

JAK/STAT3/MMP9 Pathway Inhibition by Epigallocatechin-3-Gallate Alleviates Experimental Abdominal Aortic Aneurysm

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Background: Epigallocatechin-3-gallate (EGCG) exhibits protective effects against abdominal aortic aneurysm (AAA), although its specific molecular mechanism remains unclear. Aberrant activation of the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway mediates matrix metalloproteinase 9 (MMP9) expression and cell apoptosis, which are critical to aortic wall damage and AAA progression. This study investigated whether EGCG mitigates AAA by targeting the JAK/STAT3/MMP9 axis.

Methods: Rat *in vivo* AAA models were established via elastase infusion and CaCl₂ exposure, with EGCG administered by daily oral gavage from 7 days before modeling to 28 days after modeling. Angiotensin II (Ang II)-treated rat aortic smooth muscle cells (RASMCs) were used for *in vitro* assays, combined with pretreatment with gradient concentrations of EGCG and rescue experiments using the JAK/STAT3 pathway activator RO8191. Morphological, histological and molecular analyses were systematically conducted to assess aortic pathological changes, RASMC viability, pathway activation and the expression of apoptosis- and extracellular matrix-related molecules.

Results: EGCG significantly attenuated *in vivo* AAA progression, as shown by reduced aortic dilatation, attenuated aortic wall injury, restored elastic fiber integrity and Collagen I deposition, as well as suppressed JAK/STAT3 phosphorylation, cell apoptosis and MMP9 upregulation in aortic tissues ($p < 0.05$). *In vitro*, EGCG exerted no obvious cytotoxicity on RASMCs but concentration-dependently suppressed Ang II-induced JAK/STAT3 activation, cell apoptosis and MMP9 expression at both the protein and mRNA levels ($p < 0.05$). These protective effects were effectively reversed by RO8191 ($p < 0.05$).

Conclusion: Collectively, EGCG alleviates experimental AAA progression through inhibition of the JAK/STAT3 signaling pathway and associated downregulation of MMP9 expression, providing a novel molecular basis for EGCG as a potential preventive strategy for AAA and supporting its promising clinical potential in AAA prevention.

Keywords: AAA; EGCG; extracellular matrix; matrix metalloproteinase 9; JAK/STAT3; RASMCs

Introduction

Abdominal aortic aneurysm (AAA) is a common degenerative cardiovascular disease [1]. AAA progression is associated with a markedly elevated mortality rate in Western populations, underscoring its life-threatening clinical impact [2]. Currently, surgical repair remains the only definitive treatment for AAA, and effective clinical biomarkers and pharmacological therapies to reduce the risk of AAA development or limit its progression are still lacking [3,4]. Therefore, an in-depth understanding of the specific mechanisms underlying AAA pathogenesis and progression is of great importance for identifying efficient agents for its prevention.

The pathological changes in AAA tissues include chronic transmural inflammation, destructive remodeling of the extracellular matrix (ECM), and apoptosis of vascular

smooth muscle cells [5], among which ECM degradation in the aneurysmal wall is considered one of the pivotal mechanisms [6]. A study has shown that loss of arterial wall elasticity caused by ECM degradation contributes to AAA rupture, which is the most serious complication and a major cause of death in patients with AAA [7]. The matrix metalloproteinase (MMP) family contributes to the degradation of various ECM components (non-collagenous proteins, collagen and elastin), thereby participating in ECM remodeling and destruction of the arterial vessel wall [8,9]. Overexpression of MMPs, including MMP2 and MMP9, has been demonstrated in AAA tissues [6]. In particular, elevated MMP9 expression is associated with increased proteolytic enzyme activity, and higher MMP9 levels are correlated with an increased risk of AAA rupture [10,11]. Therefore, therapeutic strategies targeting MMP9 have become an important focus of aneurysm research [12]. A previous study

reported that signal transducer and activator of transcription 3 (STAT3) activation is involved in AAA progression by promoting MMP9 transcription and translation [13].

Epigallocatechin-3-gallate (EGCG), a major bioactive component of green tea, has shown great promise owing to its anti-cancer, anti-inflammatory and cardiovascular protective effects in recent studies [14,15]. A previous study has revealed that EGCG can mitigate arthritis by inhibiting the STAT3 signaling pathway and alleviating lipopolysaccharide-induced inflammation [16]. Moreover, EGCG has been demonstrated to effectively delay the progression of AAA [17]; however, its underlying mechanism remains to be further explored.

In the present study, we investigated the effect of EGCG on MMP9 expression during AAA development and whether the inhibitory effect of EGCG on AAA progression is mediated through suppression of the Janus kinase (JAK)/STAT3 signaling pathway, using *in vivo* and Ang II-induced RASMCs modeling.

Materials and Methods

Experimental Animals

A total of 18 male Sprague-Dawley (SD) rats (305–350 g; Hangzhou Medical College, China) were housed in a specific pathogen-free environment at 25 °C and 50–55% relative humidity under a normal day-night cycle, with free access to food and drinking water for one week before *in vivo* AAA modeling. All animal-related experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the Zhejiang Center of Laboratory Animals (Approval No. ZJCLA-IACUC-20020226) and were performed in accordance with the guidelines of the China Council on Animal Care and Use. This study was reported in accordance with the ARRIVE Guidelines 2.0.

