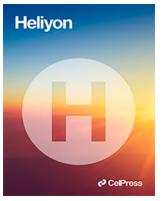
Heliyon 10 (2024) e39987

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Guilu Erxian glue reduces endoplasmic reticulum stress-mediated apoptosis and restores the balance of extracellular matrix synthesis and degradation in chondrocytes by inhibiting the ATF6/GRP78/CHOP signaling pathway

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ARTICLE INFO

Keywords:
Guilu Erxian glue
Chondrocyte
Apoptosis
Knee osteoarthritis
ATF6/GRP78/CHOP signaling pathway

ABSTRACT

Knee Osteoarthritis (KOA) is characterized by phenotypic alterations, apoptosis, and the breakdown of the extracellular matrix (ECM) in the superficial articular cartilage cells. The inflammatory response activates the Endoplasmic Reticulum Stress (ERS) signaling pathway, which plays a critical role in the pathophysiology and progression of KOA. Chondrocytes stimulated by thapsigargin(TG)exhibit heightened ERS and significantly increase the expression of ERS-associated proteins. Key mediators of ERS-induced apoptosis include X-box-binding protein 1 (XBP1), elevated levels of the protein transport protein Sec61 subunit (SEC61), and C/EBP homologous protein (CHOP).

While the precise mechanism of action of Guilu Erxian Glue (GEG), a medication commonly used in the clinical treatment of KOA, remains to be fully elucidated, our research has shown that GEG mitigates the imbalance between ECM synthesis and degradation, as well as chondrocyte apoptosis resulting from ERS. This effect is likely achieved through the suppression of the Activating Transcription Factor 6 (ATF6)/Glucose-Regulatory Protein 78 (GRP78)/CHOP signaling pathway.

In summary,our research results indicate that GEG can activate the ATF6/GRP78/CHOP signaling pathway to restore endoplasmic reticulum (ER) homeostasis in chondrocytes, thereby reducing chondrocyte apoptosis and ultimately promoting the balance between ECM synthesis and degradation.

1. Introduction

Knee Osteoarthritis (KOA) is a prevalent chronic degenerative joint disease, often associated with factors such as age, obesity, trauma, genetic predisposition, and joint overuse. This condition leads to the gradual degradation of cartilage and the loss of

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https://doi.org/10.1016/j.heliyon.2024.e39987
Received 15 February 2024; Received in revised form 28 September 2024; Accepted 29 October 2024
Available online 31 October 2024

chondrocytes [1,2]. Chondrocytes, the sole cell type found in articular cartilage, play a crucial role in maintaining the dynamic equilibrium of this tissue by regulating the synthesis and breakdown of the extracellular matrix (ECM) [3,4].The apoptosis of chondrocytes and the disruption of the balance between ECM catabolism and anabolism are pivotal in the development of KOA. Apoptosis, a gene-regulated biological process, ultimately results in programmed cell death. Among the known triggers of apoptosis, the Endoplasmic Reticulum Stress (ERS)-mediated apoptosis pathway has garnered significant attention from researchers in recent years [5–8].

The Endoplasmic Reticulum (ER) is a crucial membrane-bound organelle that plays essential roles in processes such as protein secretion, folding, lipid synthesis, and distribution [9,10].Within chondrocytes, the ER is vital for the production and turnover of extracellular matrix (ECM) molecules. However, prolonged Endoplasmic Reticulum Stress (ERS) disrupts the balance between ECM degradation and synthesis, potentially leading to chondrocyte degeneration and, in severe cases, apoptosis [4].In response to ERS-induced apoptosis, cells activate the Unfolded Protein Response (UPR) as a protective mechanism to restore cellular equilibrium [11].As research advances, future therapeutic strategies for osteoarthritis may focus on directly targeting ERS or modulating UPR signaling pathways to alleviate chondrocyte apoptosis [12].

After the initiation of the unfolded protein response (UPR), a cascade of signaling pathways is activated to address endoplasmic reticulum (ER) stress.The ATF6-GRP78-CHOP pathway, a key component of the UPR, plays a pivotal role in maintaining intracellular balance, managing stress, and regulating cell fate [13].By modulating this pathway, cells can enhance their ability to respond to ER stress, thereby sustaining normal function and survival.While the ATF6/GRP78/CHOP pathway helps protect cells from stress-induced damage, its prolonged activation may lead to apoptosis under certain conditions, highlighting the importance of balance and regulation within the pathway [14]. Studies suggest that apoptosis can be mitigated through this signaling pathway [14,15]; however, the specific molecular processes that control the ATF6/GRP78/CHOP signaling pathway to prevent chondrocyte apoptosis remain unclear.

From the perspective of traditional Chinese medicine (TCM), several factors contribute to knee osteoarthritis (KOA), including blood stasis, wind-cold dampness, phlegm, and deficiencies in the liver and kidneys.When signs of wind-cold dampness, phlegm, and blood stasis are absent, treatment typically focuses on strengthening the liver and kidneys to address the underlying causes.Guilu Erxian Glue (GEG), noted in "Medical Formulae Investigations," features Testudinis Plastrum and Cornu Cervi as key ingredients. Testudinis Plastrum nourishes Yin and blood, while Cornu Cervi warms the kidneys and enhances vitality, together providing a dual supplement to harmonize Yin and Yang.Additionally, Ginseng Radix replenishes vitality and supports Qi and blood biochemistry, while Lycii Fructus nourishes the liver and kidneys, enhancing the overall formula's effectiveness in addressing liver and kidney deficiencies in KOA.Clinical trials indicate that GEG positively impacts KOA management, particularly in reducing symptoms like muscle atrophy and joint pain in elderly male patients [16].Furthermore, in vivo studies suggest GEG's potential in alleviating joint pain and modifying the course of KOA [17].However, the precise mechanism by which GEG inhibits chondrocyte apoptosis remains unclear and warrants further investigation. Understanding how GEG functions at the molecular level to prevent programmed cell death will enhance its therapeutic applications.

In summary, we hypothesized that reducing chondrocyte degradation is linked to the inhibition of the ATF6/GRP78/CHOP pathway.To support this theory, we conducted in vitro research to evaluate whether GEG could prevent TG-induced apoptosis in SD rat chondrocytes and restore the balance of extracellular matrix (ECM) synthesis and degradation through the ATF6/GRP78/CHOP signaling pathway. We treated chondrocyte degeneration with a combination of the established ATF6 inhibitor ceapin-A7 and GEG. This study aims to enhance our understanding of GEG's therapeutic effects and mechanisms in knee osteoarthritis (KOA) and provide a theoretical framework for the future optimization of KOA treatments.

2. Materials and methods

2.1. Preparation of GEG liquid, GEG drug-containing serum and blank serum

To prepare the GEG liquid, we used a blend of medicinal herbs: Testudinis Plastrum, Cornu Cervi, Lycii Fructus, and Ginseng Radix in the ratios of 10:5:1.3:1, as detailed in "The Golden Mirror of Medicine."Lycii Fructus and Ginseng Radix were decocted twice, then mixed, followed by the addition of Testudinis Plastrum and Cornu Cervi. This mixture was concentrated to yield GEG liquid at a concentration of 1 g/mL crude drug using a rotary evaporator.Ten 2-month-old male SD rats were divided into two groups: one received 0.9 % normal saline (blank serum group), while the other was administered GEG (GuiLu serum group) via intragastric administration for one week.Blood was then collected from the abdominal aorta to obtain both blank serum and GEG drug-containing serum, which were stored at −20 °C for future cell experiments.

Table 1
Conditions for HPLC.

Time (min)	Volume flow (mL·min −1)	Detection wavelength (nm)	Injection volume (uL)	Column temperature (°C)	A% Ethyl Qing	B% 0.1%Phosphoric acid
0–5	1.0	254	10	25	25	75
5–15	1.0	254	10	25	33	67
15–25	1.0	254	10	25	37	73
25–40	1.0	254	10	25	25	75

2.2. High performance liquid chromatographic (HPLC)analysis

To prepare the GEG solution for measurement, dilute the 1 g/mL GEG solution with methanol/water (1:1, V:V) to achieve a concentration of 80 mg/mL crude drug. For the serum analysis, combine 400 μL of both GEG medicated serum and blank serum with 1200 μL of methanol, vortex for 30 s, and then refrigerate for 10 min at 10,000 rpm. Afterward, collect 1200 μL of the supernatant, dry it under nitrogen, and redissolve it in 150 μL of methanol/water (1:1, V:V). Centrifuge this solution for 10 min at 10,000 rpm, and then take an appropriate amount of the supernatant for HPLC analysis [18]. The HPLC conditions are detailed in Table 1.

