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Original Research

Research on the Mechanisms Involving Endoplasmic Reticulum Stress in the Comorbidity of Atopic Dermatitis and Psychiatric Disorders

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Abstract

Objective: This study aimed to investigate the role of endoplasmic reticulum stress (ERS) in atopic dermatitis (AD) and its comorbid mental disorders and evaluate the therapeutic potential of mesencephalic astrocyte-derived neurotrophic factor (MANF). **Methods**: Clinical samples from patients with AD and comorbidities were analyzed for ERS and apoptosis markers using quantitative polymerase chain reaction and western blotting. Mouse models of depression were developed using 28 days of chronic unpredictable mild stress (CUMS), and dermatitis was induced using 0.2% 2,4-dinitrofluorobenzene. Adenovirus-mediated MANF overexpression was induced via local injection to develop comorbidities, comorbidities + negative control overexpression, and comorbidities + MANF overexpression groups. Further, the ERS inhibitors of 4-phenylbutyric acid were employed for the comorbidities + inhibitors group. Dermatitis scoring, the sucrose preference test for behavioral changes, and ERS and apoptosis assessments using transmission electron microscopy, terminal deoxynucleotidyl transferase dUTP Nick-End labeling staining, hematoxylin and eosin staining, and immunohistochemistry were conducted. **Results**: Clinical data demonstrated significant increases in ERS-related markers (*GRP78*, *P-IRE1*, and *Caspase-12*) and apoptosis marker of Bax, whereas Bcl-2 was decreased in comorbidities compared with those with AD alone. In mouse models, increased Bax and decreased Bcl-2 indicated apoptotic pathway activation in the comorbidities group. MANF overexpression reduced ERS-related factors and skin pathology while improving behavioral indicators. Further, MANF reduced skin cell apoptosis by downregulating Bax and upregulating Bcl-2. ERS inhibitors similarly alleviated cell apoptosis, thereby confirming the critical role of ERS in disease progression. **Conclusion**: MANF overexpression reduces ERS and apoptosis, which improves AD symptoms and comorbid mental disorders, thereby highlighting its therapeutic target potential.

Keywords: endoplasmic reticulum stress; atopic dermatitis; psychiatric disorders; apoptosis; comorbidities

1. Introduction

The endoplasmic reticulum (ER) is a crucial organelle that is connected to the nuclear envelope, which is key for lipid synthesis, Ca^{2+} regulation, and protein processing in cells. Properly folded proteins are crucial for cellular functions, making the ER vital for quality control [1]. However, conditions, such as gene mutations, nutrient deprivation, hypoxia, and oxidative stress, trigger ER stress (ERS), leading to protein misfolding in the ER, toxin stimulation, Ca^{2+} metabolic imbalance, and sustained oxidative stress stimulation [2]. Further, cells that undergo severe ERS frequently cause cell death through various pathways (e.g., apoptosis), causing significant harm to the organism [3]. To survive, cells trigger self-protection events, including upregulating ERS-related proteins PERK, IRE1, and ATF6 to refold misfolded or unfolded proteins and inhibiting ERS-induced pro-apoptosis proteins, including caspase and Bax proteins [4]. A substantial body of research indicates that ERS is closely associated with several diseases, particularly inflammation, neurodegenerative condition progression, and metabolic syndrome [5].

Atopic dermatitis (AD) is a chronic, recurring, noninfectious skin inflammation disorder characterized by continuous skin itching and eczema-like symptoms, including redness and papules, which differ in terms of age and skin dryness [6]. Chronic inflammation and scratching cause skin thickening and lichenification, and persistent itching disrupts daily activities and sleep, reduces quality of life, and leads to serious mental disorder complications (e.g., depression) and autoimmune diseases [7]. Atopic dermatitis is frequently comorbid with depression. The brain-skin axis mediates this comorbidity, and the combined chronic unpredictable mild stress (CUMS) + 2,4-dinitrofluorobenzene (DNFB) model is a validated one that recapitulates skin inflammation, pruritus, and depressive-like behaviors [8,9]. AD involves complex interactions among genetic abnormalities, altered immune responses, epidermal barrier defects, and disrupted skin microbiome, all contributing to its complexity [10]. Recent studies have demonstrated that sebaceous glands produce Th2-related mediators via interleukin-4 receptors, a process regulated by galectin-12, which also inhibits ERS and promotes salivary gland enlargement and AD-like phenotype, thereby

implying the crucial role of ERS in AD progression [11]. Previous studies have revealed that ERS plays a crucial role in regulating immune responses and skin barrier function. For instance, pathway activation during ERS leads to proteolytic cleavage of precursor ATF6 in the Golgi apparatus, thereby producing an active ATF6 fragment that translocates into the nucleus [12]. In murine models of psychiatric disorders, IRE1 pathway activation under ERS conditions promotes XBP1 mRNA splicing via its endoribonuclease activity, which generates the active transcription factor XBP1s, thereby upregulating the expression of inflammation-related genes [13]. This evidence indicated that ERS helped in AD development and progression and played a role in its mental disorder complications.

Mesencephalic astrocyte-derived neurotrophic factor (MANF), a neurotrophic factor that supports the survival of midbrain dopaminergic neurons, has been upregulated and secreted in response to ERS [14]. MANF is involved in ERS-related neurological disorders, and shows its potential as a novel research target for ERS-associated metabolic diseases [15]. Recent studies have identified key AD molecular targets and therapies, including natural ingredients regulating inflammatory/oxidative stress and endothelin receptor antagonists with immunomodulatory/anti-inflammatory effects [16,17]. However, the role of MANF in AD and psychiatric disorder comorbidities remains largely unexplored, and its precise molecular mechanisms are unclear. This study investigates ERS in AD and mental comorbidities and evaluates the regulatory potential of MANF.

2. Material and Methods

2.1 Clinical Patient Sample Collection

Clinical data of patients with AD alone or AD comorbid with mental disorders—including 6 patients in the AD-alone group and 6 patients in the AD-comorbid mental disorders group—were collected from the Second Affiliated Hospital of Harbin Medical University from July 2024 to November 2024 to investigate the features of ERS and inflammation via quantitative polymerase chain reaction (qPCR) and western blotting. The baseline clinical characteristics of all enrolled patients, including gender, age, AD disease duration, and disease severity scores (SCORAD, EASI, IGA, VAS) as well as HADS anxiety and depression scores, are summarized in **Supplementary Table 1**. This study was conducted following the Declaration of Helsinki, and all participants signed written informed consent.

2.2 Inclusion and Exclusion Criteria

Diagnostic Criteria for AD: Diagnosis was based on the Chinese Guidelines for the Diagnosis and Treatment of Atopic Dermatitis, requiring pruritus as a core symptom plus at least two of the following: flexural eczematous lesions, personal or familial history of atopic diseases (e.g., asthma, allergic rhinitis), increased serum total IgE or peripheral blood eosinophil percentage, and history of skin dryness.

Diagnostic Criteria for Comorbid Psychiatric Disorders: Two attending psychiatrists made the diagnosis based on the International Classification of Diseases, 11th Revision (ICD-11), including major depressive disorder (Hamilton Depression Rating Scale [HAMD-17] score $\geq$17) and generalized anxiety disorder (Hamilton Anxiety Rating Scale [HAMA] score $\geq$14).

Exclusion criteria included comorbidity with autoimmune skin diseases or infectious skin diseases; use of systemic glucocorticoids, immunosuppressants, or psychotropic drugs within 1 month before enrollment; presence of severe hepatic, renal, or cardiovascular diseases or malignancy; pregnancy or lactation; and inability to cooperate with sample collection.

Healthy controls were enrolled if they reported no history of skin or psychiatric diseases, had not used immunomodulatory, psychotropic, or glucocorticoid drugs in the past 3 months, and had normal indicators, including liver and renal function. A 3 mm $\times$ 3 mm normal skin tissue sample was collected from nonexposed areas of each healthy control.