Establishment of AAA Rat and EGCG Administration

SD rats were randomly divided into the Sham, model (Mod) and EGCG groups with 6 rats in each group. Rats in the Mod and EGCG groups were subjected to intraluminal elastase infusion and extraluminal CaCl₂ exposure for AAA modeling, as previously described [18,19]. Briefly, rats were anesthetized with isoflurane (3% for induction, 1.5% for maintenance, delivered via a calibrated vaporizer with an oxygen flow rate of 2 L/min). A midline abdominal incision was made, and the infrarenal abdominal aorta was exposed under an operating microscope. All lumbar branches were ligated. A polyethylene catheter was inserted via the femoral artery, and the aortic segment was temporarily occluded with microvascular clamps. A piece of gauze (10 mm³) soaked in CaCl₂ (0.5 mol/L, C118444, Aladdin, Shanghai, China) was wrapped around the aorta, while porcine pancreatic elastase (20 U/mL, 30 µL, E107150, Aladdin, Shanghai, China) was simultaneously injected intra-

luminally into the aorta through the polyethylene catheter. After 20 min, the catheter was removed, the femoral artery was ligated, the clamps were released, and the incision was closed. Rats were placed in warming cages for recovery. The procedures for the Sham group were the same as those for the Mod and EGCG groups, except that porcine pancreatic elastase was replaced with 0.9% NaCl (C111547, Aladdin, Shanghai, China) and CaCl₂ exposure was omitted. The maximum diameter of the infrarenal abdominal aorta was measured at each indicated time point (pre-day 7, day 0, day 7, day 14, day 21, and day 28) using transabdominal ultrasonography without euthanizing the animals. At the experimental endpoint (day 28), the aortic diameter was further confirmed by direct measurement using a vernier caliper under a dissecting microscope. For EGCG administration, rats in the EGCG group received EGCG (50 mg/kg, E107403, Aladdin, Shanghai, China) by gavage beginning 7 days (d) before AAA modeling and continuing for 28 d after modeling [20]. Rats in the Mod group underwent the same administration procedure, except that EGCG was replaced with an equal volume of distilled water. Rats in the sham group were also given the same volume of distilled water by gavage. The macroscopic appearance of the infrarenal abdominal aorta in the three groups was photographed on day 28. Thereafter, all rats were euthanized by intraperitoneal injection of an overdose of pentobarbital sodium (2% solution, 150 mg/kg, P-010, Merck, Darmstadt, Germany). Infrarenal abdominal aorta samples were collected and stored at –80 °C.

Hematoxylin-eosin (HE) Staining

Frozen sections of infrarenal abdominal aorta samples were rewarmed and rinsed in water for 30 seconds (s). Next, Hematoxylin Solution (D005-1-3, Jiancheng Bioengineering Institute, Nanjing, China) was introduced to stain the sections for 3–5 min and washed with water. After treatment with Acid Alcohol Fast Differentiation Solution (C0163S, Beyotime, Shanghai, China) and subsequent washing with water, the sections were stained with eosin (I031-1-1, Jiancheng Bioengineering Institute, Nanjing, China) for 5 min, followed by dehydration and mounting. The stained sections were observed under a CX43 microscope (Olympus, Tokyo, Japan) at ×100 or ×200 magnifications.

Elastic Van Gieson (EVG) Staining

Elastic fibers in infrarenal abdominal aorta samples were assessed by EVG staining using a Verhoeff Staining Assay Kit (G1598, Solarbio, Beijing, China). Briefly, Verhoeff solution was first prepared by mixing hematoxylin, FeCl₂ and iodine solution at a ratio of 5:2:2. The hydrated sections were then stained with Verhoeff solution at room temperature for 30 min and washed with water, followed by treatment with Verhoeff differentiation solution for 20 s. After washing with water, the sections were treated with

95% ethanol (A112717, Aladdin, Shanghai, China) for 2–5 min and counterstained with hematoxylin for 3 min. Following dehydration and mounting, images of the stained elastic fibers were captured using a CX43 microscope at $\times 100$ or $\times 200$ magnifications.

TdT-Mediated dUTP Nick-End Labeling (TUNEL) Assay

A TUNEL Apoptosis Kit (E-CK-A320, Elabscience, Wuhan, China) was used to detect cell apoptosis in infrarenal abdominal aorta samples. After fixation with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) at room temperature, the rat sections were rinsed twice with phosphate buffered saline (PBS; P1020, Solarbio, Beijing, China) and incubated with $1\times$ proteinase K working buffer at 37 °C for 10 min. Following treatment with TdT equilibration buffer for 20-min, 50 μ L of labeling working buffer, prepared according to the manufacturers' protocol, was added to the sections, which were incubated at 37 °C in a humidified chamber for 1 hours (h) in the dark. Next, the FITC-labeled sections were stained with diamidino-phenylindole (DAPI; abs47047616, Absin, Shanghai, China) under the same conditions for 5 min. Finally, green fluorescence density was analyzed using an IX 51 fluorescence microscope ($\times 200$, Olympus, Tokyo, Japan).

Rat Aortic Smooth Muscle Cells (RASMCs) Treatment

RASMCs were commercially purchased from Lonza Cologne GmbH (Basel, Switzerland; Catalog No. R-ASM-580). All cell lines tested negative for mycoplasma contamination. The cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; 11965118, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% fetal calf serum (FCS; 10091155, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin solution (P1400, Solarbio, Beijing, China) at 37 °C in a CO₂ incubator, with the medium changed every 2 d. RASMCs at passage 5–8 were harvested and divided into five groups: The control (Con) group (unprocessed RASMCs serving as the control), the Mod group, the EGCG group, the RO8191 group and the RO8191 + EGCG group. For cell treatment, RASMCs in the EGCG group were pretreated with 20, 40 or 80 μ g/mL EGCG at 37 °C for 2 h [21]. RASMCs in the RO8191 group were pretreated with 20 μ M RO8191 (HY-W063968, MCE, Monmouth Junction, NJ, USA), a JAK/STAT3 signaling pathway activator diluted in DMEM, at 37 °C for 2 h [22]. Meanwhile, RASMCs in the RO8191 + EGCG group were pretreated with a combination of 80 μ g/mL EGCG and 20 μ M RO8191 at 37 °C for 2 h.

