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Research Article

ACE/ACE2 Steady State: A Novel Mechanism of Rosmarinic Acid in Alcohol-Associated Gastric Ulcer

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Abstract

Background: The local inhibition of the gastric renin angiotensin system (RAS) has emerged as a pivotal therapeutic target in treating gastric ulcers. Rosmarinic acid (RA), a medically significant herb belonging to the category of water-soluble polyphenolic compounds, exhibits notable antiulcer properties. Our previous research has demonstrated that the anti-ferroptosis effect of RA in lipopolysaccharide (LPS)-induced septic acute respiratory distress syndrome (ARDS) depends on the RAS. Therefore, this study aimed to assess the underlying mechanism through which RA mitigates ethanol-induced gastric ulcers in mice. **Methods**: Gastric tissue samples were collected from patients with gastric ulcers to perform RNA sequencing (RNA-seq) and bioinformatics analyses. Moreover, the gastric ulcer mouse model was induced through a single gavage of absolute ethanol at a dose of 0.1 mL/10 g. Before induction, mice were orally administered RA at doses of 4 and 20 mg/kg, or esomeprazole 3.03 mg/kg (as a reference drug), for a duration of 5 days. The anti-gastric ulcer effects were assessed through histopathological evaluations and Western blot analysis. **Results**: The RNA-seq data and bioinformatic insights revealed that the angiotensin-converting enzyme (ACE)/angiotensin (Ang) 2 converting enzyme (ACE2) balance may constitute a novel mechanism in alcohol-related gastric ulcer, which RA modulates. A total of six overlapping targets related to both RA and gastric ulcers were identified. Among them, matrix metalloproteinase-1 (MMP1), matrix metalloproteinase-3 (MMP3), and A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motif, type 4 (ADAMTS4) presented low binding energy with RA and formed a protein-protein interaction (PPI) network with ACE and ACE2. *In vivo* experiments further substantiated that RA conferred gastric protection by restoring the ACE/ACE2 balance and upregulating GPX4 expression. **Conclusions**: RA might be a potential gastroprotective agent by suppressing RAS-related ferroptosis in the gastric tissues.

Keywords: rosmarinic acid; stomach ulcer; RNA-sequencing; molecular docking simulation

1. Introduction

Gastric ulcer (GU) represents a prevalent gastrointestinal disorder worldwide, characterised by an impairment of mucosal integrity. Diverse factors cause the emergence and development of GU, including imbalance of the protective factors (mucus, tight junction, and prostaglandins) and invasive factors (gastric acid, pepsin, alcohol consumption, *Helicobacter pylori* (*H. pylori*) infection, and the extensive use of non-steroidal anti-inflammatory drugs (NSAIDs)) [1]. Recent comprehensive Mendelian randomization (MR) study highlights that alcohol consumption is associated with the occurrence of several gastrointestinal diseases [2]. Accumulating preclinical evidence has revealed the mechanism of ethanol exposure in disrupting the gastric mucosal defence systems, for example, suppressed heat shock protein 70 expression, and decreased gastric mucosal blood flow [3,4].

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure and vascular response to injury. The local inhibition of the gastric renin-angiotensin system (RAS), encompassing the suppression of angiotensin-converting enzyme (ACE) and the antagonism of angiotensin (Ang) AT-1 receptors [5,6], emerges as a crucial therapeutic target in the management of gastric ulcers, as it augments gastric microvascular perfusion and enhances gastric tissue oxygenation [7]. Conversely, recent research has demonstrated that the activation of the angiotensin (Ang) 2 converting enzyme (ACE2) pathway is linked to advantageous gastroprotective effects [8]. Thus, the restoration of the ACE/ACE2 balance is implicated in the novel approach against alcohol-associated gastric ulcers.

Rosmarinic acid (RA) is a naturally water-soluble polyphenolic compound found in several medicinal herbs, such as rosemary (Salvia rosmarinus), lemon balm (Melissa officinalis), and spearmint (Mentha spicata) [9]. RA exhibits vast potential applications in promoting human health through a diverse range of biological activities, including

anti-inflammatory, antioxidant, anti-aging, and neuroprotective benefits [10,11]. Recently, increasing studies have highlighted the significance of RA in the treatment of various gastric injuries, especially alcohol-induced gastric ulcers [12–14]. Furthermore, RA exhibits a higher binding affinity for the zinc ion center of ACE [15], as well as the capability to restore the balance between ACE and ACE2 [16]. Given these promising findings, we hypothesised that RA might provide effective protection against alcohol-associated gastric ulcers by targeting ACE/ACE2 steady state. It is noteworthy that ferroptosis plays a critical role in ethanol-stimulated gastric mucosal damage [17]. Our previous study has confirmed that RA can suppress RAS-mediated ferroptosis in the lung tissues [16]. Therefore, we further investigate whether RA ameliorates ethanol-induced gastric ulcers by suppressing RAS-mediated ferroptosis in the gastric tissues.

2. Materials and Methods

2.1 Samples and RNA-sequencing (RNA-seq)

According to protocols approved by the ethics committee of the West China Hospital of Sichuan University and 363 Hospital (No: 2024-862). A total of six samples were collected from gastric ulcer patients, including four men and two women (median age: 43 years, range: 29–58 years) as well as six samples from chronic gastritis patients, including four men and two women (median age: 49 years, range: 39–59 years) in the 363 Hospital from 1 Sep 2024 to 1 Nov 2024. Patients with other underlying conditions or infections were excluded.