2.3. Isolation and culture of chondrocyte

The experimental procedures involving animal care and use were approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine(ethics number:2023066).Each Sprague Dawley(SD)rat was housed in a specific pathogen-free (SPF) medical laboratory environment. Primary chondrocytes were extracted from the bilateral knee joints of 4-week-old SD rats obtained from Slack Laboratory Animal Co.,Ltd. in Shanghai, China. The knee cartilage was sectioned into approximately 1 mm^3 fragments and digested with a 0.2 % solution of type II collagenase for 1 h. After digestion, the chondrocyte suspension was centrifuged for 5 min at 1000 rpm and then cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin.Once 80%–90 % confluence was reached, the cells were digested with a 0.25 % trypsin solution, centrifuged, resuspended, and passaged. The complete medium was changed every two days, and only second-generation cells were used for experiments to prevent phenotypic changes.

2.4. Toluidine blue staining of chondrocytes

Chondrocytes were inoculated in 24-well plates and cultured for 24 h. Following this, they were washed three times with PBS solution, with each wash lasting 5 min. The cells were then fixed in 4 % paraformaldehyde solution for 20 min. After another wash with PBS, the chondrocytes were treated with 1 % toluidine blue solution (Solarbio, Beijing, China) for 1 h at room temperature. After washing with PBS again, the cells were dried, placed on slides, and sealed with neutral gum. All chondrocytes were observed under a microscope (Tissue FAXS Plus S; Tissue Gnostics, Vienna, Austria) and photographed.

2.5. The concentration and time of TG and GEG drug-containing serum were determined

TG and GEG concentrations and exposure times were evaluated using the CCK-8 assay. Chondrocytes were inoculated overnight in 96-well plates at a density of 5 × 10^4 cells per well. The drug treatment was initiated when the cells reached 60–70 % confluence. Chondrocytes were treated with various concentrations of TG (0, 0.5, 1, 2, 4, and 8 μmol/L) for 2, 4, 6, and 8 h. Additionally, cells were pretreated with the selected TG concentrations for the designated times before being exposed to different concentrations of GEG (0, 5 %, 10 %, 15 %, and 20 %). The GEG treatment was incubated with the cells at 37 °C for 24, 48, and 72 h. After incubation, 100 μL of CCK-8 solution (comprising 10 μL CCK-8 and 90 μL base medium) was added to each well and further incubated at 37 °C in the dark for 2 h [19].Finally, the absorbance of each sample was measured at 450 nm using a Thermomax microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.6. Experimental design

Thapsigargin (TG) is a known endoplasmic reticulum stress (ERS) inducer [20], frequently used to create degraded chondrocyte models [20–22]. In this study, we treated second-generation chondrocytes with TG (Tao Technology Biotechnology Co., Ltd., Shanghai, China) to simulate the ERS model of degenerative chondrocytes, establishing four experimental groups. The control group consisted of untreated chondrocytes, while the TG group included chondrocytes treated solely with TG. The TG + GEG group involved chondrocytes treated with TG followed by the addition of GEG-containing serum (replaced medium). The TG + GEG + Ceapin-A7 group included TG-treated chondrocytes that received both GEG-containing serum and Ceapin-A7 (500 nM) (replaced medium) [23].

2.7. Cell viability assay

The viability of articular chondrocytes was assessed using the CCK-8 assay, following the same method described in section 2.5.

2.8. TUNEL method detection

Chondrocytes in each group were treated with drug-containing serum, and apoptosis assessment was conducted using a TUNEL kit (Shanghai Yi Sheng Biological Co.,Ltd.,China).First, cells were fixed with 4 % paraformaldehyde. Staining was performed using 100 μL of Proteinase K working solution, with incubation at 37 °C for 20 min, followed by application of 1 × Equilibration Buffer at room temperature.The TdT buffer was then prepared away from light to ensure accuracy. DAPI nuclear staining was applied, and the slides were sealed to maintain sample integrity. The apoptosis rate in chondrocytes was determined using a fluorescence microscope (MacOddy Industrial Group, Xiamen, China).The experiment was conducted by three independent individuals to ensure reliability and robustness of the results.

2.9. Flow cytometry analysis

Treated chondrocytes were collected in flow tubes, washed twice with cold PBS, and resuspended in 400 μL of 1 × annexin V conjugate using the annexin V-FITC/propidium iodide double-stained apoptosis assay kit (BestBio, Shanghai, China). Next, 5 μL of annexin V-FITC staining solution was added, and the mixture was incubated for 15 min at 4 °C in light-proof conditions. Following this, 5 μL of propidium iodide (PI) staining solution was added and incubated for 3 min at 4 °C, also under light-proof conditions.Flow cytometry was then performed using a BD Celesta (San Jose, CA, United States), and flow analysis was conducted with FlowJo version 10.6.0 (FlowJo LLC, Ashland, OR, United States).

2.10. Immunofluorescence

The treated cells were washed with PBS and then fixed with 4 % paraformaldehyde for 30 min. After fixing, the cells were washed three times with PBS and permeabilized with 0.3 % Triton X-100 at room temperature for 15 min. Following permeabilization, the cells were blocked with 10 % bovine serum albumin (BSA) (Beyotime) for 1 h, then washed with PBS and incubated overnight at 4 °C in a humidified chamber with specific primary antibodies. The antibodies used were as follows: Collagen Type II Polyclonal Antibody (1:300, 28459-1-ap, Proteintech, Wuhan, Hubei), SEC61 antibody (1:300, 25352, SAB USA), anti-Bcl-2 antibody (1:300, 60178-1-lg, Sanying Biotechnology Co.,Ltd.,Wuhan, Hubei), and anti-Bax antibody (1:300, 60267-1-lg, Sanying Biotechnology Co.,Ltd.,Wuhan, Hubei).After washing the cells three times with PBS, they were incubated with fluorescein isothiocyanate (FITC) or rhodamine-labeled secondary antibodies (1:100, ZF-0311/ZF-0316, ZSBG-Bio, Beijing, China) at room temperature for 1 h, protected from light. Finally, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

2.11. RNA isolation and qPCR

Total RNA was isolated from chondrocytes using the Simply P total RNA extraction kit.RNA concentration was measured in micrograms per microliter with a UV spectrophotometer for each group. The volume of total RNA containing 500 ng was then calculated based on the measured concentration. For reverse transcription, we utilized the HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) kit (Nanjing Nuoweizan Biotechnology Co.,Ltd.,China) to convert the isolated RNA into complementary DNA (cDNA).QPCR was conducted using the Archimed X6 QPCR device (Beijing Technology Co.,Ltd.,China) with SYBR Green qPCR Mix (Biosharp Corporation,USA). All primer sequences used in the experiment are listed in Table 2. To ensure accurate comparisons, the expression levels of target genes were normalized to the control gene GAPDH.

2.12. Western blot analysis

Total proteins were extracted from cultivated chondrocytes using a protease inhibitor mixture with RIPA lysate for cell lysis. Protein concentration was then determined with a BCA protein quantification kit (Wuhan Doctor De Biological Engineering Co.,Ltd., China). The extracted proteins were separated using 10 % SDS-PAGE, followed by a wet transfer method to transfer proteins from the gel to a membrane. After transfer, the membrane was incubated with a BSA (Bovine Serum Albumin) sealant for approximately 2 h at room temperature.Western blot analysis was performed on the following proteins: CHOP (1:1000, catalog number 15204-1-ap), COL2A1 (1:2000, catalog number 28459-1-ap), XBP1 (1:1000, catalog number 24168-1-ap), MMP-13 (1:2000, catalog number 18165-1-AP), ATF6 (1:3000, catalog number 24169-1-AP), GRP78 (1:2000, catalog number 11587-1-AP), and GAPDH (1:5000, catalog number 60004-1-1G), all sourced from Proteintech (Wuhan, Hubei). The membrane was shaken at room temperature for 1 h before adding horseradish peroxidase-conjugated secondary antibodies specific to the primary antibodies. Visualization of protein bands was achieved using a highly sensitive ECL (Enhanced Chemiluminescence) kit. The bands were captured with Image Lab software, which enabled evaluation and quantification of the gray values of the protein bands. Properly exposed images were selected and saved for further analysis.