Angiotensin II (Ang II) Stimulation of RASMCs

After pretreatment with or without EGCG or/and RO8191, RASMCs in the Mod, EGCG, RO8191 and

RO8191 + EGCG groups were subjected to Ang II stimulation. Briefly, Ang II (A9290, Solarbio, Beijing, China) was dissolved in water and diluted with DMEM to prepare a 10^{-7} mol/L Ang II solution. RASMCs seeded in 24-well plates were then incubated with the Ang II solution at 37 °C for 12 h, as previously described [23].

Cell Counting Kit-8 (CCK-8) Assay

Cell viability at 0, 6, 12 and 24 h after EGCG pretreatment at different concentrations (20, 40 or 80 μ g/mL) was assessed using a CCK-8 assay. RASMCs were seeded at a density of 5×10^3 cells per well in 96-well plates and incubated at 37 °C. Subsequently, 10 μ L of CCK-8 solution (E-CK-A362, Elabscience, Wuhan, China) was added to each well. After incubation for 2 h, absorbance at 450 nm was measured using an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Immunofluorescence Assay

Ang II-induced RASMCs pretreated with or without EGCG or/and RO8191 were seeded in 6-well plates, fixed with 4% paraformaldehyde at room temperature for 15 min, and rinsed twice with PBS. The cells were then permeabilized with 0.3% Triton X-100 (abs47048168, Absin, Shanghai, China) and blocked with 5% normal goat serum (SL038, Solarbio, Beijing, China), both diluted in PBS, at room temperature. After 1 h, RASMCs were incubated overnight at 4 °C with a primary antibody against phosphorylated (p)-STAT3 (1:500, 44-384G, Thermo Fisher Scientific, Waltham, MA, USA), followed by washing with $1\times$ TBST Buffer (T1085, Solarbio, Beijing, China). Next, Goat anti-Rabbit IgG (H+L) (1:100, A-21070, Thermo Fisher Scientific, Waltham, MA, USA) was used to incubate RASMCs at room temperature for 1 h. After staining with DAPI solution, intracellular red fluorescence signals were captured using an IX 51 fluorescence microscope (Olympus, Tokyo, Japan) at $\times 200$ magnification. For quantitative analysis, the mean fluorescence intensity of p-STAT3-positive cells was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least three randomly selected fields per well were analyzed, and relative fluorescence intensity was normalized to that of the control group.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA from Ang II-induced RASMCs pretreated with or without EGCG or/and RO8191 was extracted using TRIzol (15596026, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, complementary DNA (cDNA) synthesis and template amplification were performed using a One Step RT-qPCR Kit (B639277, Sangon Biotech, Shanghai, China). An ABI7500 thermal cycler (ThermoFisher, Waltham, MA, USA) was employed for RT-PCR according to the man-

ufacturers' protocol. The specific primers used in this reaction were as follows (5'-3'): matrix metalloproteinase 9 (MMP9; GATCCCCAGAGCGTTACTCG, GTTGTGGAAACTCACACGCC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ACGGGAAACC-CATCACCATC, CTCGTGGTTCACACCCATCA). The fold change of MMP9 level was calculated using the $2^{-\Delta\Delta Ct}$ method [24].

Western Blot

Total protein from infrarenal abdominal aorta samples or RASMCs was extracted using Tissue Protein Extraction Reagent (78510, Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor (87786, Thermo Fisher Scientific, Waltham, MA, USA) and phosphatase inhibitor (78427, Thermo Fisher Scientific, USA), or RIPA buffer (20101ES60, Yeasen, Shanghai, China), followed by protein quantification using a BCA Protein Assay Kit (E-BC-K318-M, Elabscience, Wuhan, China). Subsequently, 20 μ g of protein from each sample was dissolved in 6–10% SDS-PAGE gel (E-IR-R305, Elabscience, Wuhan, China) before electrophoresis and separately transferred onto polyvinylidene fluoride membranes (FFP26, Beyotime, Shanghai, China). The membranes were then blocked with 5% TBST-dissolved bovine serum albumin (BSA, abs9157, Absin, Shanghai, China) for 1 h, followed by incubation with primary antibodies at 4 °C overnight. After washing with TBST, the membranes were incubated with secondary antibodies at room temperature for 2 h and developed using an ECL Plus kit (P0018S, Beyotime, Shanghai, China), followed by immunoblot analysis in Tanon 5200 imaging system (Tanon, Shanghai, China) and ImageJ software (National Institutes of Health, Bethesda, MD, USA). All antibodies (Abcam, Cambridge, UK) used for western blotting were as follows: p-JAK2 (1:1000, 120 kDa, ab32101), JAK2 (1:5000, 130 kDa, ab108596), STAT3 (1:1000, 88 kDa, ab68153), p-STAT3 (1:20,000, 88 kDa, ab76315), Collagen I (1:1000, 139 kDa, ab260043), intercellular adhesion molecule 1 (ICAM-1; 1:250, 57 kDa, ab171123), vascular cell adhesion molecule 1 (VCAM-1; 1:10,000, 81 kDa, ab134047), MMP9 (1:5000, 78 kDa, ab76003), BCL2 associated X (Bax; 1:1000, 21 kDa, ab32503), B cell lymphoma 2 (Bcl2; 1:1000, 26 kDa, ab194583), GAPDH (1:500, 36 kDa, ab8245), anti-rabbit IgG (1:2000, ab205718) and anti-mouse IgG (1:2000, ab205719).