The gastric tissues were collected and rapidly frozen in liquid nitrogen and sent to Hangzhou Kaitai Biotechnology Co., Ltd. for transcriptome sequencing. Samples that passed quality control were sequenced on the DNBSEQ platform. The raw sequencing data were filtered using SOAPnuke (v1.5.6) to remove (1) reads containing adapter sequences (adapter contamination); (2) reads with more than 5% of unknown bases (N); and (3) low-quality reads (reads in which bases with a quality value lower than 15 accounted for more than 20% of the total bases in the read), resulting in clean data. The clean data were then aligned to the reference genome using HISAT2 (v2.1.0, Hangzhou Kaitai Biotechnology Co., Ltd, Zhejiang, China) software. Bowtie2 (v2.3.4.3, Hangzhou Kaitai Biotechnology Co., Ltd, Zhejiang, China) was used to align the clean data to the reference gene set. Gene expression quantification was performed using RSEM (v1.3.1, Hangzhou Kaitai Biotechnology Co., Ltd, Zhejiang, China) software, and differential gene expression analysis was conducted using DESeq2 (v1.4.5) with a cutoff of Q value $\leq$0.05.

2.2 Rosmarinic Acid Target Prediction

The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was utilised to retrieve the Simplified Molecular Input Line Entry System (SMILES) expression of rosmarinic acid. Target prediction for rosmarinic acid was performed by SwissTargetPrediction (http://swisstargetprediction.ch/). DEGs from RNA-seq were intersected with rosmarinic acid target genes and visualised with Venn 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html). The Retrieval of Interacting Genes/Proteins (STRING) version 12.0 database (https://string-db.org/) was used to perform the Protein-protein interaction (PPI) network analysis.

2.3 Molecular Docking

Molecular docking was employed to investigate the potential interactions of RA with matrix metalloproteinase-1 (MMP1), matrix metalloproteinase-3 (MMP3), or A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motif, type 4 (ADAMTS4). The docking process was performed by CB-Dock2 (https://cadd.labshare.cn/cb-dock2/php/blinddock.php) to analyse the binding properties of the ligands for each protein [18]. The 3D structures of the 3SHI structural domain of MMP1, the 4DPE structural domain of MMP3, and the 3B2Z structural domain of ADAMTS4 protein were downloaded from the PDB database (https://www.rcsb.org/) and exported in PDB format.

2.4 Drugs

RA was purchased from Macklin (R817282-1g, HPLC $\geq$98%, Shanghai, China). Esomeprazole capsule was obtained from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (China).

2.5 Animals and Groups

Animal ethics and experimental procedures were approved by West China Hospital of Sichuan University and were conducted under the published guidelines of the China Council on Animal Care (No. 20240308066). Specific pathogen-free (SPF) 5-week-old Kunming mice (weight of 18–22 g) were acquired from Da-Shuo Biological Technology Co., Ltd. (Chengdu, China). After 5 days of acclimatization (Temperature: 22 °C–24 °C, relative humidity: 50%–60%, and a 12-h light/dark cycle), all mice were randomly divided into five groups: control group, model group, RA low-dose group (RAL 4 mg/kg, orally) and high-dose groups (RAH 20 mg/kg, orally), and esomeprazole group (3.03 mg/kg, orally), with 12 in each group (6 male and 6 female). RAL, RAH, and esomeprazole groups were given the corresponding drugs once daily, while the control group and model group were given an equal dose of saline for 5 days. Two hours after the final gavage, acute gastric ulcer models were established by gavage with 0.1 mL/10 g of absolute ethanol. The control group mice were given an equivalent volume of saline. One hour later, all mice were euthanised under sodium pentobarbital anaesthesia (50 mg/kg, intraperitoneally), and the gastric tissues were rapidly collected for further experiments. The dosage

basis for selecting RA was derived from our previous study and preliminary data [16].

2.6 Assessment of Gastric Tissue Injury

The gastric cavity of the mice was opened along the greater curvature and rinsed completely with 5 mL of distilled water. Then, the stomachs were blotted dry with filter paper, spread out, and photographed. The lesion length in the flattened stomach samples was measured using a vernier caliper. The gastric ulcer index (GUI) was calculated as follows: under the dissecting microscope, the amount of bleeding, the size of the ulcers, and their distribution on the gastric mucosa were observed. The longest diameter of each ulcer was measured. Ulcers with the longest diameter $\leq$1 mm were scored as 1 point; those $>$1–2 mm were scored as 2 points; those $>$2–3 mm were scored as 3 points; those $>$3–4 mm were scored as 4 points; and those $>$4 mm were scored as 5 points [19]. Additionally, ulcer inhibition rate was used to evaluate the degree of gastric mucosal injury, which was calculated as: inhibition ratio (%) = $[(GUI_{ethanol\ group} - GUI_{treated\ group})/GUI_{ethanol\ group}] \times 100$.

2.7 Detection of pH and Pepsin Activity

The pH value of gastric juice was measured by pH test strips.