2.3 Model Construction

The university ethics committee approved the *in vivo* protocol design and procedures. In this study, 4–6-week-old specific-pathogen-free BALB/c mice (Vital River, SPF level) of both sexes were randomly allocated, with 8 mice allocated to each experimental group to ensure statistical validity. Mice were randomly assigned to each experimental group using a random number table method to eliminate grouping bias. A depression model was developed via 28 days of chronic unpredictable mild stress (CUMS) [18], comprising a series of stressors applied randomly. The stressors were horizontal shaking, tilting of the cage, food and water deprivation, tail clipping, heat exposure, and cold water swimming. Each stressor was applied once per day, and the sequence was varied to prevent habituation to the stressors. The AD model was developed on day 14 of the depression model by applying a 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich, D9136) solution for sensitization. This was followed by 0.2% DNFB applications on days 5, 8, and 14 to induce dermatitis. Adenovirus-mediated MANF overexpression (negative control overexpression [oeNC] and MANF overexpression [oeMANF]) was performed via subcutaneous injection of 100 µL viral solution at the dermatitis site in the established depression model, establishing comorbidities, comorbidities + oeNC, and comorbidities + oeMANF groups. Further, the 4-phenylbutyric acid (4-PBA) as an ERS inhibitor (500 mg/kg) was administered at two separate dosages for the AD, comorbidities, and comorbidities+inhibitors [18] groups. The animal experiment was

conducted from November 2024 to January 2025, with a total duration of 3 months. The CUMS-induced depression model lasted 28 days, followed by 21 days of AD induction and intervention, with behavioral and pathological assessments conducted during the final 7 days. At the end of the animal experiment, all mice were euthanized via intraperitoneal injection of sodium pentobarbital (100 mg/kg, 50 mg/mL, Sigma-Aldrich). The skin and brain tissue were dissected within 10 min for tissue collection after euthanasia to maintain tissue integrity.

2.4 Animal Experiments Overall Evaluation

The total number of scratching bouts was counted and utilized to quantify pruritus severity. Dermatitis severity was assessed using a previously established scoring system, including five key parameters [19]: (1) edema/papules; (2) exudate/scabs; (3) epidermal erosion; (4) lichenoid change; and (5) dryness of the unaffected skin. Each item was scored on a scale (typically 0–3) according to severity, with the total dermatitis score obtained by summing the scores of all five parameters. Each parameter was scored on a scale from 0 to 3, indicating no signs or symptoms, mild manifestations, moderate severity, and severe manifestations, respectively.

The sucrose preference test (SPT) assesses an animal's emotional state by measuring its preference for sweet water, including a sucrose solution. Before testing, mice were given free access to both 1% sucrose solution and tap water. After 12 h of food and water deprivation, mice were provided with preweighed bottles containing sucrose solution and water for a 2-h testing period [20]. Fluid consumption was recorded, and the SPT was calculated utilizing the following formula:

$$\text{Sucrose Preference } (\%) = \frac{\text{Volume of sucrose solution consumed}}{\text{Total fluid intake}} \times 100$$

2.5 Quantitative PCR (qPCR)

Total RNA was extracted from treated cells or tissues using TRIzol reagent (Invitrogen, 5596026), and cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Invitrogen, N8080234, CA, USA). qPCR was performed on an ABI Step-One Plus real-time PCR system using LightCycler 480 SYBR Green I Master Mix (azyme, SGD2203, Shanghai, China). Relative RNA expression levels of *GRP78*, *P-IRE1*, *Caspase-12*, *Bax*, *Bcl-2*, *ATF4*, *CHOP*, and *P-EIF-2*α were analyzed by the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control. Primer sequences are listed in **Supplementary Table 2**.

2.6 Western Blot

Total proteins were extracted from cultured cells using RIPA lysis buffer (Sigma-Aldrich, 20-188, St. Louis, MO, USA). Protein concentration was determined using a BCA protein assay kit (Pierce™, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (30 µg) were separated by SDS-PAGE (Bio-Rad, Hercules, CA, USA) with protein markers (Beyotime, P0071, Shanghai, China; Yeason, 20350ES72, Shanghai, China), then transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk in TBST, membranes were incubated overnight at 4 °C with primary antibodies: GRP78 (Proteintech, 10768-1-AP, Rosemont, IL, USA), P-IRE1 (CST, 3294S, Danvers, MA, USA), Bax (CST, 2772S, Danvers, MA, USA), Bcl-2 (CST, 2876S, Danvers, MA, USA), ATF4 (CST, 11815S, Danvers, MA, USA), CHOP (CST, 2895S, Danvers, MA, USA), p-eIF2α (CST, 9721S, Danvers, MA, USA), and Caspase-12 (CST, 9822S, Danvers, MA, USA). Membranes were then incubated with secondary antibody (Abcam, ab205718, 1:10,000, Cambridge, UK) for 60 min. Protein bands were detected and quantified using the Odyssey Clx infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.7 Immunohistochemistry

Mice tissue samples were washed in PBS (Beyotime, ST476, Shanghai, China) for 30 min and then dehydrated through graded ethanol (Sigma-Aldrich, 459836-100ML). The tissues were cleared in xylene (Sigma-Aldrich, 339056, St. Louis, MO, USA), and stored at –20 °C. After sectioning (4 µm), sections were treated with 3% hydrogen peroxide (Sigma-Aldrich, 88597, St. Louis, MO, USA) for 10 min, followed by antigen retrieval in citrate buffer (Sigma-Aldrich, C9999, St. Louis, MO, USA) and blocking with 3% bovine serum albumin (Servicebio, GC305010-50g, Wuhan, China). Primary antibodies against Caspase-12 (Abcam, ab23352, Cambridge, UK), GRP78 (Cell Signaling Technology, 3183, Danvers, MA, USA), and P-IRE1 (Abcam, ab124945, Cambridge, UK) were incubated overnight at 4 °C. After washing, sections were incubated with secondary antibody (Cell Signaling Technology, 7074, Danvers, MA, USA) for 30 min at 37 °C. Further, 3,3'-diaminobenzidine (Sigma-Aldrich, D4293, St. Louis, MO, USA) was used for color development, followed by counterstaining with Mayer's hematoxylin (Abcam, ab220365, Cambridge, UK). Sections were dehydrated, cleared in xylene, and mounted with neutral balsam (Sigma-Aldrich, 36406, St. Louis, MO, USA).

2.8 Hematoxylin and Eosin (HE) Staining

The HE staining kit (Vectorlabs, H-3502, Burlingame, CA, USA) was used for HE staining. The fixative volume was 4–10 times the tissue volume. After fixation and dehydration, sections were trimmed, placed face down in labeled embedding cassettes, and redehydrated. Tissues were then embedded, cut into 5-µm sections, and stained with Harris HE.

2.9 Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Staining

The *In Situ* Cell Death Detection Kit, Fluorescein, which is TUNEL technology, POD (Roche, Basel, Switzerland), was performed on sections from three animals per group for tissue injury analysis in the dermatitis site. Tissues were directly analyzed under a fluorescence microscope with an excitation wavelength of 530 nm and a detection wavelength of 630 nm green.

2.10 Toluidine Blue Staining

Paraffin sections were successively immersed in xylene I and II (Selleck, 2650-17-1, Houston, TX, USA) for 10 min each. Dehydration was performed through a series of ethanol (Sigma-Aldrich, 459836-100ML) concentrations, washed with PBS (Beyotime, ST476, Shanghai, China), and stained in toluidine blue (Selleck, 92-31-9, Houston, TX, USA), followed by washing in tap water for 2 min to remove excess dye. Differentiation was performed in 95% ethanol, followed by graded ethanol dehydration, xylene clearing, and mounting.

2.11 Transmission Electron Microscopy

The samples were fixed overnight at 4 °C using PBS (Beyotime, ST476, Shanghai, China) and then washed four times with PBS. Subsequently, the samples were fixed for 2 h at 4 °C using 1% osmium tetroxide solution (Sigma-Aldrich, 251755, St. Louis, MO, USA) and then washed four times with PBS. The samples were dehydrated using an ethanol gradient. After centrifugation at 2000 g for 10 min, bacterial pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), embedded in 2% agarose, postfixed in 1% osmium tetroxide at 25 °C overnight, dehydrated with gradient ethanol, and embedded in Durcupan resin (Sigma-Aldrich, St. Louis, MO, USA). 55-nm sections were examined using a JEM-1200 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a 4-K Eagle digital camera (FEI, Hillsboro, OR, USA).

2.12 Forced Swim Test (FST)

Mice were individually placed in a water tank (24 $\pm$ 1 °C, depth 15–20 cm) for 5 min. The total immobility time was recorded. Immobility was defined as floating passively with minimal movement to maintain balance. Mice were dried and warmed immediately after testing.

2.13 Tail Suspension Test (TST)

Mice were suspended vertically by the distal 1 cm of the tail for 5 min. The immobility time was quantified, defined as the absence of active struggling movements.

2.14 Elevated Plus Maze (EPM) Test

Mice were placed in the central zone of the EPM (50 cm height) facing a closed arm and allowed to explore freely for 5 min. The percentage of time spent in the open arms was calculated as the primary index of anxiety-like behavior.