Statistical Analysis

All measurement data were presented as the mean $\pm$ standard deviation. Graphpad Prism 8.0 (GraphPad Software Inc., Boston, MA, USA) was used for statistical analysis. For comparisons of abdominal aortic diameter measured at multiple time points within the same group, repeated measures ANOVA was applied. Comparisons among multiple groups were performed using one-way

ANOVA followed by Tukey's post-hoc test for multiple comparisons. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. A $p < 0.05$ was considered statistically significant.

Results

EGCG Administration Attenuated Abdominal Aortic Degeneration and Elastic Fiber Fragmentation in AAA Rat Model

The image shown in Fig. 1A illustrates the chemical structure of EGCG, which has previously been reported to prevent the progression of AAA. However, the specific mechanism underlying the effects of EGCG in AAA remains unclear; therefore, we sought to investigate this mechanism in the present study. Following *in vivo* AAA modeling, a significant increase in abdominal aortic diameter was observed in the Mod group from day-1 to day 28 compared with the Sham group (Fig. 1B,C, $p < 0.01$). In addition, the abdominal aortic diameter of rats receiving EGCG was significantly reduced from day 21 to day 28 compared with the Mod group (Fig. 1B,C, $p < 0.05$). As illustrated in Fig. 1D, HE staining revealed thickening of the entire wall but reduced thickening of the medial layer in the Mod group. Compared with the Mod group, EGCG treatment significantly increased the thickness of the outer and middle layers and decreased the thickness of the intimal layer of the rat abdominal aorta. As shown in Fig. 1E, marked elastic fiber fragmentation in the aortic wall was observed in the Mod group after EVG staining, whereas EGCG administration markedly alleviated this injury.

EGCG Administration Inhibited JAK/STAT3 Signaling Pathway and Reduced Cell Apoptosis in the Abdominal Aorta of AAA Rat Model

Consistently, western blot analysis showed that the protein levels of p-JAK2/JAK2 and p-STAT3/STAT3 were elevated in the rat abdominal aorta after AAA modeling (Fig. 2A–C, $p < 0.001$), and these changes were reversed by EGCG administration (Fig. 2A–C, $p < 0.05$). As demonstrated in Fig. 2D,E, TUNEL assay revealed that apoptotic cells (green) were considerably increased in the rat abdominal aorta of the Mod group compared with the Sham group ($p < 0.001$), and this increase was attenuated by EGCG administration ($p < 0.05$). Moreover, upregulation of Bax and downregulation of Bcl2 were detected by western blot analysis in the Mod group (Fig. 3A–C, $p < 0.001$). Compared with the Mod group, EGCG reduced Bax protein levels and increased Bcl2 protein levels in the abdominal aorta of rats subjected to AAA modeling (Fig. 3A–C, $p < 0.05$).