Approximately 0.1 g of gastric tissue was weighed and homogenised in 1 mL of extraction buffer from the Pepsin Activity Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., Cat. No. 20210125) at 4 °C. The homogenate was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected. The protein concentration in the supernatant was determined using the BCA Protein Assay Kit at 562 nm. Pepsin activity was measured using the UV spectrophotometry method with the Pepsin Activity Assay Kit. The absorbance of all samples was measured at 275 nm, and the pepsin activity (U•mg $prot^{-1}$) was calculated using the formula: Pepsin Activity (U•mg $prot^{-1}$) = $1.31 \times (A_{sample} - A_{blank}) / Cpr$.

2.8 Hematoxylin and Eosin (HE) Staining

The gastric tissues were fixed in 4% paraformaldehyde at 4 °C for 24 hours, embedded in paraffin, and sectioned at a thickness of 4 µm. The sections were stained with HE to observe the pathological morphological changes in the gastric mucosa. For each section, 3–5 random fields were selected under a 100$\times$ microscope to examine the structure of the gastric mucosa and the infiltration of inflammatory cells in the adjacent tissues.

2.9 Immunohistochemical (IHC)

Gastric tissues were collected from mice and fixed in 10% neutral buffered formalin for 24 hours. After dehydration and clearing, the tissues were embedded in paraffin and sectioned into 4-µm slices, which were mounted on poly-L-lysine-coated slides. The sections were subjected to antigen retrieval by heating in citrate buffer for 15 min in a microwave. Following cooling, endogenous peroxidase activity was quenched with 3% hydrogen peroxide, and the sections were blocked with 5% normal goat serum for 30 min. Primary antibodies (GPX4: Abcam, #ab125066, UK), were diluted to 1:100 and incubated overnight at 4 °C. Secondary antibodies (L3032, 1:5000, Signalway Antibody, USA) were applied for 1 h at room temperature. Sections were then stained with 3,3'-diaminobenzidine (DAB), counterstained with hematoxylin, dehydrated, and mounted. Finally, the sections were observed under a microscope, photographed, and the average optical density was calculated using image analysis software for quantitative analysis.

2.10 Western Blot

Gastric tissues were lysed using Western blot lysis buffer to extract proteins. The proteins were separated by SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane for 90 minutes. The membrane was blocked with 5% skim milk for 2 hours. The primary antibodies were incubated overnight at 4 °C. The specific antibodies used were ACE (Abcam, #ab183591, 1:1000, UK), ACE2 (Proteintech, 21115-1-AP, 1:1000, USA), and β-actin (Cell Signalling Technology, 3700S, 1:1000, USA). The secondary antibodies were incubated for 90 minutes, and the signals were detected using chemiluminescence. The band densities were analysed using ImageJ software (version 1.8.0, NIH, Bethesda, MD, USA).

2.11 Statistical Analysis

All continuous values are expressed as mean $\pm$ SD and statistically analysed by one-way analysis of variance (ANOVA) with Tukey's HSD correction (GraphPad Prism version 8.0). $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1 RNA-seq and Bioinformatics Analysis

We performed transcriptome sequencing and conducted a comprehensive bioinformatics analysis on the gastric tissues between ulcer and non-ulcer gastric tissues of patients. The 261 differentially expressed genes (DEGs) were significantly downregulated in ulcer gastric tissues (Q value $<$0.05, $|\log2FC| \geq 1$). The Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis identified the transporters, PI3K-Akt signaling pathway, and gastric acid secretion were significantly enriched (Fig. 1A). Furthermore, the Gene set enrichment analysis (GSEA) indicated that DEGs were significantly enriched in oxidative phosphorylation, gastric acid secretion, alcoholism, and p53 signaling pathway (Fig. 1B). Among them, CREB3L3, GNAI3, MAOB, GRIN2D, GRIN3B, FOSB, GNGT1, PPP1R1B, DDC, SLC6A3, CALM2, NTRK2, SHC3,