2.15 Statistical Analysis

Data are presented as mean $\pm$ standard deviation. GraphPad Prism 8.0.2 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Normality and homoscedasticity were verified (Shapiro–Wilk/Levene's tests) for parametric test eligibility. Two-group comparisons used Student's *t*-test, and multi-group comparisons used one-way ANOVA with least significant difference (LSD) post-hoc test. Confounding variables (behavioral variability, disease severity) were controlled by covariate adjustment and stratification. For multiple endpoints, false discovery rate (FDR) correction was applied to control false-positive results. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Elevated ERS Gene Expression and Enhanced Apoptosis Features in Patients With Comorbidities

The ERS gene expression in clinical patients between the AD and comorbidities groups was assessed, revealing significantly increased *GRP78*, *P-IRE1*, and *Caspase-12* expressions in the comorbidities group ($p < 0.01$) (Fig. 1A). The proapoptotic gene of *Bax* increased in the comorbidities group, whereas the anti-apoptosis gene of *Bcl-2* significantly decreased compared with the AD group ($p < 0.05$) (Fig. 1B). Meanwhile, the results of western blot and IHC revealed the significantly increased proteins of GRP78, P-IRE1, and Caspase-12 in the comorbidities group ($p < 0.05$) (Fig. 1C,D). At protein expression levels, Bax was higher in the comorbidities group, whereas Bcl-2 was significantly decreased ($p < 0.01$) (Fig. 1E), indicating that the activated apoptosis pathway may be aggravating underlying cell death in patients with comorbidities.

3.2 MANF Overexpression Improved Behavior and Skin Lesions in the Comorbidities Group

Subsequently, the behavioral and dermatopathological alterations were assessed in the comorbidities, comorbidities + oeNC, and comorbidities + oeMANF groups. The SPT revealed that, by day 28, the behavioral dermatitis scoring presented that the comorbidities + oeMANF group demonstrated milder dermatitis symptoms, with significantly lower dermatitis scores compared with the comorbidities group ($p < 0.01$) (Fig. 2A,B, Table 1). The oeMANF treatment group significantly increased the SPT compared with the comorbidities group, indicating an improvement in behavioral symptoms ($p < 0.01$) (Fig. 2C). HE staining results indicated reduced skin pathology and decreased inflammatory response in the oeMANF treatment group compared with the other two groups (Fig. 2D). Further, toluidine blue staining demonstrated a marked mast cell infiltration reduction and skin hypertrophy alleviation in the oeMANF treatment group (Fig. 2E).

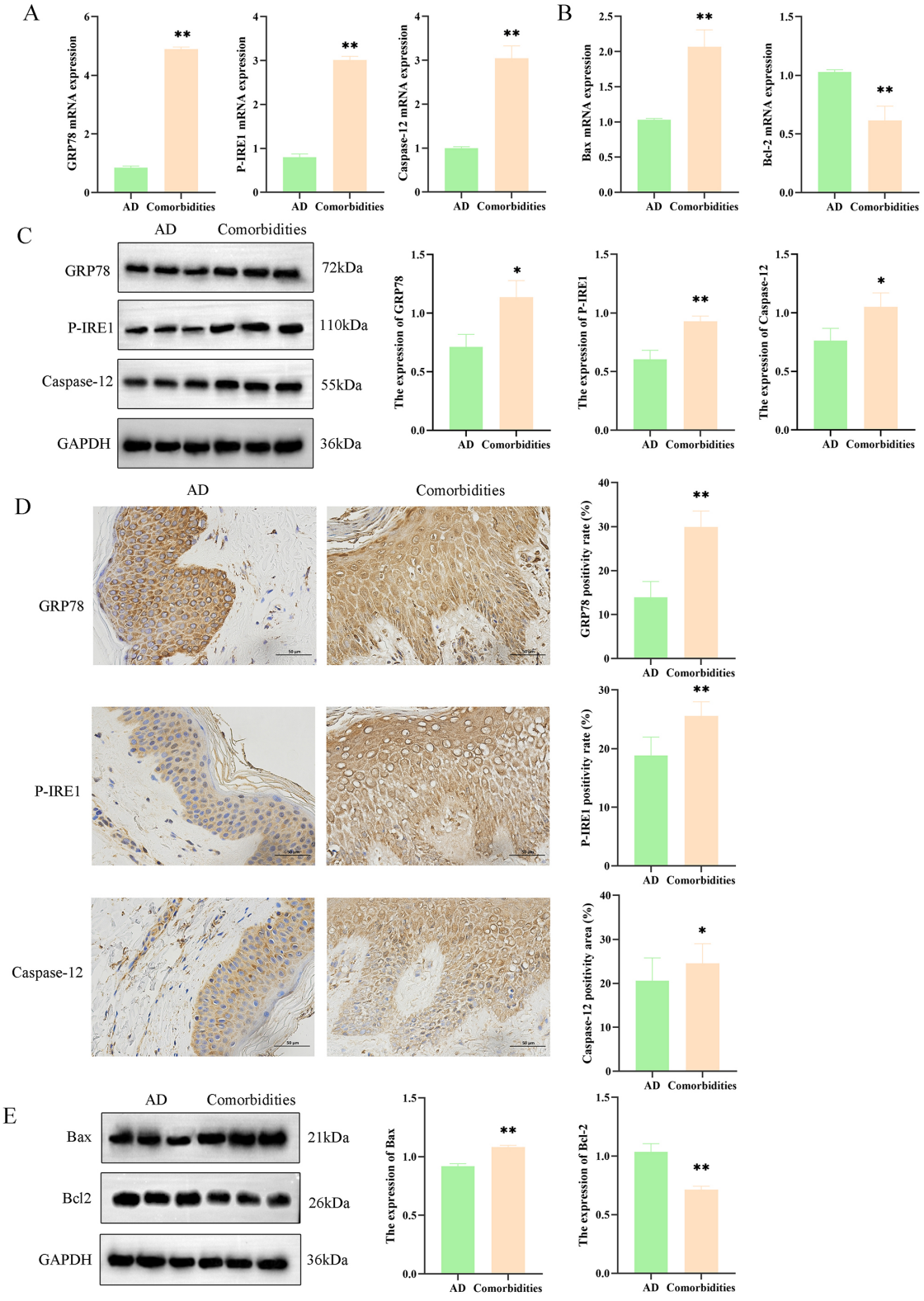

Fig. 1. Endoplasmic reticulum stress and apoptosis are increased in atopic dermatitis (AD) patients with psychiatric comorbidities. (A) mRNA levels of *GRP78*, *P-IRE1*, and *Caspase-12* in skin tissues from AD and comorbid AD patients. (B) mRNA levels of *Bax* and *Bcl-2* in the two patient groups. (C) Protein levels of GRP78, P-IRE1, and Caspase-12 detected by Western blot. (D) Immunohistochemistry of ER stress markers (scale bar = 50 μm) and quantitative analysis. (E) Protein levels of Bax and Bcl-2. Data are mean ± SD (n = 6). *$p < 0.05$, **$p < 0.01$.