2.13. Statistical analysis

All data were repeated at least three times and are presented as mean ± standard error (SE). Charts were generated using GraphPad Prism software (version 9.0,La Jolla,CA,USA). One-way analysis of variance (ANOVA) and the Kruskal-Wallis test were employed to

Table 2
Primer list for quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
COL2A1	5′-ACACCGCTAACGTCCAGATG-3′	5′-GTACGTGAACCTGCTGTTGC-3′
MMP-13	5′-TGATGCTAACCAGACTATGGACAA-3′	5′-ATGACTCTCACAATGCGATTACTC-3′
ATF6	5′-TTACGCTTCGCCTGGAAGTT-3′	5′-TCCTGCCCATTGATCACGTT-3′
GRP78	5′-CGTCGTATGTGGCCTTCACT-3′	5′-ATTCCAAGTGCGTCCGATGA-3′
CHOP	5′-CAGCGAGTCCGAGTTGA-3′	5′-CGCTACGTCGAGCATAT-3′
XBP1	5′-GACGCACTTGGAATGACCCTT-3′	5′-TTGGTTTGCCCACCTCCGAT-3′
GAPDH	5′-GGTGTGAACCATGAGAAGTATGA-3′	5′-GAGTCCTTCCACGATACCAAAG-3′

compare multiple datasets, utilizing SPSS software (version 26.0, SPSS Inc.,Chicago, Illinois, USA). A P-value <0.05 was considered as statistically significant.

3. Results

3.1. Principal ingredients in GEG, blank serum, and GEG drug-containing serum

The HPLC chromatograms of the blank serum, GEG standard, and GEG drug-containing serum indicate that the latter remains stable over a specific time period and contains active ingredients such as betaine, ginsenoside, prolyl glycine, leucyl glycine, and prolyl valine. The chromatogram of the GEG drug-containing serum closely resembles that of the GEG standard, suggesting that the purity of the extracted GEG drug-containing serum meets the experimental requirements. In contrast, the blank serum displays only the presence of betaine (Fig. 1).

3.2. Identification of isolated chondrocyte

Toluidine blue staining is a specific method for identifying chondrocytes. This technique exploits the property that proteoglycans secreted by chondrocytes exhibit metachromatic dark blue staining when treated with toluidine blue. The differentiation of soft bone cells was characterized by the presence of aggrecan and COL2A1; positive detection of these proteins confirms that the isolated cultured cells are indeed chondrocytes [24].In this study, second-generation chondrocytes were identified through both toluidine blue staining and immunofluorescence staining for COL2A1. The immunofluorescent labeling revealed strong green fluorescence in the cytoplasm and membrane of the chondrocytes, alongside clear blue fluorescence in the nucleus, indicating the expression of COL2A1—a hallmark of chondrocyte identity (Fig. 2A). Additionally, toluidine blue staining provided further evidence for chondrocyte characterization, showing light blue staining in the cytoplasm and dark blue staining in the nucleus (Fig. 2B).

3.3. In chondrocyte, GEG reversed TG-mediated growth inhibition

In studies involving chondrocytes, the impact of TG on cell survival was evaluated using the Cell Counting Kit-8 (CCK-8) assay. This assay assessed the effects of TG on chondrocyte viability at various time points (2, 4, 6, and 8 h) following treatment with different concentrations of TG (0, 0.5, 1, 2, 4, and 8 μmol/L) (Fig. 3A). The results indicated a decrease in chondrocyte survival rates as both the concentration of TG and the duration of exposure increased. This finding suggests that TG has a detrimental effect on chondrocyte viability. Consequently, we selected a treatment condition of 2 μmol/L TG for 4 h for further investigation, as this concentration resulted in approximately a 50 % reduction in cell viability.

To evaluate the impact of GEG on chondrocyte cell viability, a model simulating chondrocyte degeneration was created by exposing the cells to 2 μmol/L thapsigargin (TG) for 4 h. Following this, chondrocytes were treated with various concentrations of GEG (0 %, 5 %, 10 %, 15 %, and 20 %) for 24, 48, or 72 h (Fig. 3B). At each designated intervention time, the GEG serum concentrations showed a significant decrease in chondrocyte survival rates compared to the normal control group.Upon further analysis of the experimental groups, it was noted that the cell activity in the group treated with 10 % GEG serum peaked at the 24-h mark. Remarkably, chondrocyte activity in this 10 % GEG group was consistently higher than that of the other groups, not only at 24 h but also at both 48 and 72 h of treatment. Consequently, 10 % GEG serum was selected as the intervention condition for degenerative chondrocytes, specifically for a 24-h treatment period.

In Fig. 3C,the experimental design comprised an initial treatment of chondrocytes with TG (2 μmol/L) for 4 h, followed by exposure to 10 % GEG for 24 h.This setup was intended to evaluate the therapeutic effects of GEG on chondrocytes experiencing growth

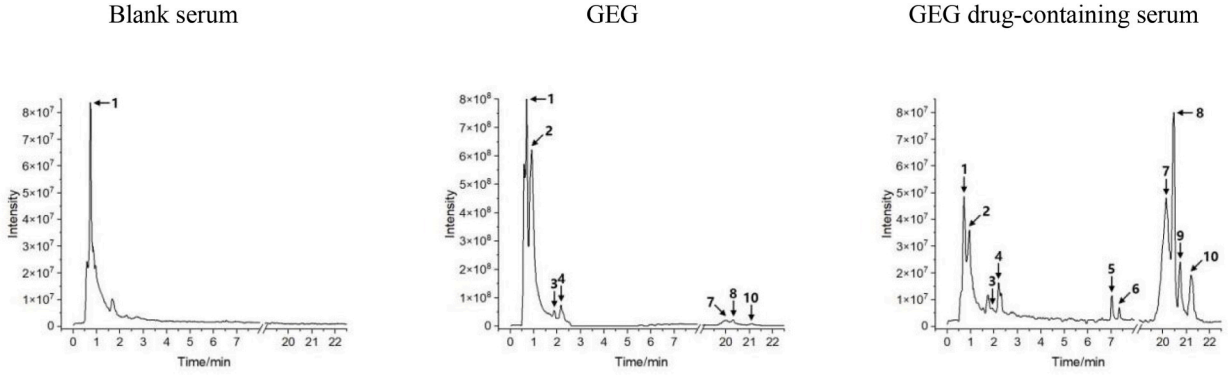

Fig. 1. Main components of GEG, GEG drug-containing serum and blank serum.
1:betaine; 2:prolyl glycine; 3: leucyl glycine; 4: prolyl valine; 5:7-hydroxy coumarin; 6:Elemenyl alcohol; 7,10: Ethyl linoleate, ethyl linoleate acetate; 8,9: Ginsenoside F2, ginsenoside Rg2, ginsenoside Rg3.

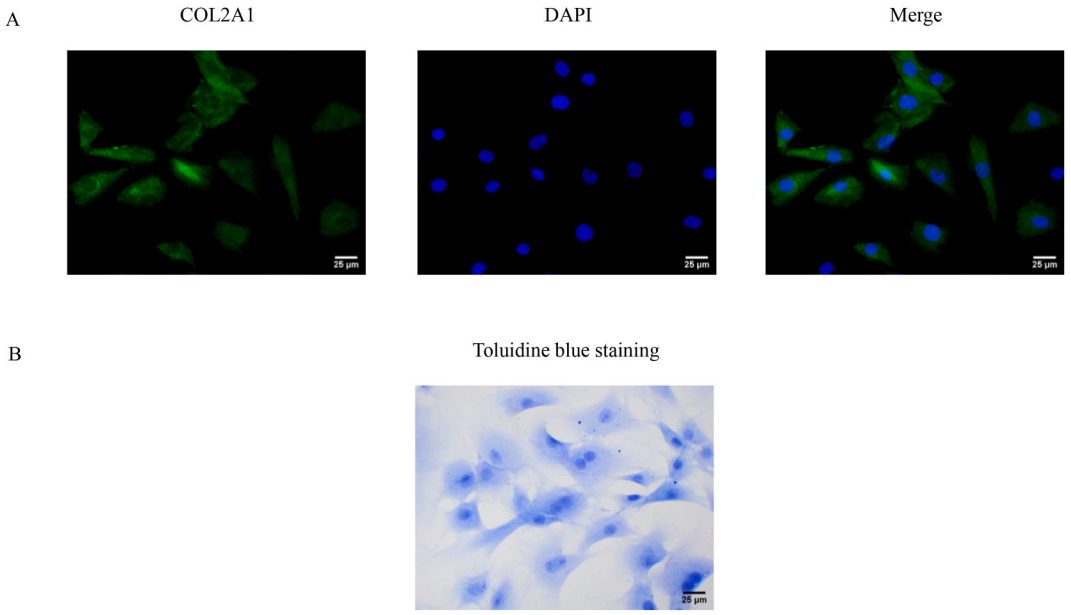

Fig. 2. Identification of isolated chondrocyte.
(A) Collagen II is stained green, while the nuclei, stained with DAPI, exhibit a clear blue fluorescence (scale: 25 μm). (B) The chondrocyte cytoplasm shows a light blue hue due to toluidine blue staining, which contrasts with the dark blue coloration of the nucleus (scale: 25 μm).