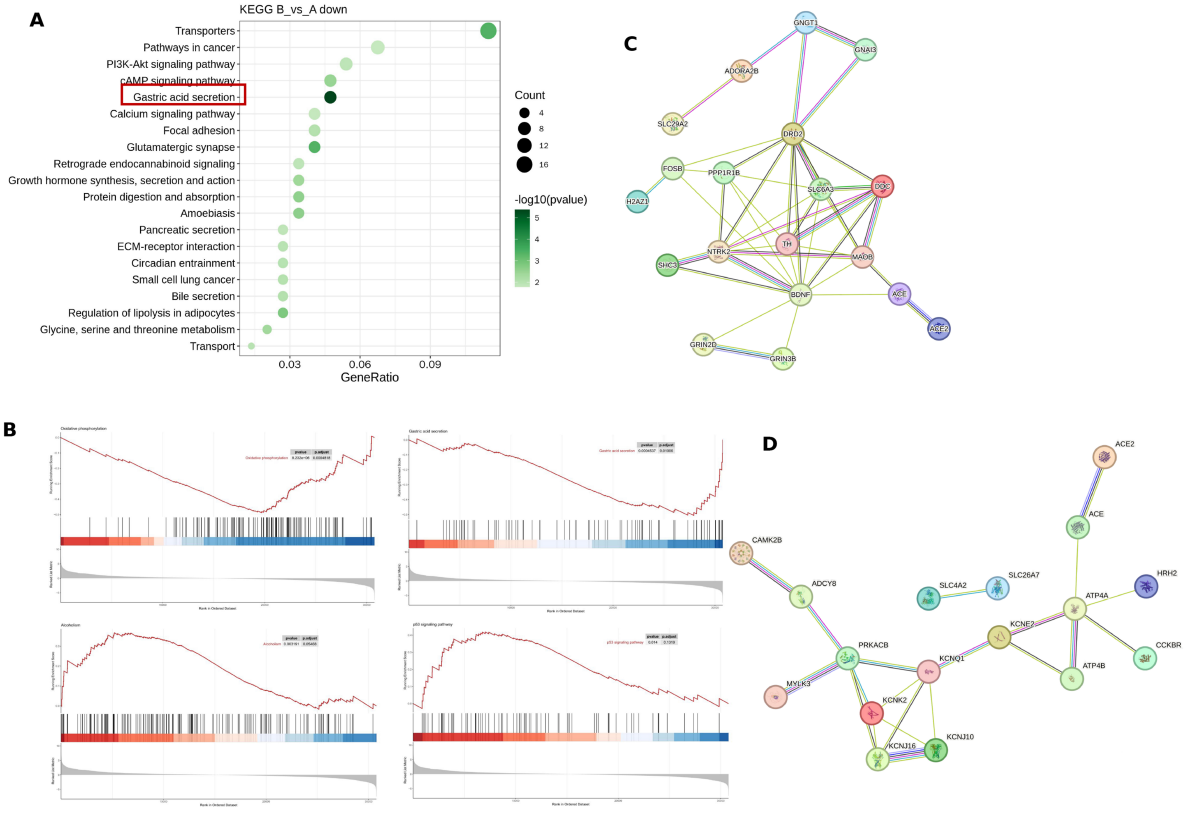

Fig. 1. RNA-seq and bioinformatics analysis. (A) KEGG pathway. (B) GSEA analysis (NES >1, p value < 0.05, p.adjust < 0.25). (C) PPI analysis of the DEGs enrichment in alcoholism with ACE and ACE2 was conducted using STRING. (D) PPI analysis of the DEGs enrichment in gastric acid secretion with ACE and ACE2 was conducted using STRING. RNA-seq, RNA-sequencing; KEGG, Kyoto Encyclopaedia of Genes and Genomes; GSEA, Gene set enrichment analysis; NES, normalized enrichment scoring; PPI, Protein-protein interaction; DEGs, differentially expressed genes; ACE, angiotensin-converting enzyme; ACE2, angiotensin (Ang) 2 converting enzyme; STRING, The Retrieval of Interacting Genes/Proteins.

DRD2, H2AZ1, ADORA2B, SLC29A2, BDNF, TH, H2AC20, H2AC21, SHC4, H2AX, H2AC7, H4C3, NRAS, H2BS1, H2BC14, H3C8, H4C13, H2BC9, H2AC14, H2AC16, H4C5, H2BC7, H4C1 were included in KEGG-alcoholism, and KCNQ1, CAMK2B, KCNK2, ATP4A, CCKBR, HRH2, CHRM3, MYLK3, PRKACB, SLC26A7, KCNJ16, ADCY8, KCNE2, SLC4A2, KCNJ10, SSTR2, ATP4B were included in KEGG-gastric acid secretion. The protein-protein interaction network constructed using STRING revealed interactions between all the DEGs with ACE and ACE2 (Fig. 1C,D). These results suggested that the ACE/ACE2 balance might be the potential regulatory mechanism in gastric ulcers, primarily contributing to alcoholism and the regulation of gastric acid secretion.

3.2 Identification of Potential Targets Between RA and Gastric Ulcer

From the RNA-seq data, 533 DEGs were identified. Utilising data from the SwissTargetPrediction, 100 RA-related targets were obtained. As shown in Fig. 2A, a total of 6 overlapping targets related to both RA and gastric ulcer were identified, including MMP1, MMP3, HCAR2, PADI4, ADAMTS4, and FOLH1, representing potential key therapeutic targets. Also, the PPI network constructed using STRING revealed interactions between MMP1, MMP3, and ADAMTS4 with ACE and ACE2 (Fig. 2B). As shown in Fig. 2C, the analysis revealed significant differences in several GO molecular functions related to metallopeptidase activity and metalloendopeptidase activity. The WikiPathways enrichment identified several pathways that were significantly altered and associated with anti-inflammatory effects and the regulation of ACE2. Importantly, molecular docking analysis revealed that RA exhibited low binding energy to MMP1, MMP3, and ADAMTS4 (Fig. 2D), predicting a possible mechanism through which RA regulates metallopeptidase activity. Given RA's capacity to modulate the ACE/ACE2 balance [16], these observations offer valuable insights into the molecular mechanisms by which RA may ameliorate alcohol-associated gastric ulcers.