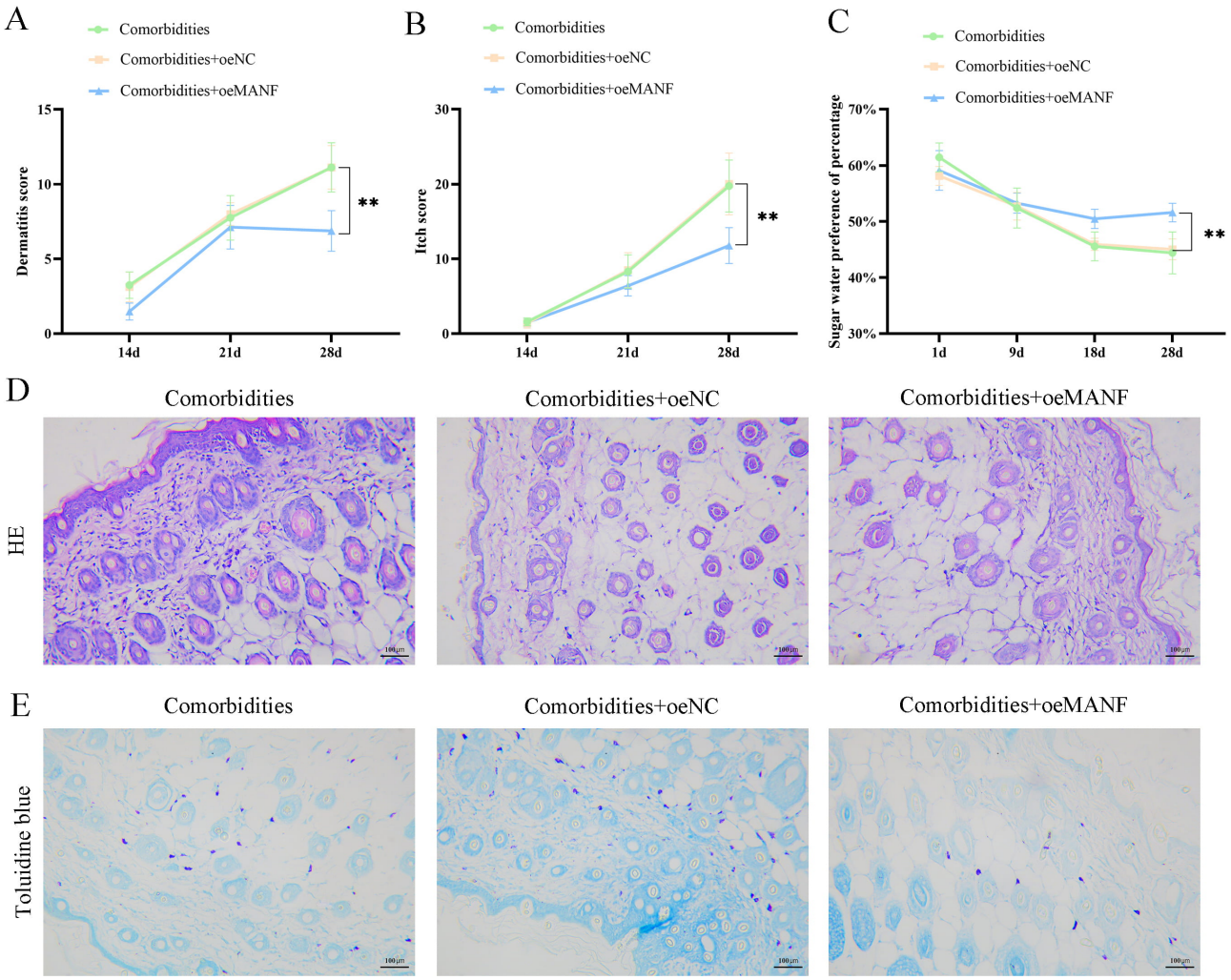

Fig. 2. Mesencephalic astrocyte-derived neurotrophic factor (MANF) overexpression improved behavior and skin lesions in comorbidities. (A) Dermatitis severity scores in comorbid, comorbid + negative control overexpression (oeNC), and comorbid + MANF overexpression (oeMANF) mice. (B) Scratching bouts in each group. (C) Sucrose preference test for depressive-like behaviors. (D) Hematoxylin and eosin (HE) staining of mouse skin tissues (scale bar = 100 µm). (E) Toluidine blue staining for mast cell infiltration (scale bar = 100 µm). Data are mean ± SD (n = 6). $^{**}p < 0.01$.

3.3 MANF Overexpression Alleviated ERS and Improved Pathology in Comorbidities Models

The underlying regulatory role of MANF in skin changes in comorbid mice under ERS and its associated pathways were explored. The oeMANF significantly reduced the expression of ERS-related factors *GRP78*, *P-IRE1*, and *Caspase-12* at both mRNA and protein levels in an AD mouse model with comorbid mental disorders (comorbidities) ($p < 0.01$) (Fig. 3A,B). Specifically, compared with the control group (comorbidities) and the empty vector control group (comorbidities + oeNC), the oeMANF group demonstrated a significant decrease in the expression of these markers (all p-values < 0.001 or lower), indicating that MANF may have a protective effect against ERS, thereby decreasing cell damage or death caused by such stress. Further, immunohistochemical analysis revealed the significantly decreased GRP78, P-IRE1, and Caspase-12 protein in dermatitis tissues ($p < 0.01$) (Fig. 3C–E). In another group of ERS-related factors, qPCR demonstrated the significantly decreased *ATF4*, *CHOP*, and *P-EIF-2*α expression ($p < 0.01$) (Fig. 4A). Further, western blot results revealed a significant reduction of these protein levels in the comorbidities + oeMANF group ($p < 0.01$) (Fig. 4B). The protein reduction is associated with ERS alleviation and improved AD with mental comorbidities. Finally, regarding changes in the morphology of ER, our electron microscopy results indicated significant expansion of the ER in the comorbidities group. Conversely, the swelling of the rough ER in the comorbidities + oeMANF group was significantly improved, with the ER morphology approaching normal and demonstrating a more elongated, tubular structure (Fig. 4C). Notably, quantitative morphometric analysis

Table 1. The detailed results of dermatitis scoring.

Condition	Edema/Papules	Exudate/Scabs	Epidermal erosion	Lichenoid change	Dryness (unaffected skin)
Comorbidity 1	2	3	3	2	3
Comorbidity 2	2	3	3	2	2
Comorbidity 3	2	3	2	2	2
Comorbidity 4	2	2	2	2	2
Comorbidity 5	3	3	2	3	2
Comorbidity 6	2	2	2	3	2
Comorbidity 7	2	2	3	2	2
Comorbidity 8	2	2	2	1	1
Comorbidity + oeNC1	2	2	3	2	2
Comorbidity + oeNC2	2	3	3	3	2
Comorbidity + oeNC3	2	3	3	3	2
Comorbidity + oeNC4	2	2	3	2	2
Comorbidity + oeNC5	2	2	2	2	2
Comorbidity + oeNC6	2	2	2	1	2
Comorbidity + oeNC7	2	3	3	2	2
Comorbidity + oeNC8	2	2	2	2	2
Comorbidity + oeMANF1	2	2	2	2	1
Comorbidity + oeMANF2	1	2	2	1	1
Comorbidity + oeMANF3	1	1	2	1	1
Comorbidity + oeMANF4	1	2	2	2	1
Comorbidity + oeMANF5	1	1	2	1	1
Comorbidity + oeMANF6	2	2	2	1	1
Comorbidity + oeMANF7	1	1	1	1	1
Comorbidity + oeMANF8	1	1	2	1	1

of ER morphology was not performed in this study; only qualitative evaluation based on transmission electron microscopy (TEM) images was conducted. The reduction in these protein levels indicates that oeMANF alleviates ERS, protects cells, and improves AD and comorbid mental disorders.

3.4 MANF Reduced Apoptosis in Comorbidities Models

Further analysis of apoptosis in mouse skin tissue revealed significantly lower skin apoptosis levels in the comorbidities + oeMANF group than in the comorbidities group ($p < 0.01$) (Fig. 5A). Further, qPCR results indicated a significant reduction in Bax gene expression ($p < 0.01$) (Fig. 5B) and a significant increase in *Bcl-2* gene expression in the comorbidities + oeMANF group. Conversely, no significant changes in the two genes were observed in the comorbidities + oeNC group. Further western blot assays exhibited a significant reduction in Bax protein expression ($p < 0.05$) and a significant increase in Bcl-2 protein in the comorbidities + oeMANF group ($p < 0.01$) (Fig. 5C). The IHC results revealed no significant differences in protein expression in the comorbidities + oeNC group relative to the comorbidity group. In addition, the IHC results revealed significant changes in the expression of Bax and Bcl-2 proteins in the comorbidities + oeMANF group compared with the comorbidity group (Fig. 5D).

3.5 An ERS Inhibitor Reduced Apoptosis in Comorbidities Models

To investigate the regulatory effects of ERS on the apoptotic pathway proteins in the skin tissues of comorbid mice, we first used the TUNEL staining to observe skin tissue apoptosis. Skin apoptosis levels were higher in the comorbidities group compared with the AD group ($p < 0.05$) (Fig. 6A) and markedly reduced in the comorbidities + inhibitors group. Subsequently, qPCR results revealed that *Bax* was increased in the comorbidities group, whereas *Bcl-2* was decreased ($p < 0.01$) (Fig. 6B), compared with the AD group. However, *Bax* was decreased, whereas *Bcl-2* was increased in the comorbidities + inhibitors group, consistent with the results in the western blot assay ($p < 0.05$) (Fig. 6C). Further IHC analysis revealed that Bax protein expression was significantly increased, whereas Bcl-2 expression was significantly decreased in the comorbidities group ($p < 0.05$) (Fig. 6D). Compared with the AD group, the comorbidities + inhibitors group demonstrated a decrease in Bax protein and an increase in Bcl-2 protein.

3.6 MANF Suppresses IRE1α-Mediated ER Stress and Apoptosis in Both Skin and Brain Tissues

To explore how MANF regulates ER stress and apoptosis in skin and brain tissues, rescue experiments were performed in four groups: AD, Comorbidity, Comorbidity + oeMANF, and Comorbidity + oeMANF + oeIRE1α.