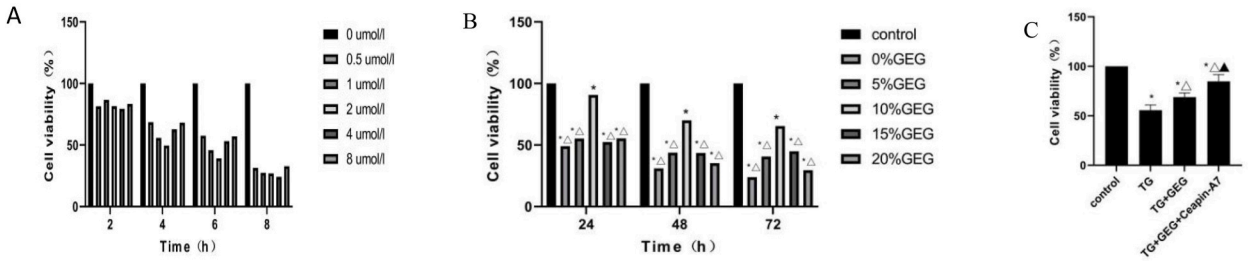

Fig. 3. In chondrocyte, GEG reversed TG-mediated growth inhibition.
(A) Effect of TG dose and time on chondrocyte viability (2 μmol/l for 4 h). (B) Effect of GEG dose and time on chondrocyte viability in degenerative chondrocyte (10 % GEG as serum for 24 h).*$P < 0.05$, compared with the control group;$^{\triangle}P < 0.05$, compared with the TG+10 % GEG group. (C) Effect of GEG treatment on the activity of TG-induced degenerative chondrocyte.These findings show the mean ± SD.(n = 6).*$P < 0.05$,Compared with control group;$^{\triangle}P < 0.05$,Compared with the TG group;$^{\blacktriangle}P < 0.05$,Compared with the GEG group.

inhibition induced by TG, thereby simulating conditions associated with chondrocyte degeneration.As previously documented, treatment with TG significantly reduced cell viability compared to the control group.In contrast, the subsequent treatment with GEG following TG exposure reversed this trend and led to an improvement in chondrocyte viability.Notably, the addition of Ceapin-A7—a blocker of the endoplasmic reticulum stress (ERS)-related pathway—alongside GEG resulted in an additional increase in chondrocyte activity.These findings suggest a potential synergistic role for GEG and Ceapin-A7 in sustaining cell viability in the context of TG-induced degeneration, this combination may offer a more effective approach for promoting chondrocyte vitality.

3.4. GEG inhibited TG-induced apoptosis In chondrocyte

Chondrocyte apoptosis has been identified as a key mechanism in the occurrence of KOA [25].To investigate the impact of GEG on reducing programmed apoptosis in chondrocytes,SD rat chondrocytes were initially treated with TG at 2 μmol/l for 4 h.Following this, the chondrocytes were cultured for an additional 24 h with either 10 % blank serum or 10 % GEG-containing serum.Results indicated that GEG treatment significantly reduced TG-induced chondrocyte apoptosis (Fig. 4).Immunofluorescence analysis revealed that TG stimulation up-regulated Bax intensity while down-regulating Bcl-2 intensity compared to the control group. GEG treatment resulted in decreased Bax levels and a notable increase in Bcl-2 expression, with Ceapan-A7 further enhancing these effects (Fig. 4A–C). Additionally, TUNEL assays and flow cytometry demonstrated that TG increased the apoptotic rate of chondrocytes, which was significantly reduced following GEG treatment.The presence of Ceapan-A7 further augmented GEG's protective effect against

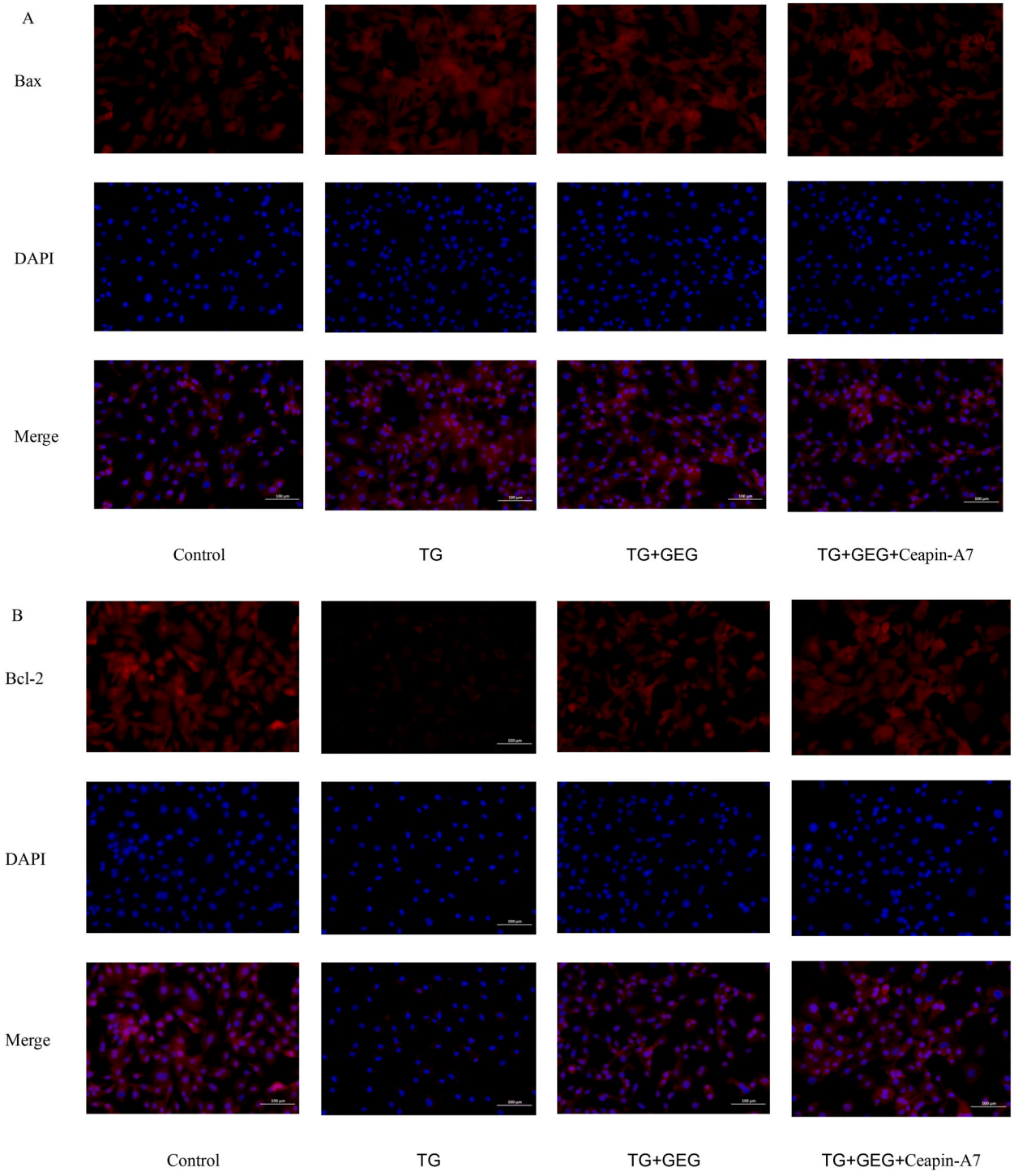

Fig. 4. GEG inhibited TG-induced apoptosis in chondrocyte.
(A-B) Following GEG treatment, immunofluorescence staining revealed alterations in the fluorescence intensity of Bax and Bcl-2 across all groups (scale bar: 50 μm). (C) The fluorescence intensity of Bax and Bcl-2 was quantitatively measured using ImageJ software (National Institutes of Health, Bethesda, USA). (D-E) Apoptotic chondrocytes were evaluated using the TUNEL assay, where apoptotic cells were visualized as green. Additionally, DAPI was employed to counterstain the nuclei, resulting in blue fluorescence (scale bar: 25 μm). (F-G) Post-treatment, flow cytometry was utilized to assess and quantify apoptotic cells within each group. The result represent mean ± SD (n = 3).$^{*}P < 0.05$,$^{**}P < 0.01$,Compared with control group;$^{\triangle}P < 0.05$,$^{\triangle\triangle}P < 0.01$,Compared with the TG group;$^{\blacktriangle}P < 0.05$,$^{\blacktriangle\blacktriangle}P < 0.01$,Compared with the TG + GEG group.