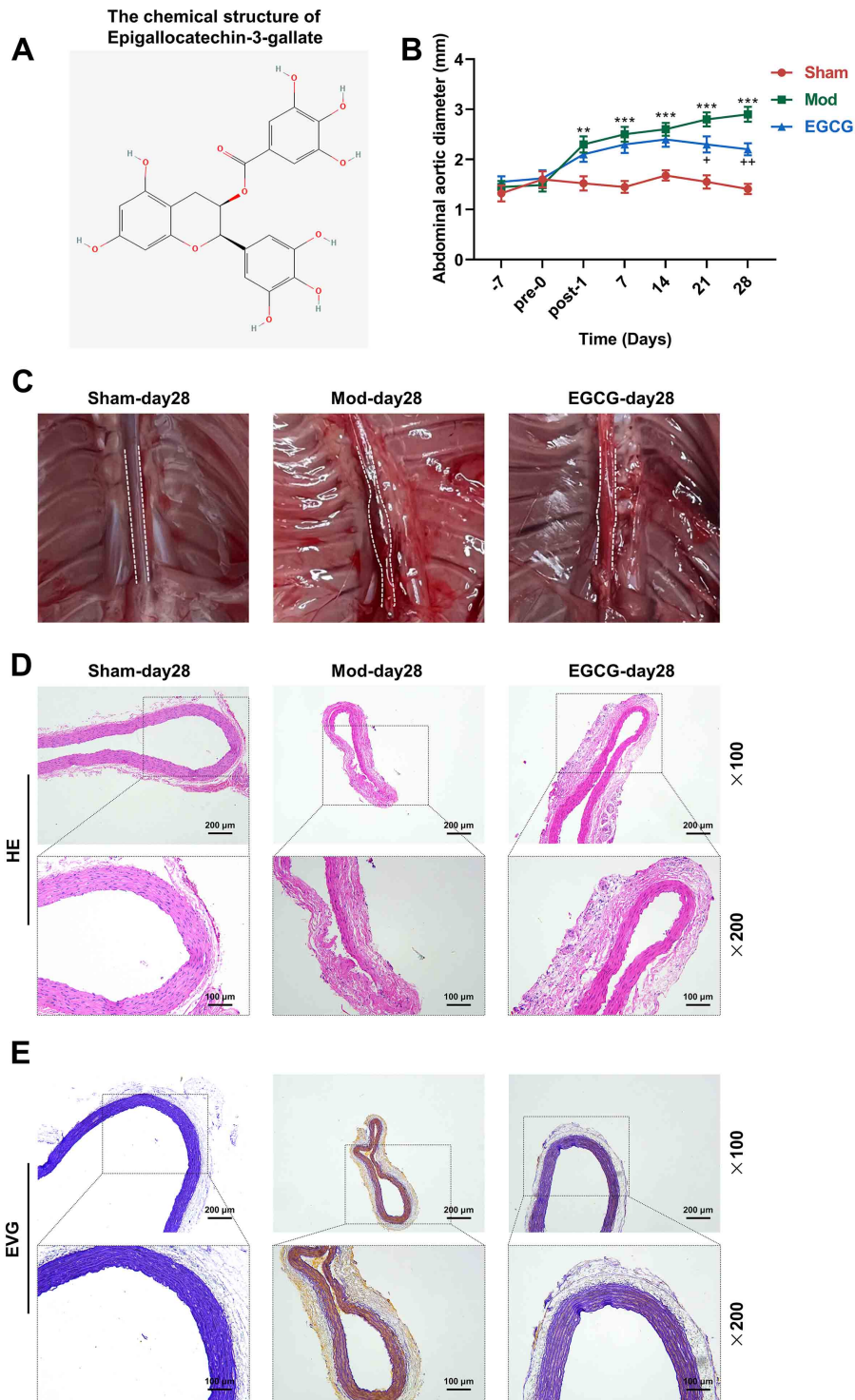


Fig. 1. The role of epigallocatechin-3-gallate (EGCG) in the progression of abdominal aortic aneurysm (AAA) *in vivo*. (A) The chemical structure of EGCG. (B) The abdominal aortic diameter from pre-day 7 to day 28 in rats subjected to AAA modeling or/and EGCG administration. (C) Images of abdominal aortic diameter on day 28 in rats with or without AAA modeling or/and EGCG administration. (D) Hematoxylin-eosin (HE) staining was used to analyze histopathological changes in abdominal aorta of rats with or without AAA modeling or/and EGCG administration ($\times 100$ or $\times 200$ magnifications, scale bar: 200 or 100 μm). (E) Elastica van gieson (EVG) staining (deep violet blue) was performed to detect the change of elastic fibers in aortic wall of rats with or without AAA modeling or/and EGCG administration ($\times 100$ or $\times 200$ magnifications, scale bar: 200 or 100 μm). For *in vivo* experiments, each group contained 6 rats. Data are presented as mean $\pm$ SD (n = 6 rats per group). ** $p < 0.01$, *** $p < 0.001$, vs. Sham; + $p < 0.05$, ++ $p < 0.01$, vs. model (Mod).