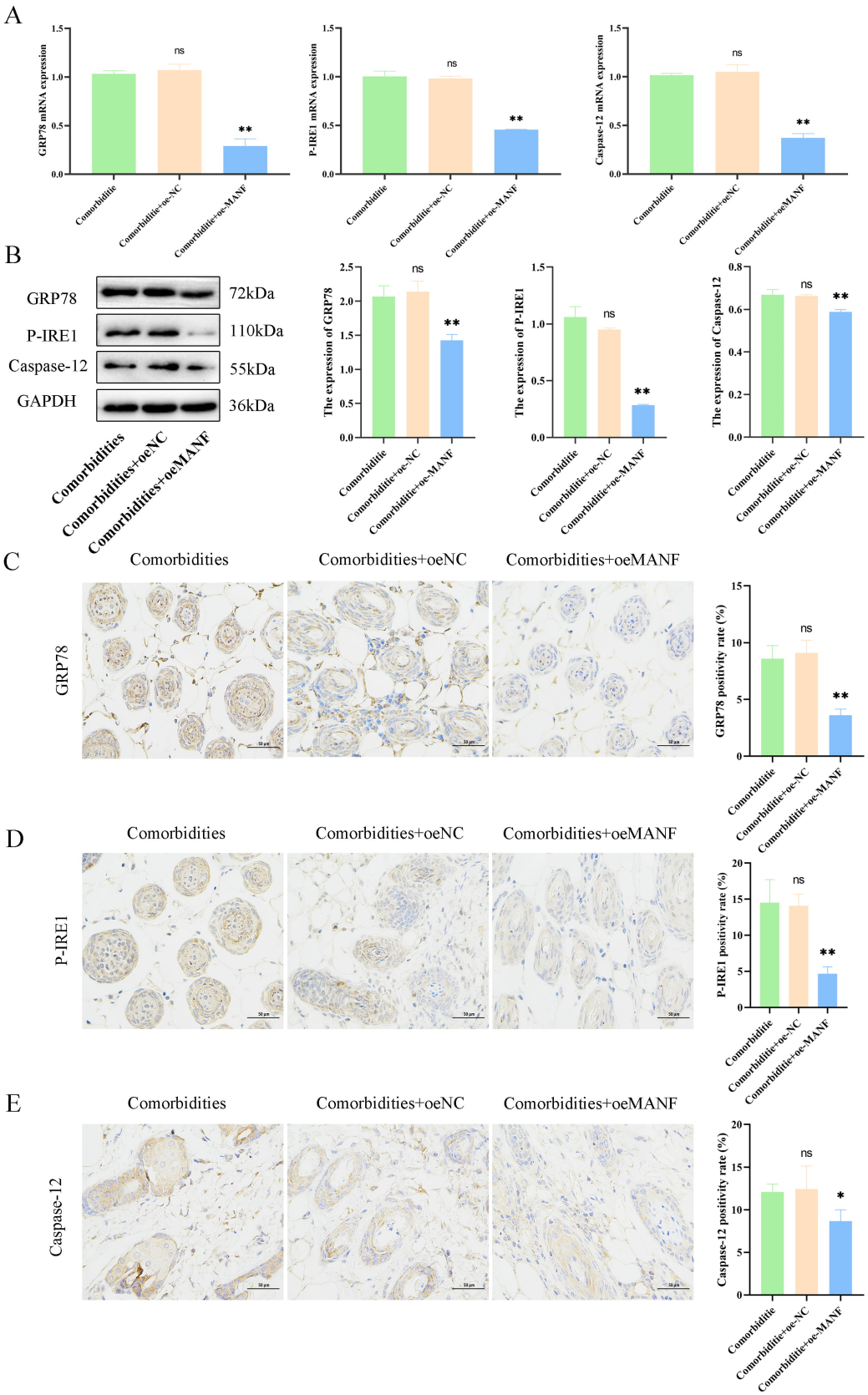

Fig. 3. MANF overexpression alleviated endoplasmic reticulum stress (ERS) and improved pathology in comorbidities models. (A) mRNA levels of *GRP78*, *P-IRE1*, and *Caspase-12* in mouse skin. (B) Protein levels of ER stress-related markers. (C–E) Immunohistochemistry and quantification of GRP78, P-IRE1, and Caspase-12 (scale bar = 50 μm). Data are mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ns, no significant difference.

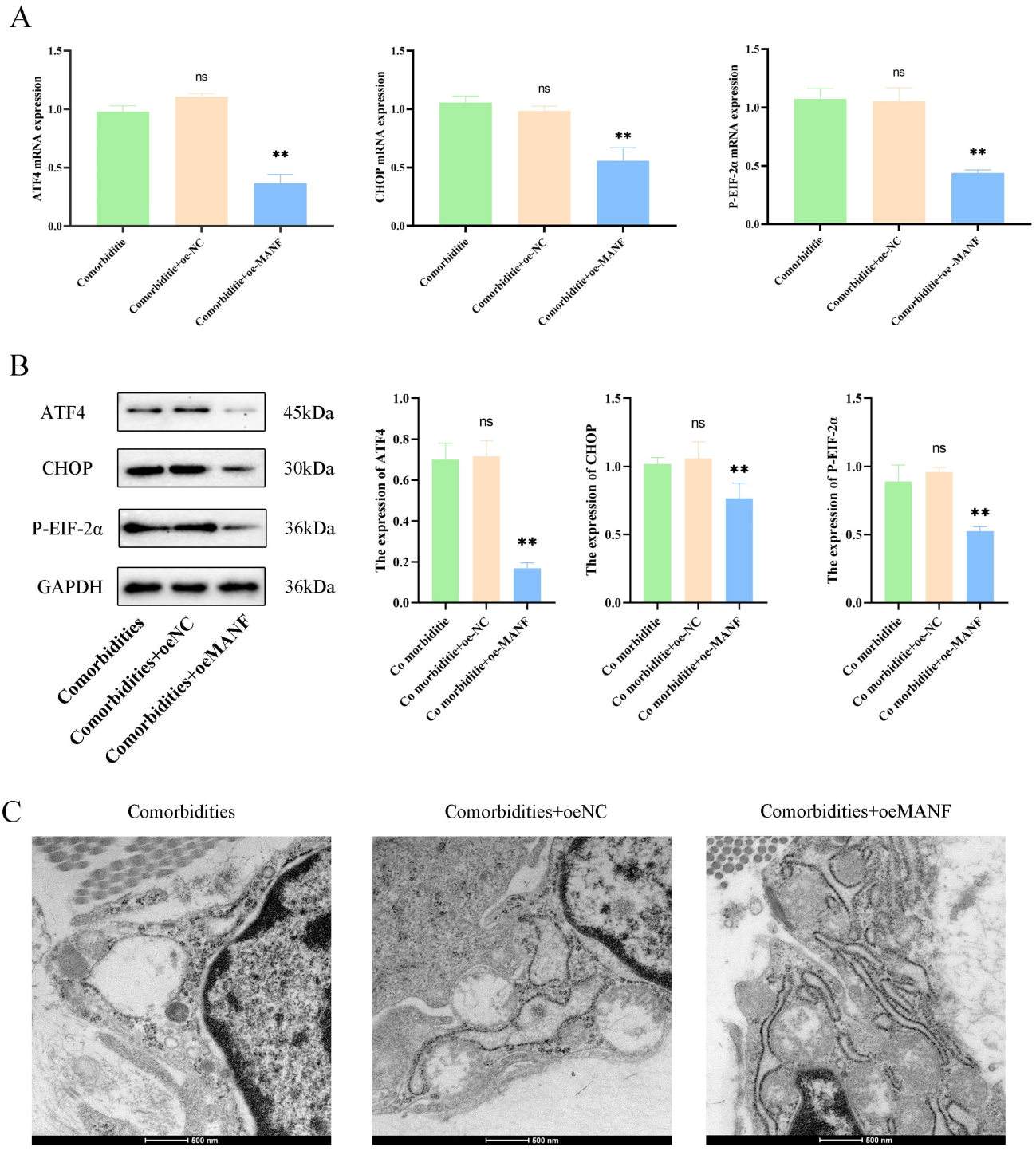

Fig. 4. MANF overexpression alleviated ERS and improved pathology in comorbidities models. (A) mRNA levels of ERS marker protein (*ATF4*, *CHOP*, and *P-eIF2α*) expression levels. (B) Protein of ERS pathway protein expression (ATF4, CHOP, and P-eIF2α). (C) Transmission electron microscopy analysis of ER morphology in skin tissue of different groups (scale bar = 500 nm). Data are mean ± SD (n = 3). $^{**}p < 0.01$, ns, no significant difference.

In skin tissues, the Comorbidity group showed markedly upregulated caspase-12, GRP78, and p-IRE1α (Fig. 7A). MANF overexpression significantly reduced these ER stress markers, whereas IRE1α overexpression abolished this effect. MANF overexpression also improved behavioral performance (Fig. 7B–D) and reduced dermatitis scores and scratching frequency (Fig. 7E), which were reversed by IRE1α overexpression.

