figure S3A). Therefore, the supervised analysis method sPLS-DA was used to analyze the data to eliminate environmental errors and systematic errors to obtain more accurate grouping results. The sPLS-DA model can effectively reduce the number of variables (metabolites) in high-dimensional metabolomics data, resulting in robust and easy-to-interpret models. The performance of the sPLS-DA model can be evaluated through CV. Our study used a five-fold CV for CV to express model fitness and plotted the average classification error rate for each sPLS-DA dimension. The validation results indicated that the estimated error rate of sPLS-DA is stable and decreases when running for a sufficient number of dimensions (Figure S3B). Therefore, the sPLS-DA model can provide satisfactory sample clustering with a low error rate, highlight relevant information among very few potential variables, and clearly

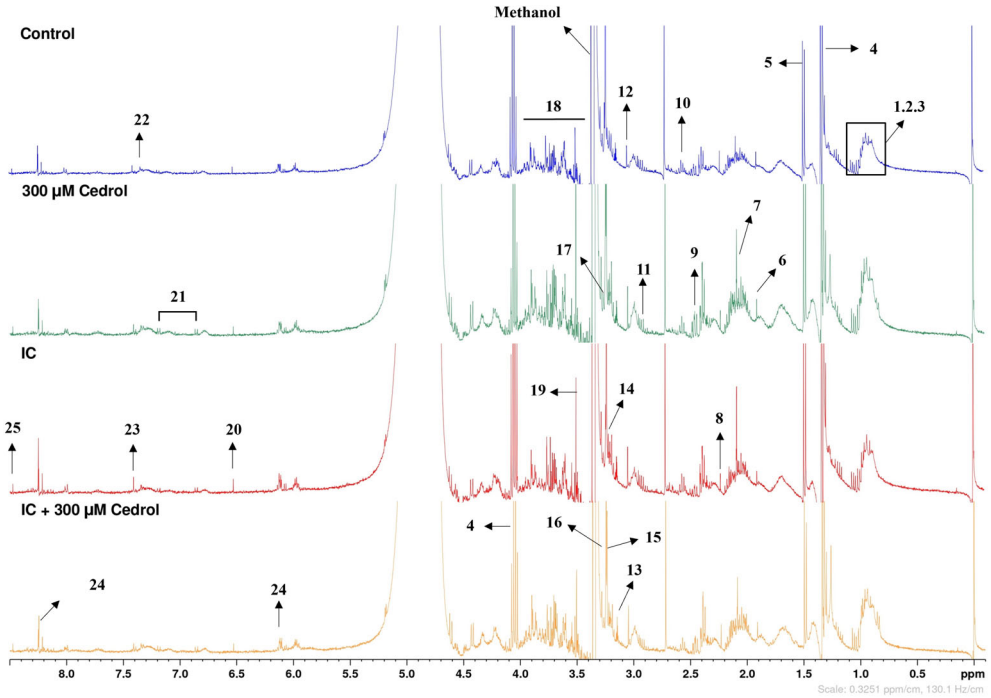

FIGURE 5 The identified metabolites of Caco-2 cells in 400 MHZ ^{1}H-nuclear magnetic resonance spectra. The characteristic peaks from the identified metabolites for the Caco-2 cell are annotated with the number. All cell extractions are prepared in 650 μL deuterium oxide + methanol-d4 (including 0.0002% TSP). Metabolites: 1, leucine; 2, isoleucine; 3, valine; 4, lactate; 5, alanine; 6, acetate; 7, homoserine; 8, acetoacetate; 9, glutamine; 10, glutamate; 11, aspartate; 12, creatine; 13, ethanolamine; 14, choline; 15, *O*-phosphocholine; 16, sn-glycerophosphocholine; 17, betaine; 18, glucose; 19, glycine; 20, fumarate; 21, tyrosine; 22, phenylalanine; 23, histidine; 24, adenosine triphosphate (ATP); 25, formate. IC, inflammatory cocktail; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt.

distinguish the control, IC, and IC + cedrol groups (Figure 6A). The loadings plot in Figure 6B highlights the metabolites with differences between the groups in the sPLS-DA model. The differences in all metabolites between groups are depicted in Figure S4, with six metabolites showing significant differences, namely glucose, leucine, alanine, acetate, choline, and tyrosine (Figure 7). In comparison to the control group, the IC group decreases the levels of acetate, choline, and tyrosine. However, after cedrol treatment (IC + cedrol), their levels are restored and can increase the levels of leucine and alanine. Figure 8 presents a schematic representation of the biosynthetic metabolic pathways based on group metabolite differences. It is evident from the figure that metabolites are downregulated or upregulated by IC induction and can be reversed and restored to control group levels through cedrol treatment (IC + cedrol), indicating that IC induction leads to metabolic disruptions in Caco-2 cells, and cedrol treatment can balance this metabolic imbalance.