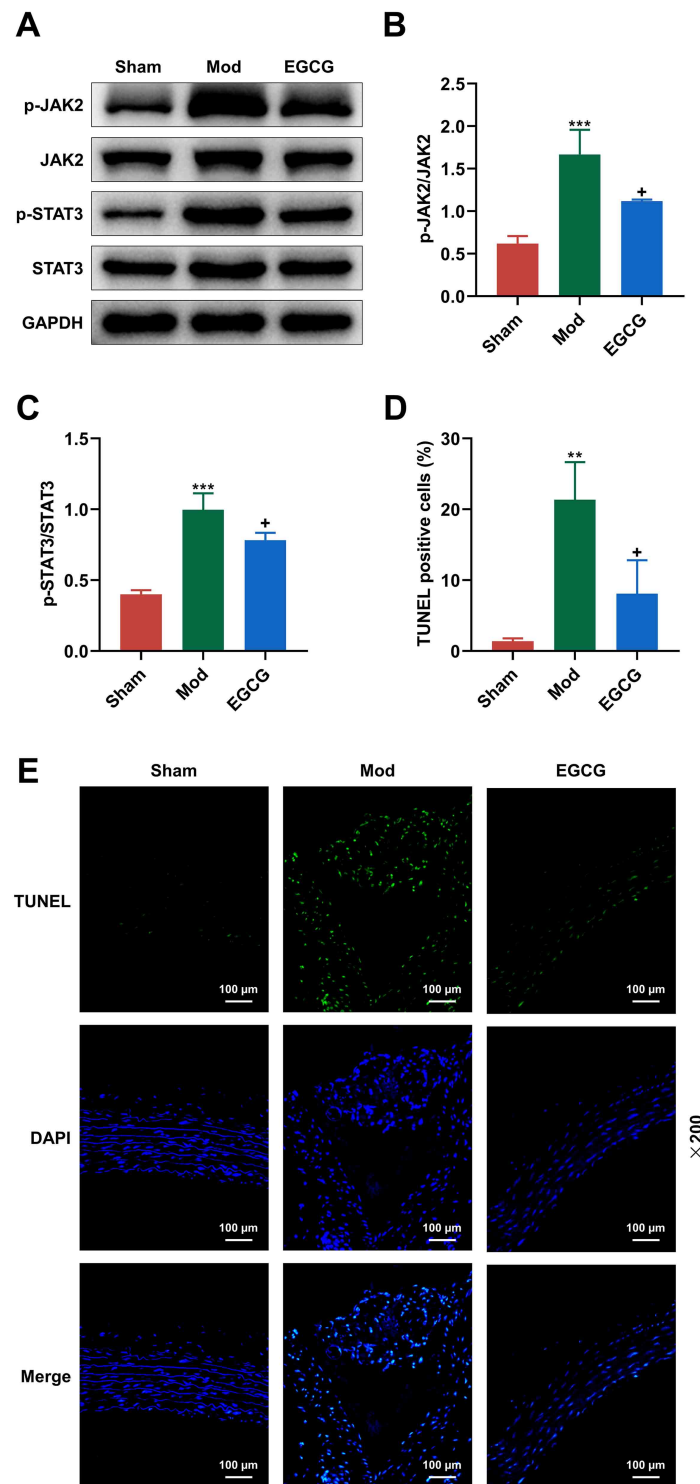


Fig. 2. The effect of EGCG on Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling and abdominal aortic tissue damage in AAA *in vivo*. (A–C) Western blot was used to analyze the activation of JAK2/STAT3 signaling pathway in the abdominal aorta of rats with or without AAA modeling or/and EGCG administration. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control. (D,E) TdT-mediated dUTP nick-end labeling (TUNEL) assay was employed to examine cell apoptosis (green) in abdominal aorta of rats with or without AAA modeling or/and EGCG administration ($\times 200$ magnification, scale bar: 100 μm). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, blue) staining of nuclei. For Western blot and TUNEL analyses of aortic tissues, samples from 3 rats per group were used. Data are presented as mean $\pm$ SD ($n = 3$ rats per group for Western blot and TUNEL; $n = 6$ rats per group for aortic diameter measurements). ** $p < 0.01$, *** $p < 0.001$, vs. Sham; + $p < 0.05$, vs. Mod.

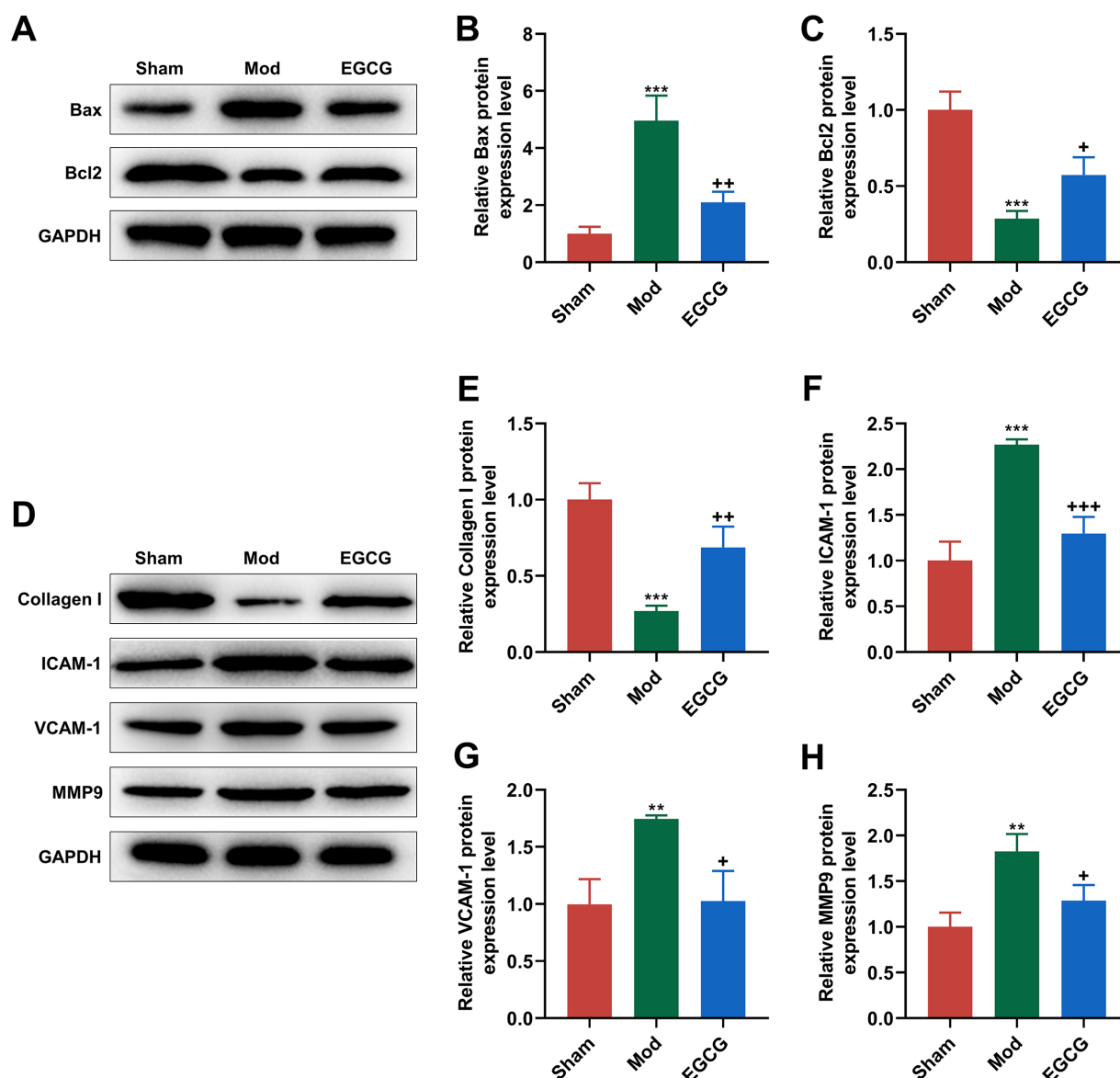


Fig. 3. The effect of EGCG on apoptosis- and extracellular matrix (ECM)-related proteins in AAA rat model. (A–H) Protein levels of apoptosis markers (BCL2 associated X (Bax), B cell lymphoma 2 (Bcl2)) and ECM-related molecules (Collagen I, Inter cellular Adhesion Molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and matrix metalloproteinase 9 (MMP9)) in the abdominal aorta of rats with or without AAA modeling or/and EGCG administration were measured by western blotting. GAPDH served as the loading control. Data are presented as mean $\pm$ SD (n = 3 rats per group). ** p < 0.01, *** p < 0.001, vs. Sham; + p < 0.05, ++ p < 0.01, +++ p < 0.001, vs. Mod.