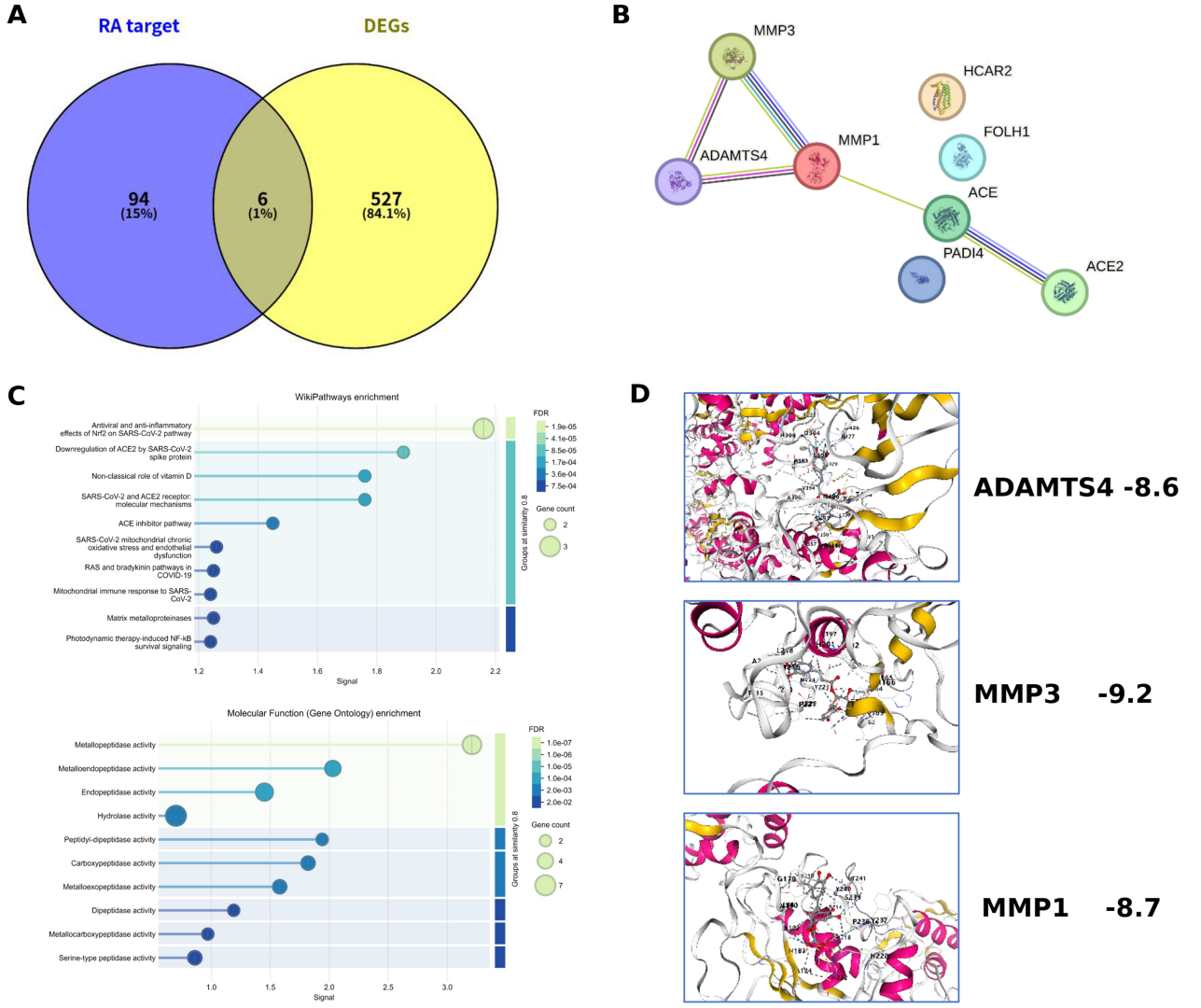

Fig. 2. Identification of potential targets between RA and gastric ulcer. (A) Venn diagram showing 6 DEGs were the potential targets of RA. The yellow circle represents 533 DEGs between groups, blue circle represents 100 potential RA target genes. (B) PPI analysis was conducted using STRING between ACE, ACE2, and the overlapped genes. (C) Wikipathway enrichment and Molecular function enrichment were analysed using STRING. (D) Molecular docking (CB-Dock2) among RA with ADAMTS4, MMP3, and MMP1. RA, Rosmarinic acid; MMP1, matrix metalloproteinase-1; MMP3, matrix metalloproteinase-3; ADAMTS4, A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motif, type 4.

3.3 RA Prevents Alcohol-Associated Gastric Ulcers in Mice

Compared with the control group, the gastric ulcer model mice exhibited significant gastric mucosal ulcers and bleeding, with a markedly elevated ulcer index (Fig. 3A, $p = 0.0048$), indicating successful model establishment. After the RA treatment at the dose of 4 or 20 mg/kg, damage to gastric mucosal bleeding and gastric ulcers was significantly relieved in a dose-dependent manner (Fig. 3A, $p = 0.0013$), which is consistent with the previous report [14]. The ulcer index and ulcer inhibition rate verified the protective effect of RA against gastric ulcers, as well as esomeprazole. Interestingly, our results demonstrated that the pH was increased after alcohol exposure when compared to the control group (Fig. 3B, $p = 0.0008$). Although RA and Esomeprazole significantly reduced the alcohol-induced pH increase, there was no significant difference in pH levels compared to the control group (Fig. 3B, $p > 0.05$). Moreover, the pepsin activity assay did not reveal any significant differences, suggesting that alcohol-induced gastric ulcers may have minimal impact on pepsin activity within a short period (Fig. 3C, $p = 0.96$).