4 | DISCUSSION

Treatment options for IBD are constrained by its complexity and unknown etiology. The current standard of care involves corticosteroids, amino-salicylates, and immunosuppressive agents. While these therapies may mitigate symptomatic complications, they are often accompanied by severe side effects, such as immune tolerance and diarrhea, nausea, and vomiting.[36] Thus, there is an urgent imperative to develop novel treatments and alternative medications. IBD manifests as a chronic inflammatory condition of the GI tract characterized by the disruption of the intestinal barrier function. The intestinal epithelial barrier functions primarily operation through tight junction, which prevent the passage of intestinal bacteria, toxins, and cellular inflammatory factors. Dysfunction of these TJ is associated with various GI disorders, and maintaining their function may delay disease progression.[37] Maintenance and restoration of the intestinal barrier necessitate considerable energy expenditure.[38] Bacallao et al. have demonstrated that ATP depletion adversely affects epithelial cell TJ.[39] Hence, we aimed to expolre the impact of cedrol on ATP levels and barrier function in Caco-2 cells. Our results indicate that cedrol significantly elevates ATP content in Caco-2 cells (Figure 2) and enhanced the TEER value, thus fortifying intestinal barrier function (Figure 3). Conversely, treatment with an IC markedly decreases the TEER value, indicating that induced with IC can reduce the permeability of the cell

TABLE 1 The different metabolites in Caco-2 cells were identified by ^{1}H-nuclear magnetic resonance (NMR).

No.	Metabolite name	Con versus IC		IC versus IC + cedrol	
		Trend	*p*-value	Trend	*p*-value
1	Leucine	↑		↑	**
2	Isoleucine	↑		↑	*
3	Valine	↑		↑	
4	Lactate	↑		↓	
5	Alanine	↑		↑	**
6	Acetate	↓	*	↑	*
7	Homoserine	↑		↓	
8	Acetoacetate	↓		↑	
9	Glutamine	↓		↑	
10	Glutamate	↓		↑	
11	Aspartate	↓		↓	
12	Creatine	↓	**	↑	
13	Ethanolamine	↓		↑	
14	Choline	↓	*	↑	**
15	*O*-Phosphocholine	↓		↓	
16	sn-Glycerophosphocholine	↓		↑	
17	Betaine	↓	*	↑	
18	Glucose	↑	**	↑	
19	Glycine	↓		↑	*
20	Fumarate	↑		↓	
21	Tyrosine	↓		↑	*
22	Phenylalanine	↓		↑	
23	Histidine	↑		↑	
24	ATP	↑		↑	
25	Formate	↓		↑	

Note: Different metabolites in Caco-2 cells were identified through ^{1}H-NMR, and the data were analyzed through partial least squares discriminant analysis to find differential metabolites between groups. The data was performed based on a parametric Student's *t*-test. The arrows indicate the trend of up- and downregulations of metabolites between the two groups.

Abbreviations: ATP, adenosine triphosphate; IC, inflammatory cocktail.

*$p < 0.05$; **$p < 0.01$.

monolayer permeability. Notably, cedrol treatment significantly restore the TEER value (Figure 3B) and upregulate the messenger ribonucleic acid (mRNA) expression of TJ proteins (ZO-1, Occludin, and Claudin-1), mitigating IC-induced intestinal barrier dysfunction (Figure 4A–C). Previous research has elucidated that inhibiting ATP synthase diminishes cellular orientation persistence and migration, underscoring the role of mitochondrial ATP production in epithelial wound healing and barrier restoration.[40] Additionally, adenine nucleotides ADP and ATP have been found to notably stimulate epithelial cell restitution,[41] emphasizing the significance of intestinal epithelial cell proliferation and migration in the wound-healing process. Our study demonstrates that cedrol promotes the proliferation of Caco-2 cells (Figure 1A–C). Therefore, it demonstrated that cedrol could enhance the mRNA expression of the TJ protein and promote the proliferation of intestinal epithelial cells by increasing the ATP content of Caco-2 cells.