In skin tissues, the Comorbidity group displayed severe morphological injury, increased mast cell accumulation, and elevated apoptosis (Fig. 8A–C). MANF overexpression alleviated these pathological changes. Meanwhile,

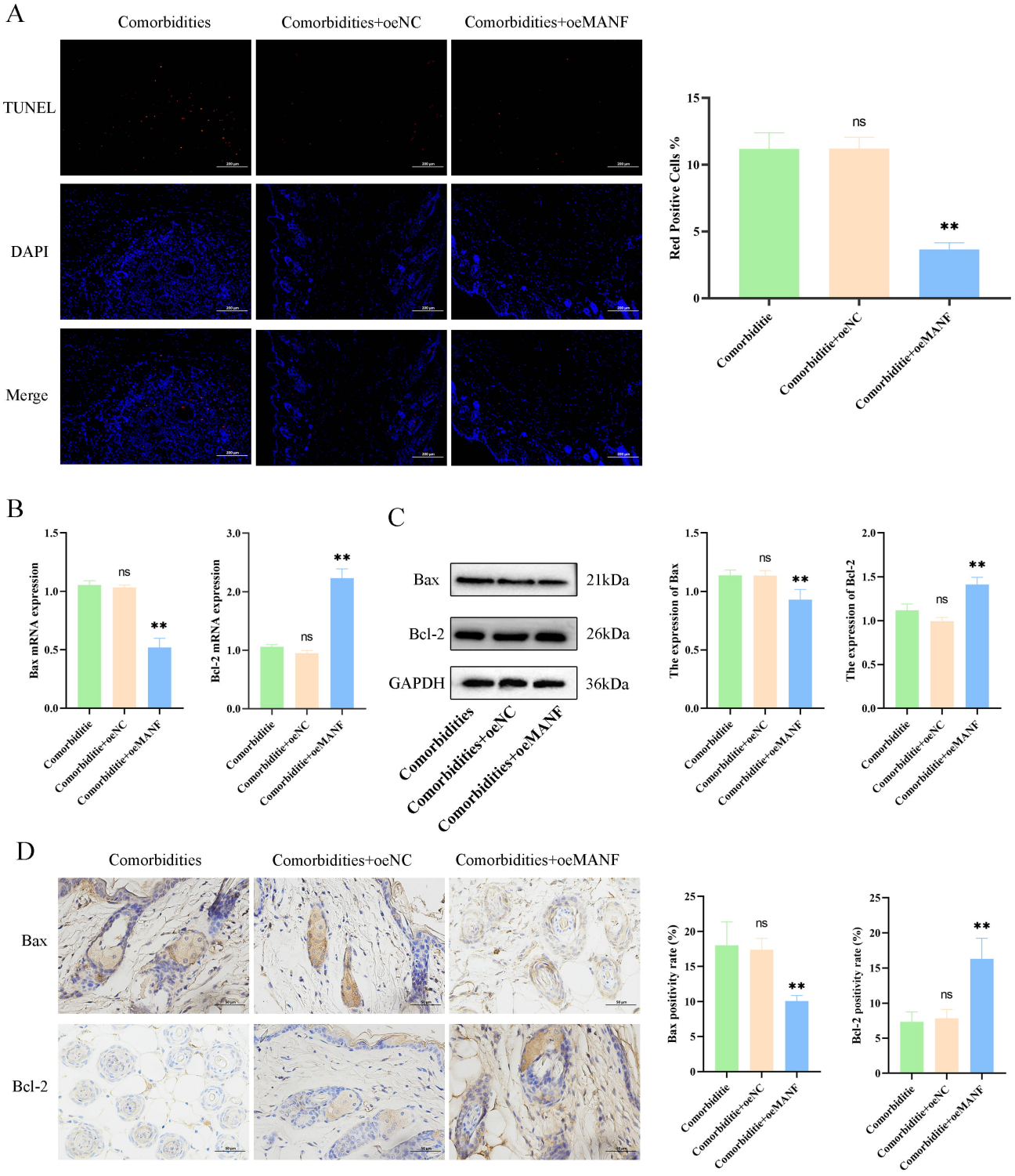

Fig. 5. MANF overexpression reduces skin cell apoptosis in comorbid mice. (A) Terminal deoxynucleotidyl transferase dUTP Nick-End labeling (TUNEL) staining and apoptotic index (scale bar = 200 µm). (B) mRNA levels of *Bax* and *Bcl-2*. (C) Protein levels of Bax and Bcl-2. (D) Immunohistochemistry and quantification of Bax and Bcl-2 (scale bar = 50 µm). Data are mean ± SD (n = 3). $^{**}p < 0.01$, ns, no significant difference.

the ratios of cleaved caspase-3/pro caspase-3 (Fig. 8D) and Bax/Bcl-2 (Fig. 8E) were increased in the Comorbidity group; MANF overexpression normalized these ratios, and these effects were blocked by IRE1α overexpression.

In brain tissues, the Comorbidity group exhibited elevated caspase-12, GRP78, and p-IRE1α (**Supplementary Fig. 1A**), increased cleaved caspase-3 (**Supplementary Fig. 1B**), and a higher Bax/Bcl-2 ratio (**Supplementary**

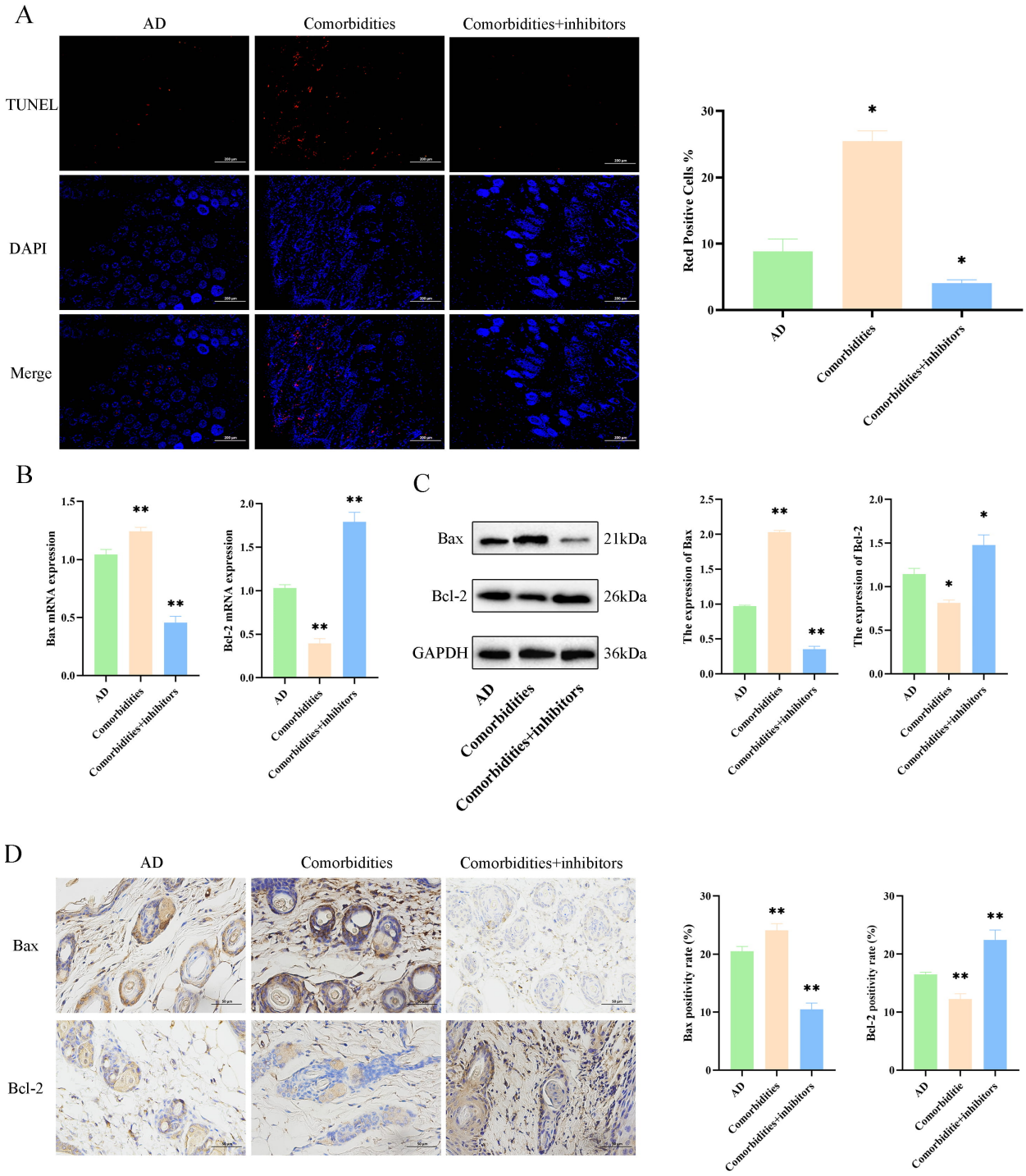

Fig. 6. 4-phenylbutyric acid (4-PBA)-mediated inhibition of ER stress reduces apoptosis in comorbid mice. (A) TUNEL staining and apoptotic index (scale bar = 200 μm). (B) mRNA levels of *Bax* and *Bcl-2*. (C) Protein levels of Bax and Bcl-2. (D) Immunohistochemistry and quantification of Bax and Bcl-2 (scale bar = 50 μm). Data are mean ± SD (n = 3). $*p < 0.05$, $**p < 0.01$.