To observe the degree of damage to the gastric tissue, macroscopic gastric biopsies were performed. As shown

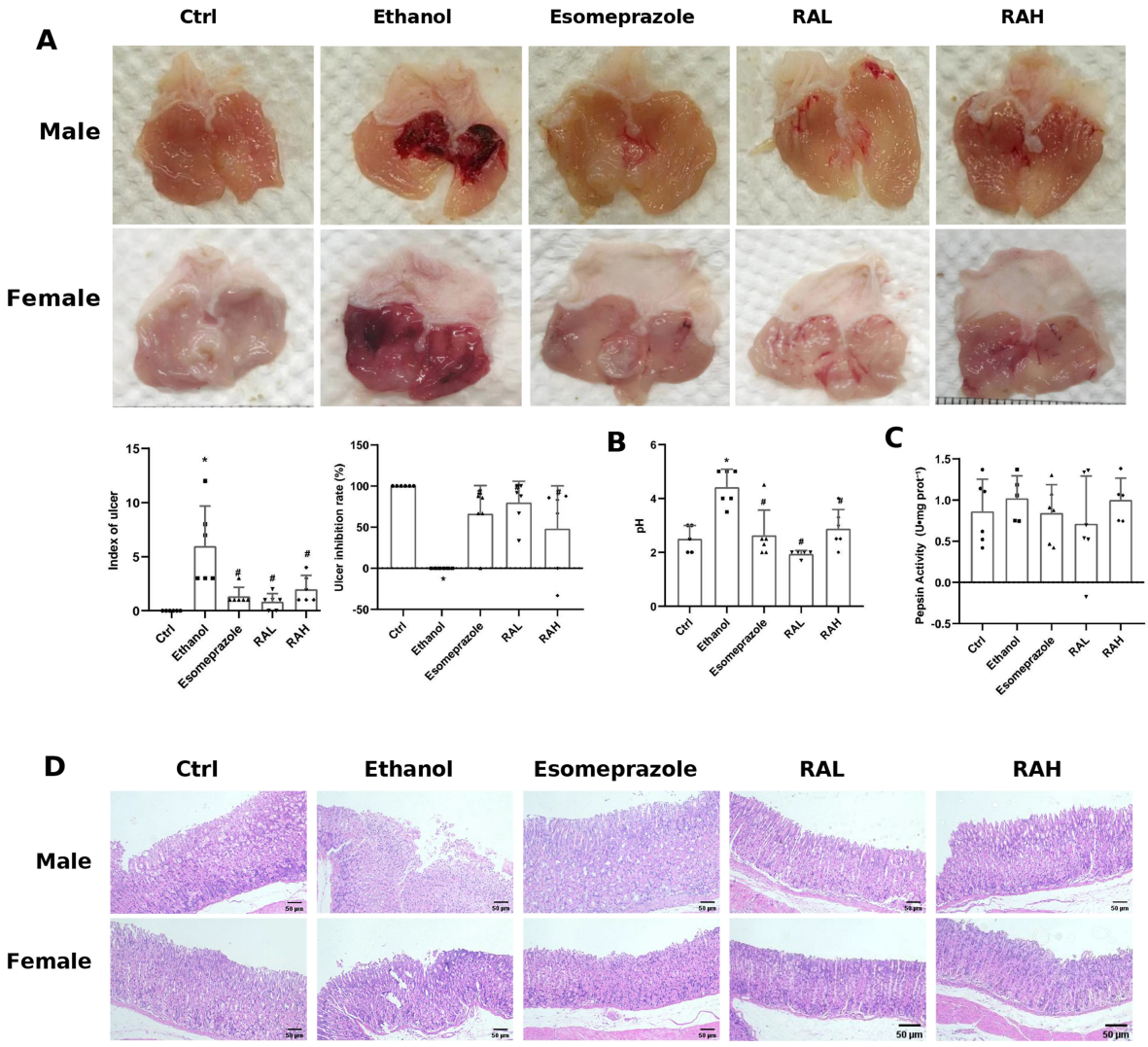

Fig. 3. Protective effect of RA on alcohol-associated gastric ulcer in mice. (A) Macroscopic images of the gastric mucosa, gastric ulcer index, and ulcer inhibition rate (n = 6). (B) pH (n = 6). (C) Pepsin activity (n = 6). (D) Representative microscopic images of the gastric mucosa by HE staining (100× magnifications). Data are means ± SD and statistically analysed by one-way analysis of variance (ANOVA) with Tukey's HSD correction (GraphPad Prism version 8.0). *$p < 0.05$, versus the Ctrl group; $^{\#}p < 0.05$, versus the Ethanol group. The scale bar: 50 μm. HE, Hematoxylin and Eosin; SD, standard deviation; HSD, Honestly Significant Difference.

in Fig. 3D, the control group mice exhibited intact gastric mucosa with well-aligned gastric glands. The base of the glands mainly consisted of chief cells, which appeared conical or columnar, with no evident hyperplasia. In contrast, the model group mice showed interrupted mucosa with ulcer formation, atrophic gastric glands, disordered gland structure, and localised necrosis. Inflammation and bleeding were observed in the mucosa and submucosa. Pretreatment with RA and Esomeprazole capsules reduced these changes in the gastric mucosa and protected against gastric mucosal injury.