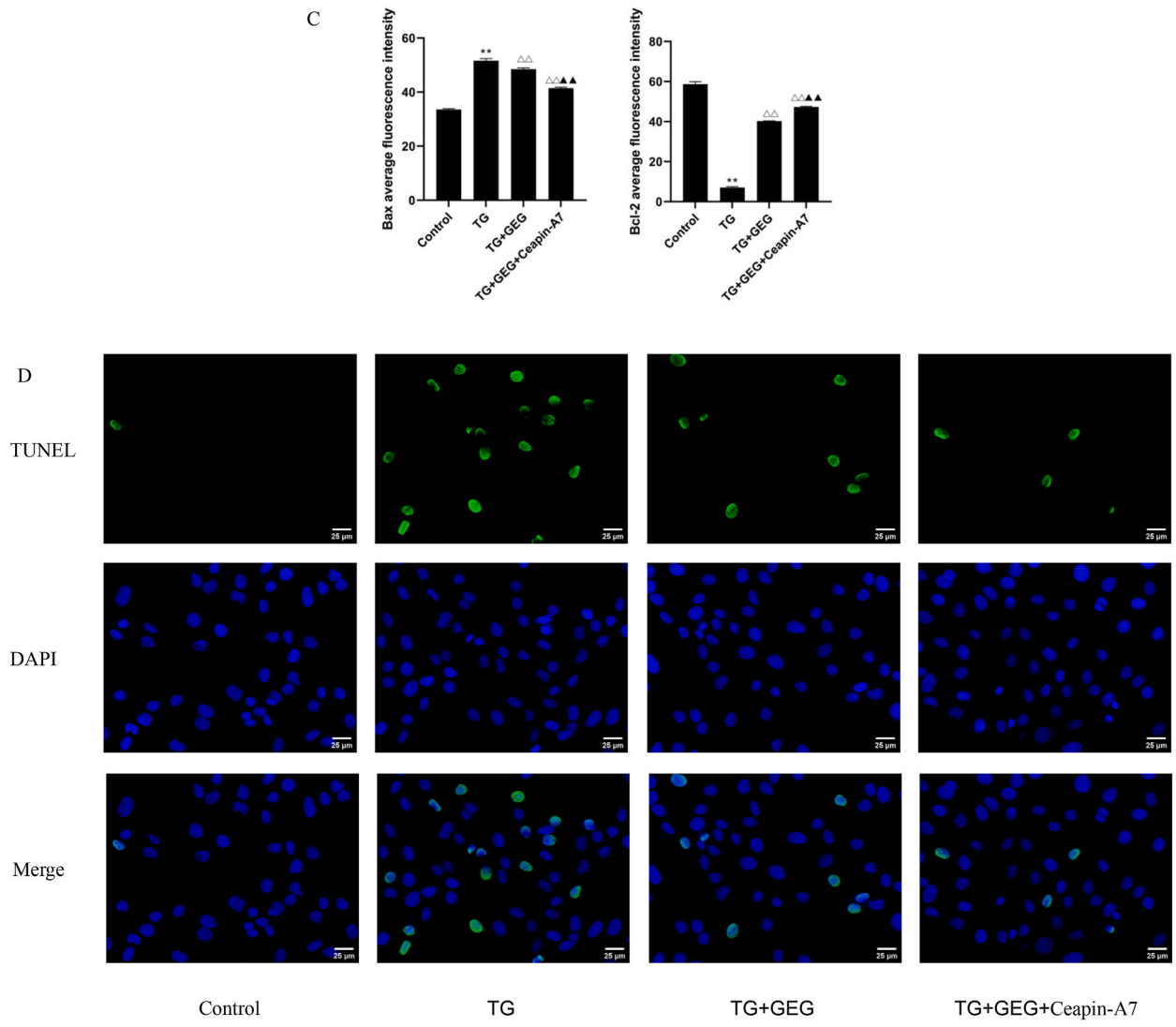

Fig. 4. (*continued*).

apoptosis (Fig. 4D–G).These findings suggest that GEG exhibits an anti-apoptotic effect in degenerative chondrocytes.

3.5. GEG alleviates TG-induced ECM degradation in chondrocytes

The investigation into the protective effects of GEG on chondrocytes utilized Western blot and qPCR techniques to analyze specific markers associated with extracellular matrix (ECM) degradation (MMP-13) and synthesis (COL2A1) (Fig. 5A–C).Both Western blot and qPCR analyses demonstrated that chondrocytes subjected to TG treatment exhibited increased levels of MMP-13 and COL2A1, both in protein and mRNA expression, compared to the control group.However, GEG treatment significantly influenced these expression changes.In contrast to the model group, the GEG group showed a marked decrease in MMP-13 protein and mRNA levels in TG-induced degenerative chondrocytes.Simultaneously, there was a significant increase in COL2A1 mRNA and protein expression.Further comparisons between the GEG and Ceapin-A7 groups revealed that Ceapin-A7 treatment resulted in a notable decrease in MMP-13 protein and mRNA levels, along with an increase in COL2A1 protein and mRNA expression, compared to the GEG-treated group.Overall, the results indicate that GEG effectively restored anabolic and catabolic balance in chondrocytes affected by TG, highlighting its role in mitigating TG-induced ECM degradation.