Moreover, prior research has demonstrated alteration in metabolic profiles among IBD patients, encompassing changes in amino acids, short-chain fatty acids, bile acids, and lipid metabolism.[42,43] Consequently, metabolomics emerges as a viable tool for assessing metabolic product variations in Caco-2 cells. Within this context, metabolomics can be discerned through NMR and MS techniques. NMR offers advantages such as reproducible, quantitative, and non-destructive nature toward samples. Notably, NMR has been employed recently to investigate

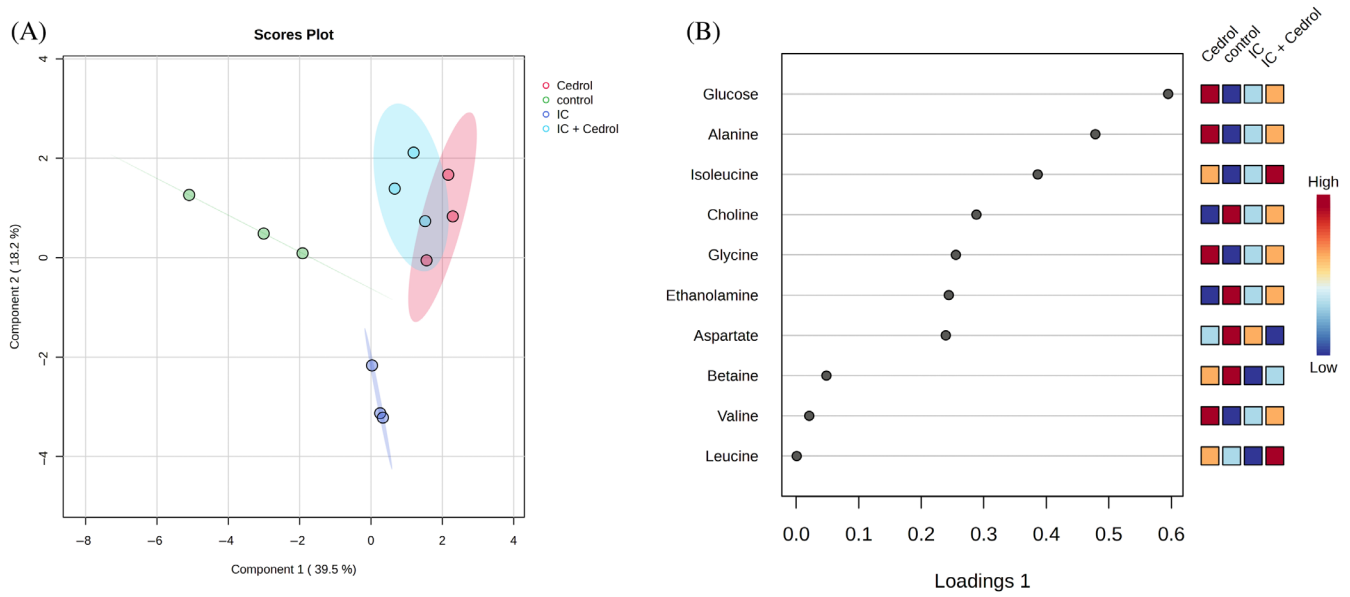

FIGURE 6 Sparse partial least squares-discriminant analysis (sPLS-DA) of ^{1}H nuclear magnetic resonance spectral data from the four groups of Caco-2 cells. (A) The score plots were separated by components 1 (39.5%) and 2 (18.2%) of the total variance in the four groups of Caco-2 cell sample extracts. (B) The loadings plot shows the variables selected by the sPLS-DA model for a given component. IC, inflammatory cocktail.