3.4 Protective Effect of RA on Alcohol-Associated Gastric Ulcer via Restoring ACE/ACE2 Balance and Upregulating GPX4

Our previous research has indicated that RA's capacity to modulate the ACE/ACE2 balance [16]. Compared with the control group, Western blot revealed a significant increase in the ratio of ACE/ACE2 in the gastric tissues of ethanol-induced mice (Fig. 4A, $p = 0.05$). Pretreatment with RA at doses of 4 and 20 mg/kg significantly reduced the ratio of ACE to ACE2, whereas no significant difference was observed between esomeprazole-treated mice and those induced with ethanol (Fig. 4A, $p = 0.71$).

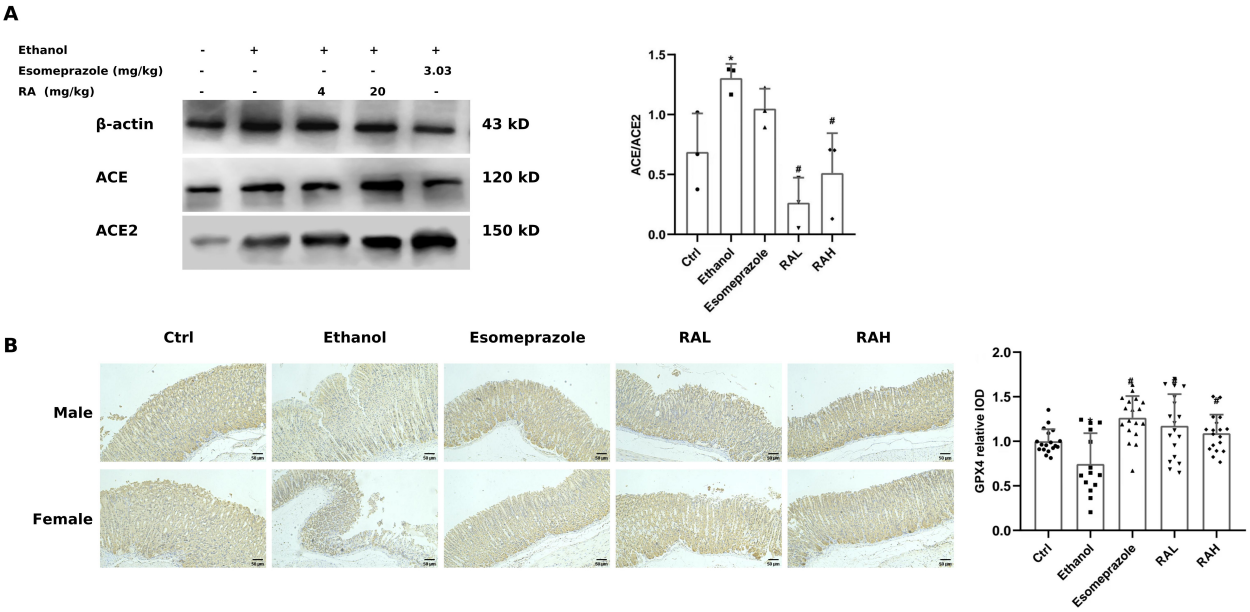

Fig. 4. RA reduced ACE/ACE2 and upregulated GPX4 in the stomach. (A) Representative Western blotting images and quantitative analysis of ACE and ACE2 in the gastric tissues. β-actin was used as an internal standard (n = 3). Data are means ± SD and statistically analysed by one-way analysis of variance (ANOVA) with Tukey's HSD correction (GraphPad Prism version 8.0). (B) Representative IHC staining of GPX4 expression in gastric tissues (100× magnifications) (n = 6). The scale bar: 50 µm. Data are means ± SD and statistically analysed by one-way analysis of variance (ANOVA) with Tukey's HSD correction (GraphPad Prism version 8.0). *$p < 0.05$, versus the Ctrl group; $^{\#}p < 0.05$, versus the Ethanol group.

Furthermore, based on the enrichment of the p53 and oxidative phosphorylation pathways in GSEA, and the fact that the RAS signalling pathway can mediate ferroptosis [16], we evaluated GPX4 through IHC staining, a key molecule involved in ferroptosis. Our data showed that GPX4 expression was markedly reduced in the model group ($p = 0.04$), while RA pretreatment significantly increased GPX4 expression (Fig. 4B, $p = 0.004$). Similarly, esomeprazole treatment also elevated GPX4 expression compared with the model group (Fig. 4B, $p = 0.001$). Moreover, no significant difference was observed between the RA and esomeprazole treatment groups (Fig. 4B).