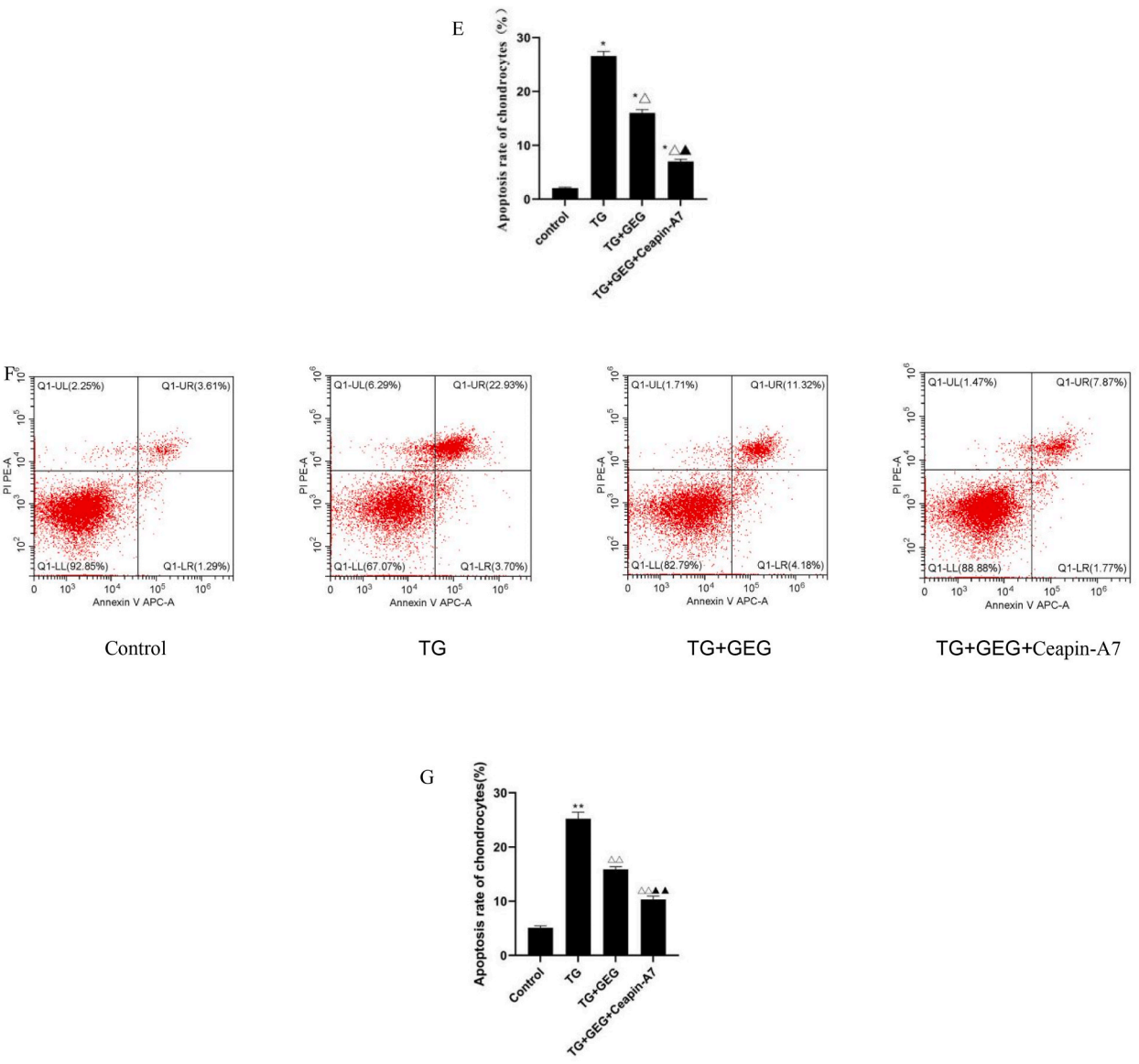

Fig. 4. (*continued*).

3.6. GEG inhibited of TG-induced ERS in chondrocyte

We utilized Western blot and qPCR to examine the expression of endoplasmic reticulum stress (ERS)-related genes and proteins, including XBP1, to investigate the potential relationship between the anti-apoptotic effects of GEG and ERS suppression (Fig. 6A–C). Additionally, we conducted immunofluorescence to detect SEC61 expression (Fig. 6D and E).Our results showed that TG-induced degenerative chondrocytes exhibited a significant increase in XBP1 levels; however, GEG treatment effectively reduced this TG-induced increase. Notably, the combination of GEG with Ceapin-A7 led to a marked decrease in XBP1 expression compared to GEG treatment alone (Fig. 6A–C).The immunofluorescence findings aligned with the trends observed in Western blot and qPCR analyses (Fig. 6D and E).These results suggest that GEG exerts an anti-apoptotic effect on chondrocytes, likely through the suppression of ERS, as indicated by the downregulation of XBP1 and SEC61. Furthermore, the combination of GEG with Ceapin-A7 appears to enhance this suppressive effect, offering promising insights into potential therapeutic strategies for addressing degenerative processes in chondrocytes.

3.7. GEG alleviated TG-induced apoptosis in degenerative chondrocytes by inhibiting ERS

To further investigate whether GEG inhibits endoplasmic reticulum stress (ERS) in TG-treated chondrocytes, we activated ERS

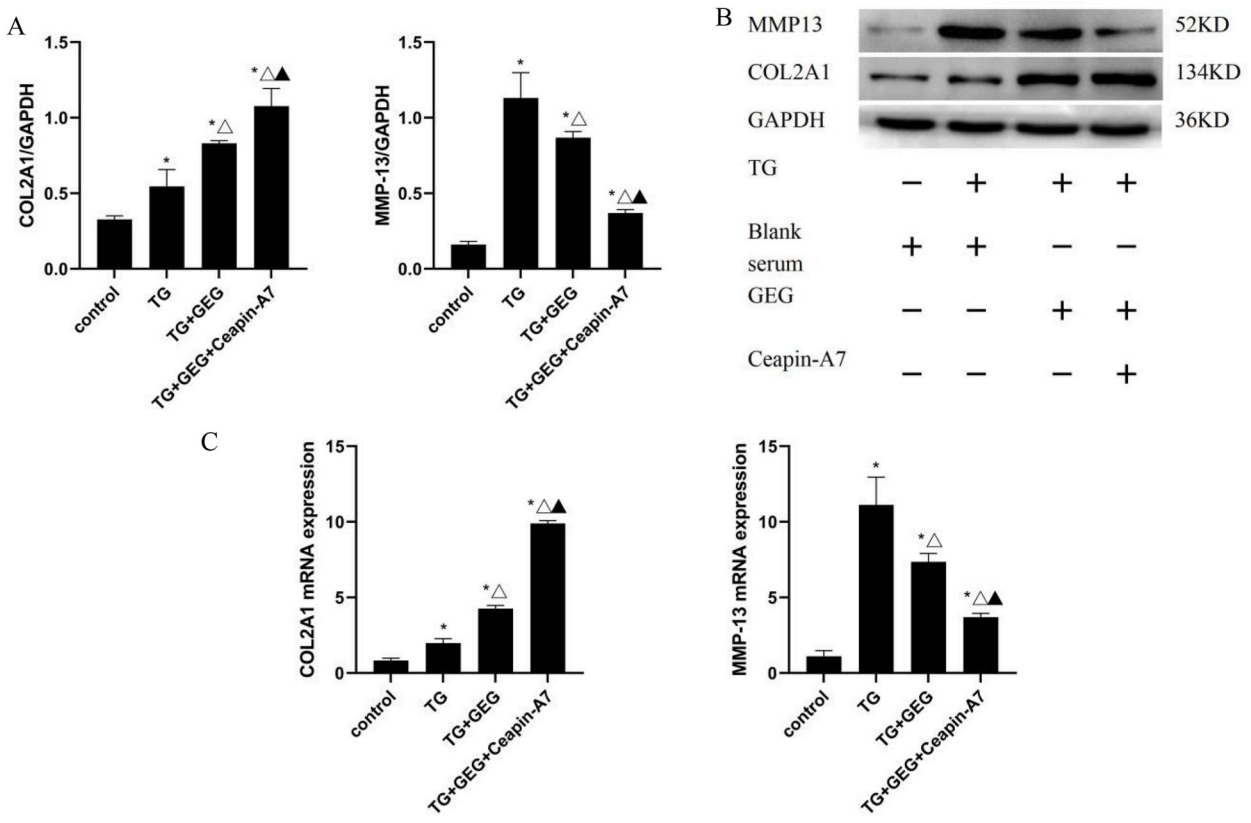

Fig. 5. GEG inhibited TG-induced MMP-3 activation and promoted COL2A1 expression.
(A, B) The protein levels of MMP-13 and COL2A1 were measured in each group.(C) qPCR was utilized to assess the mRNA expression levels of MMP-13 and COL2A1 in each group.The results represent mean ± SD (n = 3).*$P < 0.05$,Compared with blank group;$^{\triangle}P < 0.05$,Compared with the model group;$^{\blacktriangle}P < 0.05$,Compared with the GEG group.

using TG.The Western blot results (Fig. 6B–C) and qPCR analysis (Fig. 6A) indicated that the expression level of XBP1 was significantly increased in TG-treated chondrocytes compared to those treated with GEG.Additionally, immunofluorescence analysis revealed that GEG reduced the fluorescence intensity of SEC61, which was significantly upregulated by TG (Fig. 6D–E).These findings demonstrate that GEG protects chondrocytes from TG-induced apoptosis.To further confirm whether GEG inhibits TG-induced chondrocyte apoptosis via ERS suppression, we again activated ERS with TG and measured the expression levels of the pro-apoptotic biomarker Bax and the anti-apoptotic biomarker Bcl-2 (Fig. 4A–C).Flow cytometry (Fig. 4F–G) and TUNEL assays (Fig. 4D–E) were employed to detect apoptosis in chondrocytes induced by TG.In summary, TG reduces the anti-apoptotic effects of GEG, while GEG mitigates apoptosis by inhibiting ERS.