metabolism and metabolic mechanisms in both human cellular and animal models.[44] Thus, NMR served as the technique for analyzing intestinal disease metabolomics in our investigation. The findings revealed significantly reduced acetate, creatine, and betaine levels in the IC group compared to the control group (Table 1). Acetate, a short-chain fatty acid, is known for its potential to evaluate intestinal epithelial barrier gene expression and suppress inflammation.[45] Additionally, acetate has been observed to inhibit TNF-α-mediated activation of the nuclear factor kappa B (NF-κB) pathway in a human colon adenocarcinoma cell (Colo320DM).[46] In contrast, oral acetate administration has demonstrated the ability to restore intestinal permeability for treating inflammatory disorders.[47] Notably, treatment with cedrol effectively restored acetate levels in our study. Previous research highlights creatine as a crucial energy metabolite governing intestinal barrier function.[48] Dietary supplementation with creatine has shown significantly improvement inflammatory responses in a mouse colitis model.[49] Similarly, betaine, a natural compound with anti-inflammatory effects, has been found to alleviate colitis in mice by modulating the inflammatory response, enhancing the intestinal barrier, and altering the intestinal flora.[50,51] Furthermore, reduced choline and tyrosine levels were observed in the IC group compared to the control group (Figure 7). Choline deficiency exacerbates the severity of IBD, while choline supplementation reduces susceptibility to colitis in mice.[52,53] Moreover, Williams et al. indicate that L-tyrosine levels decreased in dextran sulfate sodium (DSS)–induced mice, with other studies indicating the involvement of *Bifidobacterium* in synthesizing various antimicrobial peptides and enhancing intestinal barrier function through L-tyrosine production.[52,54] Importantly, in our study, treatment with cedrol successfully restored acetate, creatine, betaine, choline, and tyrosine levels.

Currently, numerous studies suggest that amino acids may influence the intestinal barrier, potentially altering TJ and intestinal permeability. Prior research has demonstrated that glutamate, glutamine, arginine, and leucine can mitigate the reduction in TEER induced by MTX in Caco-2 cells and prevent the decrease in expression of TJ protein (ZO-1 and Occludin). This reaffirms amino acids' restorative and regulatory role in the intestinal mucosal barrier function.[55] In our investigation, NMR analysis revealed a significant increase in amino acid levels (leucine, Isoleucine, alanine, glycine, and tyrosine) in the treatment group (IC + cedrol) compared to the IC group. Additionally, levels of glutamate, glutamine, and phenylalanine were also restored compared to the IC group (Table 1; Figure S4). Previous studies suggest that leucine enhances intestinal barrier function in fish by elevating levels of TJ proteins (ZO-1, Occludin, and Claudin-2).[56] Moreover, it maintains the integrity of the intestinal cellular barrier in LS174T cells by increasing the expression of Mucin 2 and occludin proteins.[57] D-alanine has been shown to protect the barrier function of *Streptococcus*