Fig. 1C). MANF overexpression significantly ameliorated these changes, which were fully reversed by IRE1α overexpression.

Collectively, MANF inhibits IRE1α-mediated ER stress and apoptosis in both skin and brain tissues, thereby alleviating skin lesions and psychiatric disorders in the comorbid mouse model.

4. Discussion

AD pathogenesis is complex involving genetics, immunity, and environment, and recent studies indicate that ERS aggravates skin damage and promotes psychiatric symptoms by regulating immunity, apoptosis, and inflammation [21]. Grounded on this understanding, this study constructed a mouse AD model with comorbid psychiatric

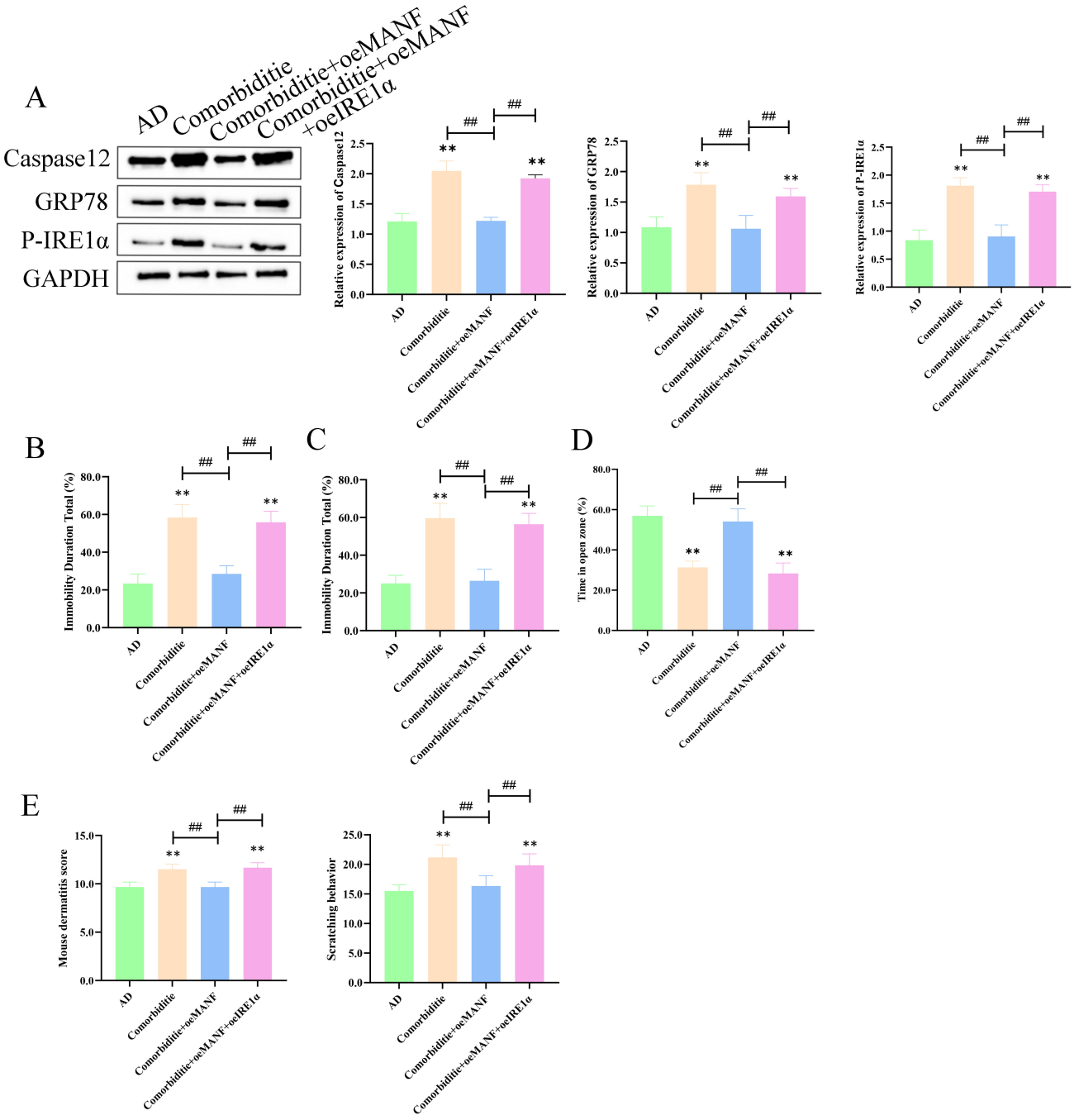

Fig. 7. MANF suppresses IRE1α-mediated ER stress and improves behavioral phenotypes. (A) Protein levels of Caspase-12, GRP78, P-IRE1α in mouse skin tissues. (B) Forced swim test for depressive-like behaviors. (C) Tail suspension test for depressive-like behaviors. (D) Elevated plus maze test for anxiety-like behaviors. (E) Dermatitis severity scores and scratching frequency in each group. Data are mean $\pm$ SD (n = 3). $^{**}p < 0.01$, $^{\#\#}p < 0.01$.

disorders to analyze the role of ERS in this process and explore the potential role of MANF in regulating ERS.

In this study, ERS-related proteins (GRP78, P-IRE1, Caspase-12) were significantly upregulated, particularly in AD patients with psychiatric disorders ($p < 0.01$). GRP78, a key ER chaperone, mediates protein folding and initiates ERS upon unfolded protein accumulation [22]. Previous studies have pointed out that GRP78 plays a crucial role in skin inflammation and immune responses [23]. GRP78 in both the skin and immune cells of patients with AD, with its expression correlating positively with skin inflammation severity [24]. Further, excessive activation of GRP78 exacerbates AD by regulating skin immunity and promoting inflammatory factor release; P-IRE1, a key ERS sensor, enhances immune inflammation via the IRE1/XBP1 pathway and mediates skin immune damage [25]. In AD, overacti-