3.8. In degenerate chondrocyte, GEG alleviates TG-induced apoptosis by inhibiting the ATF6/GRP78/CHOP pathway

The ATF6-XBP1-CHOP signaling pathway is a well-established pathway that plays a critical role in initiating apoptosis. Suppression of the ATF6-GRP78-CHOP signaling pathway has been linked to reduced apoptosis [14].To explore the potential connection between the anti-apoptotic mechanism of GEG and the ATF6-GRP78-CHOP signaling pathway, we conducted Western blot and qPCR analyses to evaluate the expression levels of ATF6,GRP78,and CHOP.Our investigation revealed that treatment with TG significantly activated the ATF6/GRP78/CHOP signaling pathway, resulting in increased expression of ATF6, GRP78, and CHOP compared to the control group. In contrast, the introduction of GEG-containing serum to TG-induced degenerative chondrocytes led to a notable down-regulation of ATF6, GRP78, and CHOP expressions (Fig. 7A–C).Furthermore, when Ceapin-A7 was used in combination with GEG,the inhibitory effects on the ATF6/GRP78/CHOP signaling pathway were significantly enhanced. These results suggest that GEG can inhibit chondrocyte degeneration by downregulating ATF6 expression, thereby modulating the ATF6/GRP78/CHOP signaling pathway.

4. Discussion

KOA is the most prevalent joint disease worldwide and a leading cause of disability.It significantly limits patients' daily activities,

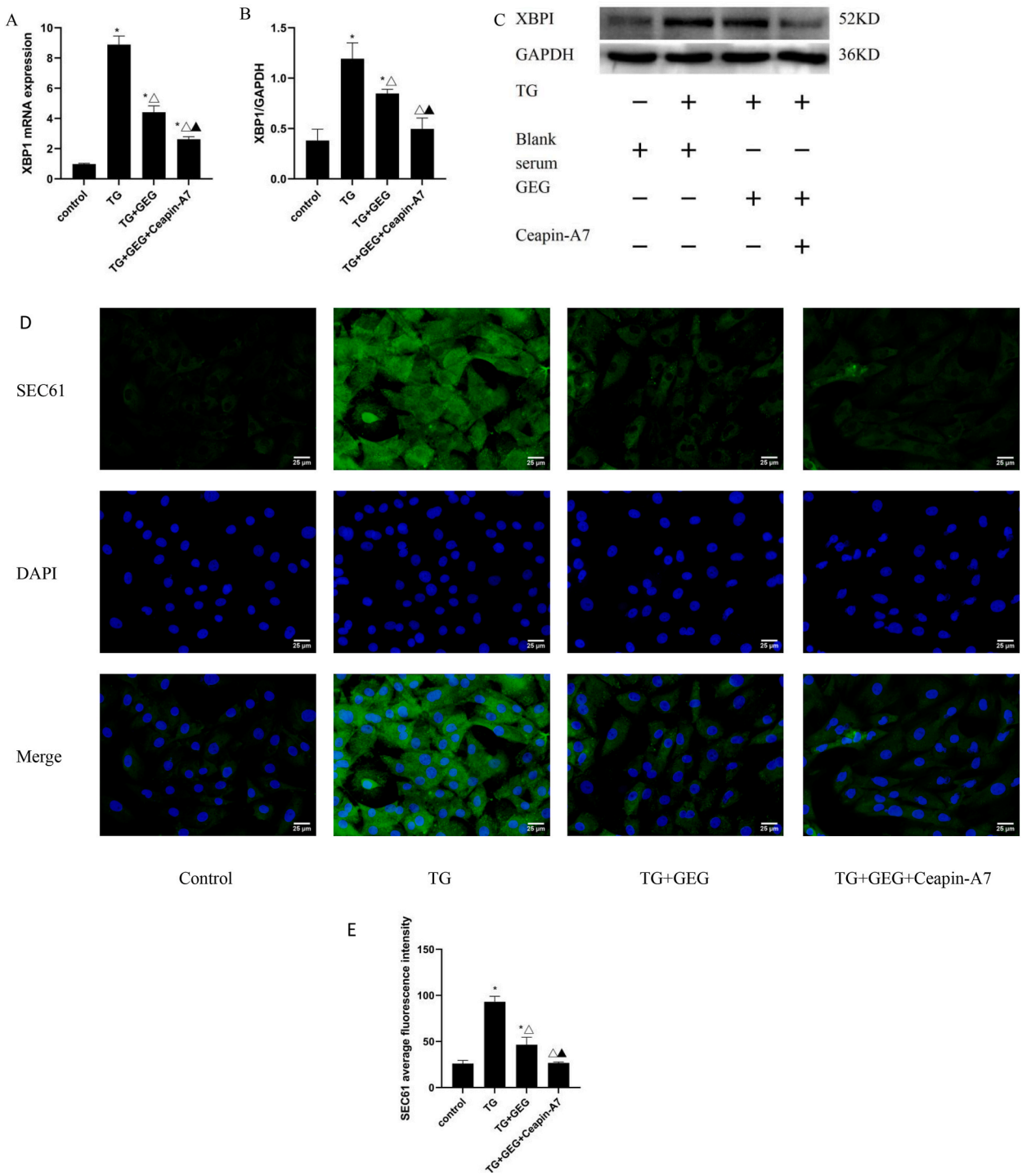

Fig. 6. GEG Inhibited of TG-induced ERS in chondrocyte.
(A) The qPCR method was used to evaluate XBP1 mRNA expression. (B-C) The Western blot technique was applied to detect XBP1 protein levels. (D-E) Immunofluorescence was employed to assess SEC61 expression.The results represent mean ± SD(n = 3).*$P < 0.05$,Compared with Control group;$^{\triangle}P < 0.05$,Compared with the TG group;$^{\blacktriangle}P < 0.05$,Compared with the GEG group.

including walking and running, adversely affecting their quality of life and imposing a considerable social burden.The prevalence of KOA in the elderly population is particularly high, with recent epidemiological studies reporting rates of 9.6 % in elderly men and 18 % in elderly women [26,27].The Traditional Chinese Medicine (TCM) perspective holds that the syndrome of KOA is characterized by a