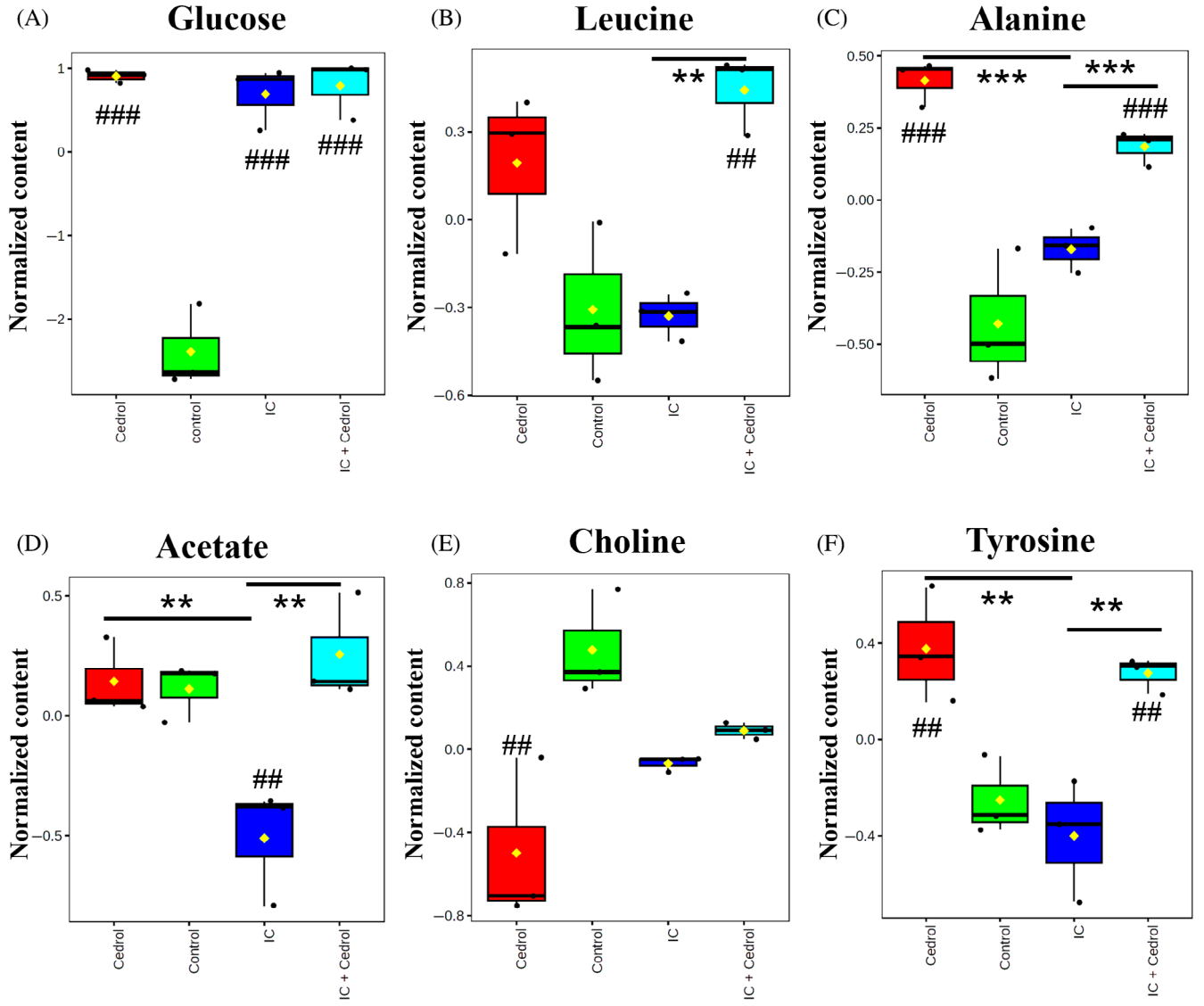

FIGURE 7 The quantitative levels of significant metabolites detected in Caco-2 extracts. The values are expressed as mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with control group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with inflammatory cocktail (IC) group.

thermophilus ATCC 19258^{T} against Caco-2 cells,[58] and it can prevent DSS-induced colitis in mice.[59] Hence, it is hypothesized that cedrol may restore intestinal cell barrier integrity by enhancing amino acid content and increasing the TJ gene expression.

In recent years, studies have found that many natural products and herbs have shown efficacy against colitis in experimental models and clinical trials.[60–62] Previous studies have confirmed that cinnamaldehyde in cinnamon essential oil can increase TEER value and the expression of TJ proteins (Claudin-4, ZO-1, ZO-2, and ZO-3) to promote intestinal barrier function.[63] Other studies have indicated the protective effect of essential oil of *Zanthoxylum bungeanum* pericarp on DSS-induced colitis in mice.[64] Cedrol is the main component of *C. lanceolata* var. *konishii* essential oil. Previous studies have demonstrated that it has anti-inflammatory, antibacterial, anti-anxiety, and anti-cancer effects.[20,22,65,66] It can also increase the length of hair follicles in mouse skin and promote the extracellular matrix formation of human dermal fibroblasts to accelerate the growth of dermal fibroblasts.[26,67] The structure of cedrol contains hydroxyl groups, which can be rapidly metabolized by uridine diphosphate glucuronosyltransferase and glucuronic acid. Additionally, liver enzymes can efficiently metabolize cedrol due to its interaction with plasma proteins and hydrophobicity.[21] Therefore, cedrol has excellent potential for drug research. Furthermore, modifying