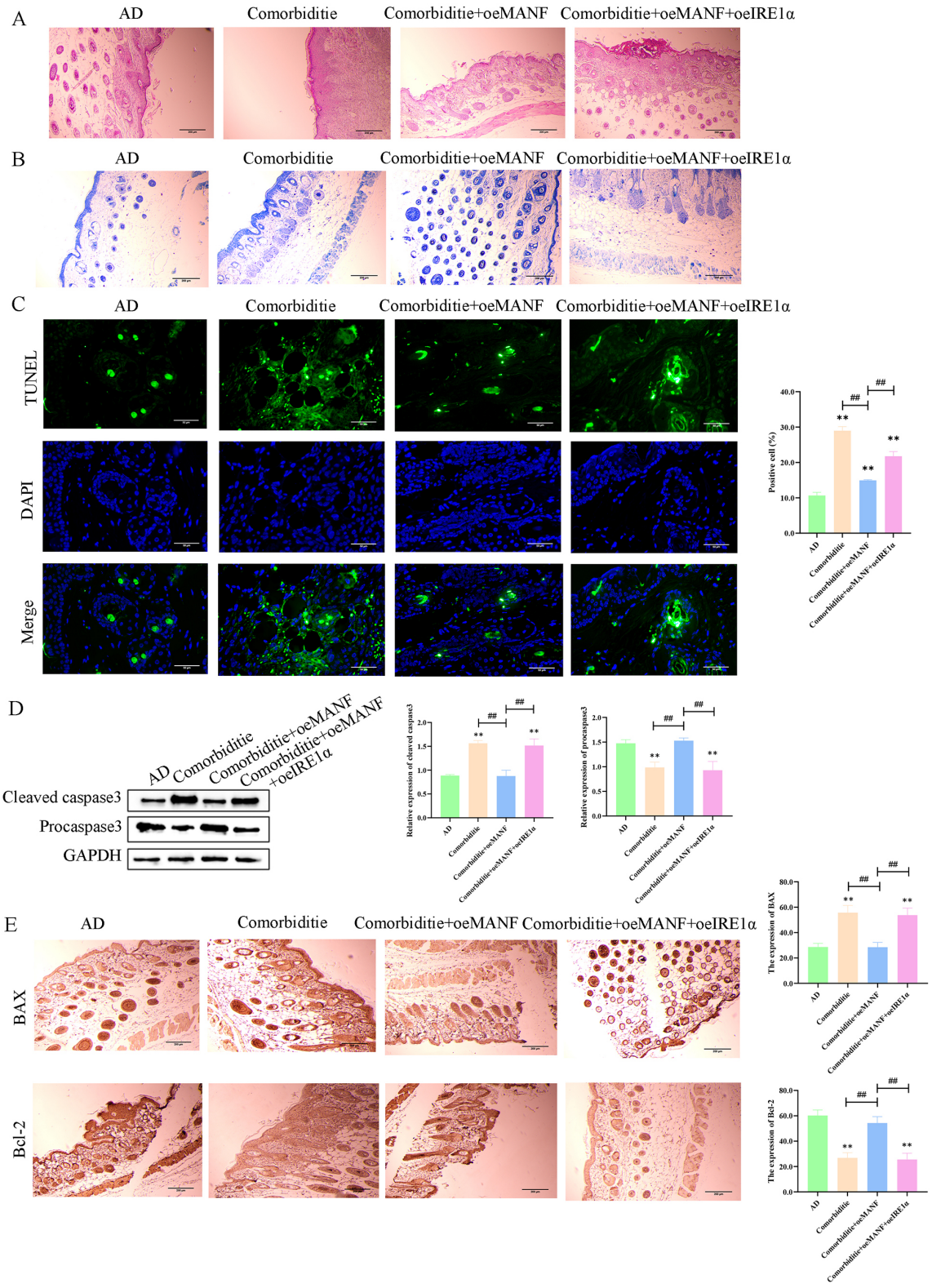

Fig. 8. MANF alleviates skin pathology and inhibits apoptosis via IRE1α. (A) HE staining of mouse skin tissues (scale bar = 200 µm). (B) Toluidine blue staining of mast cells (scale bar = 200 µm). (C) TUNEL staining of apoptosis (scale bar = 50 µm). (D) Protein levels of pro-caspase 3 and cleaved-caspase 3 detected by Western blot. (E) Immunohistochemistry and quantification of Bax and Bcl-2 in skin tissues (scale bar = 200 µm). Data are mean ± SD (n = 3). $^{**}p < 0.01$, $^{\#\#}p < 0.01$.

vated P-IRE1 aggravates skin inflammation and triggers or worsens psychiatric symptoms through systemic inflammatory responses. As an ERS-related pro-apoptotic protein, highly expressed Caspase-12 promotes apoptosis of skin, neuronal and immune cells, exacerbating tissue injury and psychiatric disorders [26]. Overall, our results revealed the activated ERS pathway affecting AD with comorbid psychiatric disorders.

ATF4, CHOP, and P-eIF2α form a critical signaling pathway in the ERS response for another group of ERS-related factors, thereby managing cellular adaptation and apoptosis [27]. During ERS, phosphorylation of eIF2α inhibits global protein synthesis to relieve ER stress and promotes ATF4 translation. As a transcription factor, ATF4 supports cell survival under mild stress but induces apoptosis under severe stress [28]. CHOP, a key downstream target, increases under persistent ERS, causing mitochondrial dysfunction, oxidative stress elevation, and proapoptotic protein upregulation, leading to apoptosis [29]. This pathway helps cells adapt to short-term stress and, if recovery fails, guides them toward programmed cell death [27]. The activation of the PERK-mediated CHOP pathway has a detrimental effect in rats and HK-2 cells by promoting apoptosis (Bax), whereas this cytotoxic effect was inhibited by the ERS inhibitor of 4-PBA with lower PERK, eIF2α, CHOP, and ATF4 expression levels [30]. Importantly, our results demonstrated the crucial role of ERS in regulating AD in the mental disorders model.

ERS inhibitors (4-PBA) effectively regulate Bax/Bcl-2 expression to reduce skin cell apoptosis, offering a novel strategy for ER-related skin diseases; Bax promotes apoptosis while Bcl-2 exerts anti-apoptotic effects [31]. Our results showed abnormal apoptosis-related protein expression in AD mice with psychiatric disorders, characterized by significantly upregulated Bax and downregulated Bcl-2. Previous studies confirmed that ERS promotes apoptosis via regulating Bax/Bcl-2, thus exacerbating tissue injury [32]. This indicates that in the AD mouse model, ERS may lead to excessive skin cell apoptosis by promoting Bax activation, thereby exacerbating skin inflammation and barrier dysfunction. Finally, this study confirmed that MANF significantly improved skin damage and immune responses in the AD with psychiatric disorders mouse model. Specifically, similar to the clinical sample results, GRP78, P-IRE1, and Caspase-12 expression was significantly reduced, thereby alleviating ERS responses, with MANF playing an intermediary regulatory role. Studies have demonstrated that P-IRE1 plays a driving role in the pathological process of AD, particularly in exacerbating local inflammation by regulating immune cell function [33]. Another study revealed that MANF interacts with ER sensor IRE1α, which reduces unfolded protein response (UPR) signaling and protects neurons from ERS-induced death, which is crucial for neuronal survival *in vitro* and in Parkinson's disease models [34]. In this study, it alleviates skin damage and immune responses in the AD with psychiatric disorders mouse model by weakening the excessive immune response activation, thereby improving skin barrier function. MANF overexpression for apoptotic proteins in this study reduced apoptosis by regulating the Bax/Bcl-2 ratio and promoted the recovery of skin tissue. Similar studies have revealed that MANF alleviates ERS in AD mice, inhibits skin cell apoptosis, and improves skin barrier function. This result can be disrupted by adding a MANF inhibitor. Uniquely, MANF is involved in restoring ER morphology. Our results indicate the significantly improved ER morphology in the comorbidities + oeMANF group mice, displaying more elongated tubular structures, close to normal morphology. Moreover, from a behavioral perspective, MANF alleviated behavioral changes in the depression mouse model and improved their neurological functions. The study reveals that MANF alleviates ERS, improves pathology, and reduces apoptosis via IRE1α, thereby providing a new AD and mental disorder treatment target. Systemic administration can serve as a secondary option for severe cases with widespread skin lesions and prominent psychiatric symptoms. Given the systemic immune disorder and cross-tissue pathological link of the comorbidity [35], systemic delivery can simultaneously modulate ER stress and inflammatory responses in both skin and central nervous system, which may produce a synergistic therapeutic effect on both AD lesions and depressive-like behaviors.

5. Limitations

A limitation of this study is that 4-PBA has broad chaperone activity, and potential non-specific effects cannot be completely excluded despite its well-characterized role as an ER stress inhibitor. However, insufficient recruitment of eligible healthy volunteers precluded molecular marker testing and group comparisons, reducing conclusion specificity. Moreover, only 6 cases per clinical group (due to the difficulty in screening AD patients with psychiatric comorbidities) led to insufficient statistical power and limited generalizability. Future studies will expand recruitment, conduct multicenter research to supplement healthy control data and increase sample size, thereby enhancing study rigor and conclusion applicability. Further, expression changes of ERS-related factors in skin tissues were only evaluated at the whole-tissue level in this study, with no identification of their specific cellular sources via techniques. This precluded accurate cellular target localization for MANF-mediated ERS regulation and hindered in-depth clarification of MANF's molecular mechanisms. Future studies are recommended to employ cell-specific detection techniques to address this gap.

6. Conclusion

In summary, our study indicates that changes in the ERS and apoptosis pathways in patients with AD and psychiatric disorders may play a crucial role in disease pro-

gression. MANF, as an ERS-related protein, helps improve skin damage and immune abnormalities in AD and comorbid psychiatric disorder mice by regulating these pathways.

Availability of Data and Materials

The datasets generated and analyzed during the current study are not publicly available due to ethical restrictions, but will be made available on reasonable request from the corresponding author.

Author Contributions

HC: Data curation, Formal analysis, Writing—original draft, Writing—review and editing; JZ, YG: Conceptualization; GY: Data curation; YZ: Obtaining funding, performed the research; SH and YL: Investigation, analyzed the data. 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

The animal experiment was approved by the Second Affiliated Hospital of Harbin Medical University (YJSDW2024-001); The clinical research was approved by The Second Affiliated Hospital of Harbin Medical University (YJSKY2024-277). All animal procedures were strictly performed in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of China, as well as the ARRIVE guidelines and international animal welfare standards. All efforts were made to minimize animal suffering and the number of experimental animals used. The study was carried out in accordance with the guidelines of the Declaration of Helsinki, and written informed consent was obtained from all participants.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL47821.

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