EGCG Administration Alleviated ECM Degradation in the Abdominal Aorta of AAA Rat Model

To determine the effect of EGCG on ECM degradation during AAA development, we subsequently measured the expression of Collagen I, MMP9 and the adhesion molecules (ICAM-1, VCAM-1) in the rat abdominal aorta. As shown in Fig. 3D–H, the protein level of Collagen I was decreased, whereas those of ICAM-1, VCAM-1 and MMP9 were increased in the Mod group (p < 0.01). These changes were counteracted by EGCG administration (p < 0.05).

EGCG Treatment Suppressed JAK/STAT3 Signaling Pathway, Cell Apoptosis and MMP9 Expression in Ang II-Induced RASMCs

RASMCs were treated with different concentrations of EGCG for 0, 6, 12 and 24 h, and no difference in cell viability was observed following treatment with 20, 40 or 80 μ g/mL EGCG (Fig. 4A). We then induced RASMCs with Ang II with or without EGCG treatment (2 h) for subsequent *in vitro* assays. At the molecular level, 12 h of Ang II induction markedly increased the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 and the expression of MMP9 and Bax,

but reduced Bcl2 expression in RASMCs (Fig. 4B–H, $p < 0.001$). These changes were partially reversed by EGCG at concentrations of 20, 40 and 80 $\mu\text{g/mL}$, although 20 $\mu\text{g/mL}$ EGCG had no significant effect on the p-JAK2/JAK2 and p-STAT3/STAT3 ratios or Bax and Bcl2 levels (Fig. 4B–H, $p < 0.05$). Likewise, qRT-PCR results showed that the Ang II-induced upregulation of MMP9 was inhibited by EGCG at concentrations of 20, 40 and 80 $\mu\text{g/mL}$ (Fig. 4I, $p < 0.01$). Taken together, 80 $\mu\text{g/mL}$ EGCG exerted the most pronounced inhibitory effects on JAK/STAT3 signaling, cell apoptosis and MMP9 expression in Ang II-induced RASMCs, and was therefore selected for subsequent experiments.

RO8191 Treatment Reversed the Effects of EGCG in Ang II-Induced RASMCs

To further investigate whether EGC regulates the JAK/STAT3 signaling pathway during AAA progression, we treated RASMCs with RO8191, an activator of JAK/STAT3 signaling, for 2 h. As shown in Fig. 5A,B, p-STAT3 positive staining (red) was observed in Ang II-induced RASMCs and was further enhanced by RO8191 pretreatment but attenuated by EGCG pretreatment. Moreover, the inhibitory effect of EGCG on p-STAT3 positive staining in Ang II-induced RASMCs was reversed by RO8191. Furthermore, the Ang II-induced increases in the p-JAK2/JAK2 and p-STAT3/STAT3 ratios and MMP9 and Bax expression, as well as the decrease in Bcl2 expression, were further enhanced by RO8191 (Fig. 6A–G, $p < 0.05$), whereas these effects were attenuated by EGCG (Fig. 6A–G, $p < 0.05$). Compared with the EGCG group, pretreatment with RO8191 also reversed the regulatory effects of EGCG on the p-JAK2/JAK2 and p-STAT3/STAT3 ratios and MMP9, Bax and Bcl2 expression (Fig. 6A–G, $p < 0.05$). Similarly, the expression of MMP9 was higher in RASMCs pretreated with RO8191 and induced with Ang II than in those treated with Ang II alone (Fig. 6H, $p < 0.001$), and this increase was counteracted by EGCG (Fig. 6H, $p < 0.001$). In addition, the EGCG-induced downregulation of MMP9 in Ang II-induced RASMCs was reversed by combined RO8191 pretreatment (Fig. 6H, $p < 0.001$).

Discussion

Although the pathogenesis of AAA remains incompletely understood, it is well established that the loss of elasticity of the abdominal aortic wall play a key role in progressive aortic dilation and AAA progression [1]. Therefore, preserving and enhancing arterial wall elasticity represents an important therapeutic strategy for slowing AAA progression.

An appropriate AAA animal model is essential for investigating disease pathogenesis, diagnosis and treatment. Anidjar et al. [25] were the first to establish a rat model of AAA using trypsin infusion, which mimicked the patho-

physiological features of AAA by inducing vascular wall inflammation through elastin degradation. Subsequently, Tanaka et al. [19] established a rat model of AAA using intraluminal elastase infusion combined with extraluminal CaCl_2 exposure. By taking advantage of the high affinity of vascular wall elastin for Ca^{2+} , this method induces Ca^{2+} deposition and accelerates the inflammatory response, thereby shortening the elastase exposure time and reducing the failure rate of AAA induction. Based on abdominal aortic diameter measurements and HE and EVG staining, we observed marked arterial diameter expansion, increased intimal thickness, decreased medial thickness, and loss of elastic fibers in infrarenal abdominal aortic samples after AAA modeling using a combination of porcine pancreatic elastase infiltration and CaCl_2 exposure. These findings confirmed the successful establishment of the *in vivo* AAA model in the present study. A previous study by Setozaki et al. [18] reported that EGCG, a polyphenol derived from green tea, attenuated the development of AAA *in vivo* by preserving abdominal aortic wall thickness and promoting elastin regeneration in the medial layer, which is consistent with our current findings. More recently, Hosseini et al. [26] further suggested that phytochemicals, including EGCG, may have potential benefits in preventing AAA progression. However, the specific mechanisms underlying these effects of EGCG against AAA remains poorly understood.