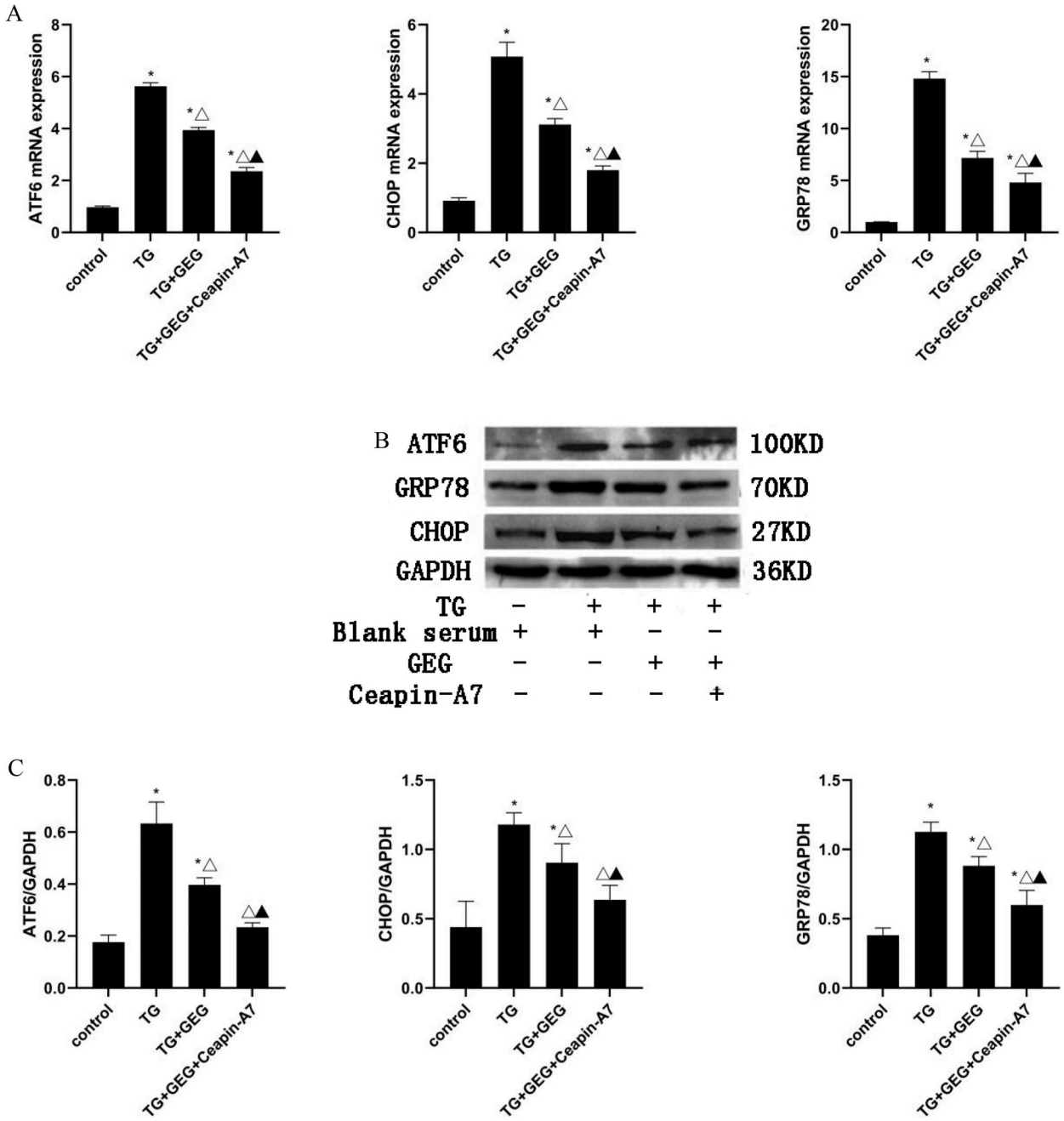

Fig. 7. In degenerative chondrocytes, GEG alleviates TG-induced apoptosis by inhibiting the ATF6/GRP78/CHOP pathway. (A) qPCR was utilized to measure the mRNA expression levels of ATF6, GRP78, and CHOP across all groups. (B–C) The protein expression levels of ATF6, GRP78, and CHOP were assessed using Western blot and quantitative methods.The findings are shown as mean ± SD(n = 3).*$P < 0.05$,Compared with control group;△$P < 0.05$,Compared with the TG group;▲$P < 0.05$,Compared with the GEG group.

deficiency of the fundamental essence and an excess of secondary symptoms, where a deficiency in Yang transforming qi is the underlying issue, and excessive Yin formation is the manifestation.By addressing the root causes of KOA through an understanding of its pathogenesis, treatments can enhance Yang qi and inhibit the progression of Yin formation, thereby restoring a dynamic balance between Yin and Yang.The GEG prescription embodies the principles of Yin and Yang harmony, achieving a balance between Yang tonifying herbs and Yin tonifying herbs, as well as between qi tonifying and blood nourishing ingredients.The ultimate goal is to ensure that the body's muscles, qi, blood, and meridians attain a state of self-harmony between Yin and Yang.ERS is aligned with the TCM concept of "Yang transforming qi," and the synthesis of macromolecular substances regulated by ER resident proteins correlates with the idea of "Yin formation."Therefore, GEG offers a theoretical foundation for treating KOA through its influence on ERS.In this study,

we developed an in vitro degenerated chondrocyte model using TG and employed techniques such as CCK-8, flow cytometry, TUNEL assays, immunofluorescence,qPCR,and Western blotting to investigate the chondroprotective mechanisms of GEG.Our results demonstrated that GEG not only inhibited TG-induced ERS and apoptosis in chondrocytes but also promoted chondrocyte proliferation and restored the balance of ECM synthesis and degradation.Mechanistically, we found that GEG regulates KOA progression through the ATF6/GRP78/CHOP signaling pathway, thereby modulating ERS effectively.

According to reports, chondrocytes are the only cell type that constitutes articular cartilage and are responsible for the synthesis and secretion of large molecules in the cartilage ECM, such as COL2A1 [28].In addition, chondrocytes can synthesize matrix metalloproteinases, such as MMP-13 [29].Currently, the synthesis and degradation status of the cartilage matrix is mainly reflected indirectly by detecting the anabolic and catabolic marker proteins in chondrocytes [30,31].Under normal physiological conditions, chondrocytes maintain a balance between the synthesis and degradation of ECM components to ensure the structural and functional integrity of cartilage [32]. However, when chondrocytes are exposed to various stress stimuli, either directly or indirectly, proteins may misfold or unfold, leading to ERS [33].Stress cells subsequently activate downstream adaptive mechanisms to alleviate stress and restore ER homeostasis.This adaptive mechanism is called the UPR. Mild ERS helps misfolded and unfolded proteins to fold correctly or be degraded, thereby restoring normal ER function to support cell survival. However, if the stressors persist, ERS will exceed the UPR threshold and induce apoptosis, disrupting the delicate balance between ECM synthesis and degradation [34–36].In recent years, an increasing number of studies have shown that the ERS marker XBP1 is crucial for the pathophysiology of KOA [37–39].Within the framework of KOA pathogenesis, the atypical increase in XBP1 protein expression activates the pro-apoptotic protein Bax, which translocates from the cytoplasm to the ER membrane and undergoes a series of reactions with the anti-apoptotic protein Bcl-2, antagonizing Bcl-2's original inhibitory effect on apoptosis, ultimately causing an imbalance between ECM synthesis-related factors COL2A1 and degradation-related factors MMP-13, leading to cell apoptosis [8,40].In current studies, apoptosis biomarkers Bax, ECM degradation-related factor MMP-13, and ER stress marker XBP1 are upregulated in chondrocytes stimulated by TG, while the anti-apoptotic protein Bcl-2 and ECM synthesis-related factor COL2A1 are downregulated. However, GEG can reverse the effects of TG on chondrocyte ECM, ERS, and apoptosis, and Ceapin-A7 can enhance GEG's effects on chondrocytes. These findings suggest that GEG may exert its effects by inhibiting ATF6 expression, thereby suppressing ER stress-mediated apoptosis, promoting a balance between ECM synthesis and degradation-related factors, and ultimately delaying cartilage degeneration. However, further experimental studies are needed to determine whether its protective effect on KOA chondrocytes is related to the apoptosis-related signaling pathway mediated by ATF6.

Several pathways contribute to the apoptosis of chondrocytes, includg endoplasmic reticulum stress (ERS)-related apoptotic inpathways and mitochondrial apoptotic pathways [8,11,12,41,42].In recent years,the ERS apoptosis pathway has garnered increasing attention from researchers.Targeting ERS and intervening in the unfolded protein response (UPR) signaling to reduce chondrocyte apoptosis are considered effective strategies for treating KOA [12].Among these pathways,ATF6 is one of three receptor proteins involved in the ERS-mediated UPR response [43].While GRP78 serves as a crucial molecular chaperone in the ERS process, playing a key role in protein synthesis and folding [44,45].Under normal physiological conditions,the levels of GRP78 are low; however, its expression significantly increases during ERS, making the upregulation of GRP78 an effective marker for detecting ERS.In a healthy state, GRP78 binds to downstream ATF6 in a non-activated form.When ERS persists, GRP78 regulates protein flux through the Sec61 complex, leading to the dissociation of ATF6.The dissociated ATF6 is then transported to the nucleus, where it activates downstream transcription and translation of CHOP, initiating the apoptotic process [13,43,46]. Our study demonstrates that GEG (a specific compound or drug) can activate the ATF6/GRP78/CHOP signaling cascade by inhibiting the expression of ATF6,thereby alleviating ERS in chondrocytes.

5. Conclusion

The experimental results suggest that GEG may have the potential to slow the progression of KOA.In in vitro studies, GEG exhibited protective effects on articular cartilage by inhibiting ERS-induced chondrocyte death and restoring the balance between ECM production and degradation.Furthermore, the study found that GEG can inhibit the expression of ATF6 and suppress the ATF6/GRP78/CHOP signaling pathway, further reducing cell apoptosis and promoting the balance between ECM synthesis and degradation, thereby protecting articular cartilageThese findings indicate that GEG could ultimately be developed as a treatment for KOA.Future research should explore the impact of the various components of GEG on its overall efficacy.This will enhance our understanding of its mechanisms of action and improve treatment strategies for patients with KOA.

CRediT authorship contribution statement

Qiudong Geng: Writing – review & editing. **Weixin Wu:** Writing – original draft. **Meixin Yang:** Visualization. **Fucheng Gu:** Resources, Conceptualization. **Weijun Cai:** Data curation. **Yangyi Qin:** Data curation. **Lifang Wei:** Methodology. **Heming Wang:** Validation. **Nan Li:** Validation.

Data availability

Data supporting the findings of this study are available from the first author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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