4. Discussion

Gastric ulcer is a chronic condition resulting from damage to the gastric mucosa caused by various factors. Proton pump inhibitors (PPIs) are currently the first-line agents for treating peptic ulcers. It has been used in clinical over forty years since first developed in the 1980s [20]. However, long-term use of PPIs may result in a range of adverse reactions, including gastric polyps and enterochromaffin-like cell hyperplasia, thereby necessitating the urgent exploration of alternative therapeutic strategies. In this study, firstly, we determined that DEGs from ulcer patients were significantly enriched in alcoholism. Then, we found the targets that overlap with both RA and gastric ulcers form a PPI network with ACE and ACE2. Finally, absolute ethanol-induced gastric mucosal ulcers and bleeding, increased ACE/ACE2 ratio, and down-regulated GPX4, all of which were notably ameliorated through RA treatment. Based on these findings, it is plausible to suggest that RA may as a therapeutic intervention for the prevention of gastric ulcers.

Excessive alcohol consumption is associated with the occurrence of several gastrointestinal diseases, including ulcers and cancer [2], due to the damage to the epithelial and vascular endothelial cells of the gastric mucosa. This damage impairs the protective role of the epithelial cells, enabling gastric acid and other detrimental substances to more easily permeate the submucosa, subsequently inducing inflammation and ulcer development. Upon the entry of ethanol into cells, a substantial quantity of reactive oxygen species (ROS) is produced during the ensuing metabolic processes, thereby eliciting oxidative stress responses and ferroptosis [17,21]. Our GSEA data consistently demonstrated that DEGs were significantly enriched in pathways related to oxidative phosphorylation, gastric acid secretion, alcoholism, and the p53 signalling pathway.

Local inhibition of gastric ACE or activation of ACE2 augments gastric microvascular perfusion and enhances gastric tissue oxygenation, thereby exerting gastroprotective effects [7,8]. RA possesses potential as an ACE in-

hibitor, owing to its unique characteristics as both a phenolic acid and a flavonoid-related compound, which allow it to bind to metal ions, including the zinc ion center of ACE [15]. This binding enables the suppression of free radical generation and effectively halts oxidative chain reactions. Our study revealed that RA treatments afforded dose-dependent protection for the gastric mucosa. When subjected to acute ethanol stimulation, RA treatment markedly decreased the incidence of gastric ulcers and the prevalence of gastric mucosal bleeding, with these effects being consistent across both female and male mice. Moreover, the protective efficacy of RA was comparable to that of the positive control drug, esomeprazole. Interestingly, no notable disparities were discernible in pepsin activity, potentially owing to the experiment's comparatively short timeframe. We hypothesise that RA may augment the body's resilience to alcohol consumption, consequently mitigating the irritation induced by alcohol and safeguarding the gastric mucosa.

Ferroptosis is a type of programmed cell death triggered by lipid peroxidation and iron overload [22]. p53 serves as a crucial regulator of ferroptosis by binding to the promoter region of SLC7A11, thereby suppressing its transcriptional expression and subsequently inhibiting the expression levels and enzymatic activity of GPX4, which in turn facilitates ferroptosis [23]. Given our GSEA data, the p53 signalling pathway was found to be enriched in GU patients. We subsequently assessed the expression of GPX4, as the pivotal and distinctive biomarker of ferroptosis in gastric tissues by IHC. Consistent with our previous finding that RA can suppress the RAS-mediated ferroptosis in the lung tissues [16], in this study, RA notably augmented the expression of GPX4 in gastric tissues, concurrently with the restoration of the ACE/ACE2 balance. Importantly, molecular docking analysis revealed that RA exhibited low binding energy to MMP1, MMP3, and ADAMTS4, all of which are DEGs that form PPI networks with ACE and ACE2. These results predicted another possible mechanism of RA against GU in regulating metallopeptidase activity. Perhaps it could serve as a new avenue for future research.

However, there are many limitations in the present study. Firstly, the pathway analysis of RNA-seq results failed to directly reveal the correlation among RAS, ferroptosis, and gastric ulcers. Secondly, the molecular docking results were not validated by experiments. Finally, future studies should investigate the regulatory effects of MMP1, MMP3, and ADAMTS4 on the ACE/ACE2 balance.

5. Conclusion

Collectively, the ACE/ACE2 balance might be the potential regulatory mechanism in gastric ulcers, primarily contributing to alcoholism and the regulation of gastric acid secretion. Protective effect of RA on alcohol-associated gastric ulcer via restoring ACE/ACE2 balance and upregulating GPX4.

Availability of Data and Materials

Data will be made available on request.

Author Contributions

LZ, ZY, and LW conceived the idea and designed the study; LW wrote and revised the manuscript; TL, XZ, and TZ contributed to most of the animal and molecular biology experiments; XZ, FL, and ZZ assisted in specimen collection and participated in data analysis. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

These protocols were approved by the ethics committee of the West China Hospital of Sichuan University and 363 Hospital (No: 2024-862). The study was conducted in accordance with the principles of the Declaration of Helsinki, and written informed consent was obtained from all participants. This research was led by West China Hospital of Sichuan University, so West China Hospital of Sichuan University and 363 Hospital share the same ethical approval number. Only the West China Hospital of Sichuan University has officially issued the approval. The Animal ethics and experimental procedures were approved by West China Hospital of Sichuan University and the guidelines of the China Council on Animal Care (No. 20240308066).

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Conflict of Interest

The authors declare no conflict of interest.

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