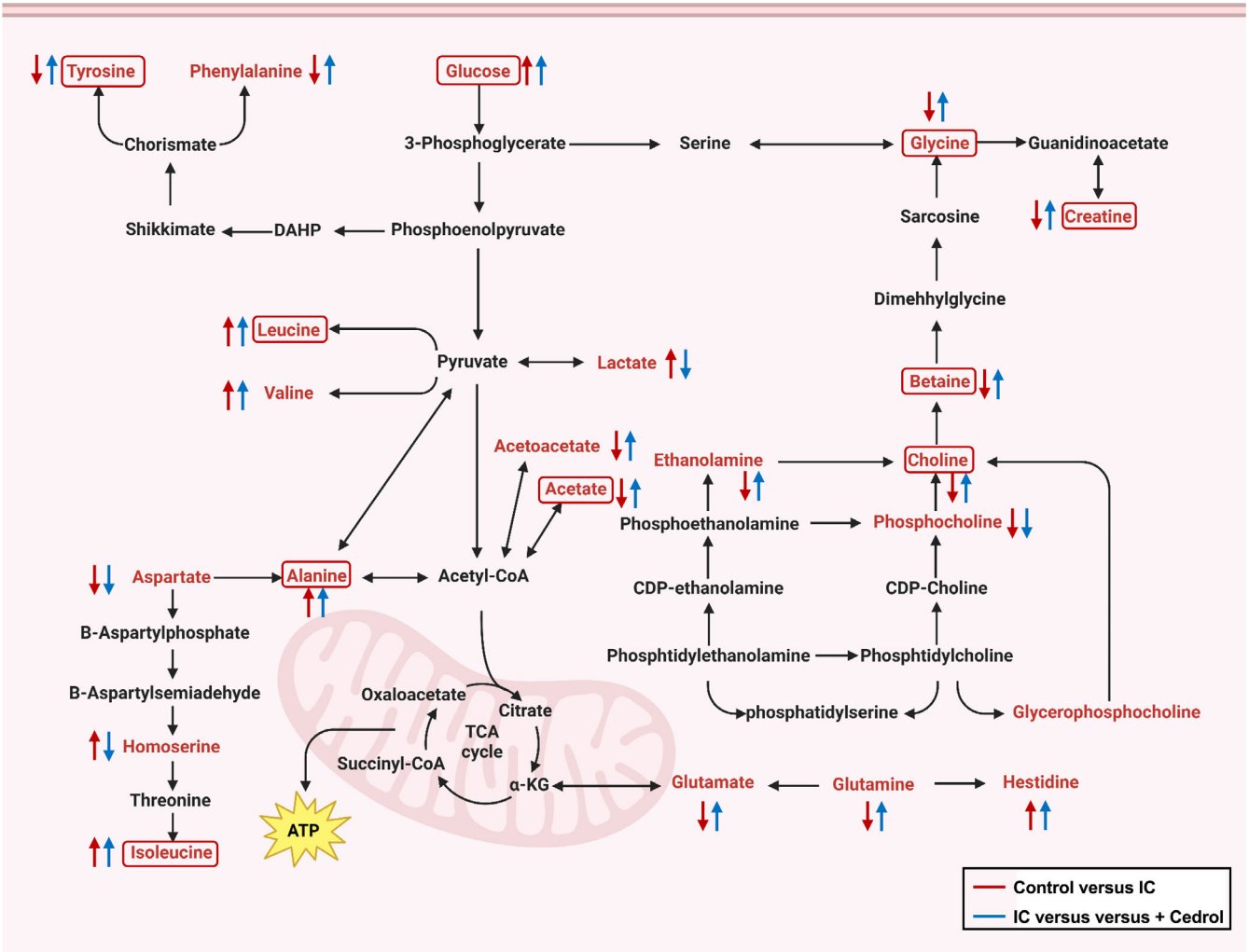

FIGURE 8 Schematic diagram of the different metabolic pathways. Relevant metabolic pathways were altered after inflammatory cocktail (IC)-induced and treatment with cedrol in Caco-2 cells. Red letters represent metabolites detected in this study, and red boxes represent metabolites with significant differences between groups. The red arrow represents the metabolite changes between the control and IC groups, while the blue arrow represents the metabolite changes between the IC and IC + cedrol groups. ATP, adenosine triphosphate.

cedrol's dosage form, such as recombinant high-density lipoprotein nanoparticles and nanostructured drug delivery systems, can also improve its bioavailability and absorption.

5 | CONCLUSION

In conclusion, the mechanism of cedrol involves repairing the IC-induced intestinal barrier by increasing ATP levels to promote cell proliferation and increasing amino acid content to influence TJ mRNA expression in Caco-2 cells. Therefore, cedrol has excellent potential to be developed into a drug for the treatment of IBD in the future. In addition, this study is the first to use NMR metabolomics to analyze the metabolite changes in Caco-2 cells induced by IC. These metabolites may be used as diagnostic targets for monitoring intestinal diseases and can be used as a diagnostic platform for intestinal diseases in the future.

AUTHOR CONTRIBUTIONS

Conceptualization and supervision and funding: Sheng-Yang Wang. Methodology: Mo-Rong Xu and Chung Hsuan Wang. Investigation and formal analysis: Mo-Rong Xu and Chia-Hsin Lin. Validation and writing—original draft: Mo-Rong Xu. Writing—review and editing: Mo-Rong Xu and Sheng-Yang Wang.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available in this article's supplementary materials.

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SUPPORTING INFORMATION

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