It has been demonstrated that the loss of arterial wall elasticity is closely associated with ECM damage, including elastic fiber fragmentation and the degradation of collagen fibers and proteoglycans [1]. In particular, decreased elastin production in the medial layer of the vascular wall is considered a critical factor contributing to aortic dilation and rupture [27]. During AAA progression, the inflammatory response has been shown to promote elastin degradation and cell apoptosis by stimulating the secretion of MMPs, such as MMP2 and MMP9, from ASMCs and inflammatory cells [28]. Accumulating evidence has demonstrated that STAT3 activation plays an important role in intracellular inflammatory responses and tumorigenesis [29]. The JAK/STAT3 signaling pathway has been found to be abnormally activated in human AAA tissues [30], and Bernal et al. [31] showed that blockade of this signaling pathway could significantly alleviate the inflammatory response and preserve aortic elasticity in AAA. EGCG is well known for its natural anti-inflammatory activity and has been shown to exert protective effect against inflammatory diseases [32] and inhibit the progression of several tumors by suppressing p-STAT3 activation [33,34]. In this study, we first demonstrated that the elevated expression of p-JAK2 and p-STAT3 was suppressed by EGCG in a rat model of AAA, indicating that the JAK/STAT3 signaling pathway may be involved in the mechanisms underlying EGCG in AAA. It has been shown that MMPs are closely associated with degradation and rupture of the arterial wall

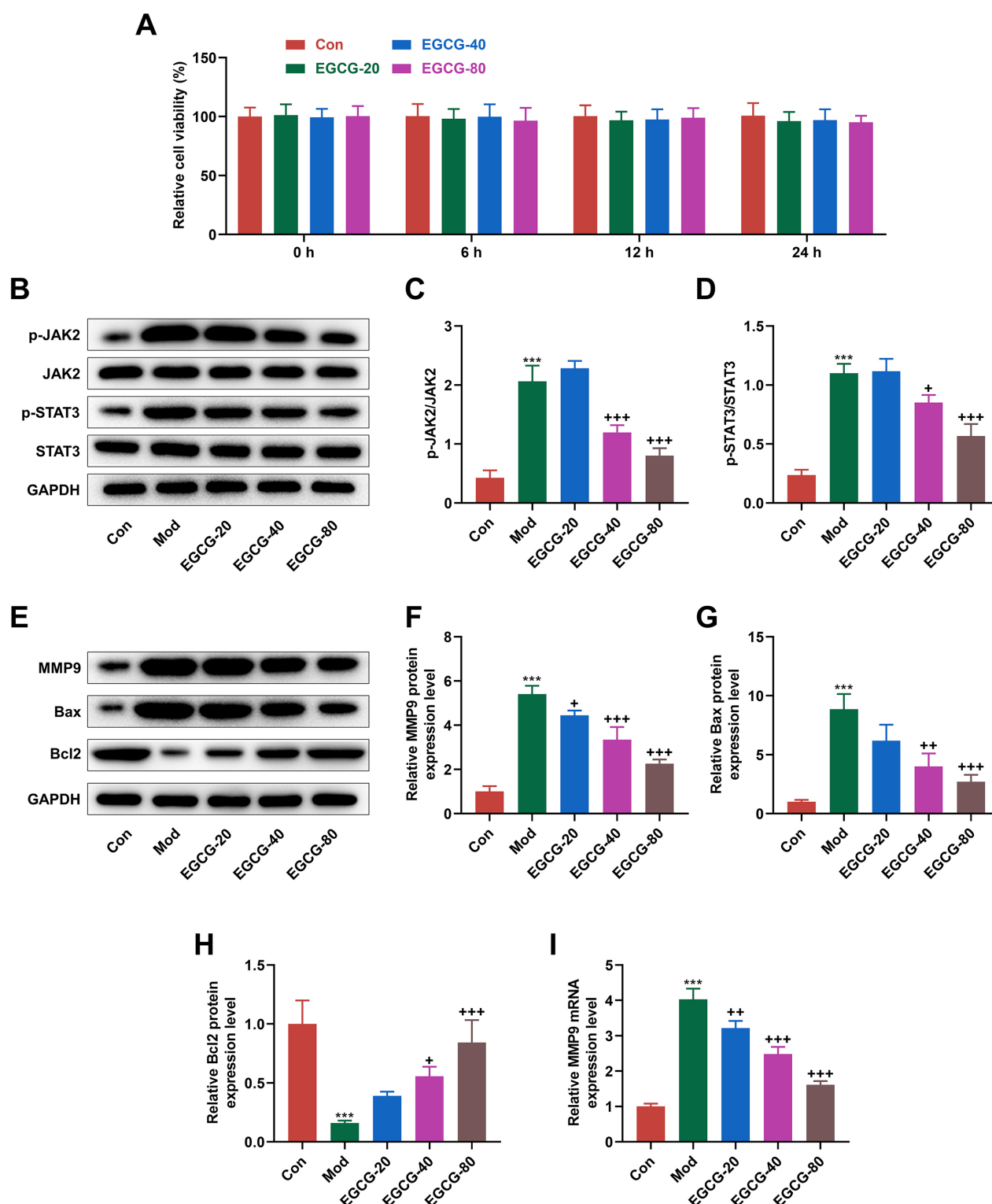


Fig. 4. The molecular mechanism of EGCG in Ang II-induced RASMCs. (A) Rat aortic smooth muscle cells (RASMCs) were treated with 20, 40 or 80 $\mu\text{g/mL}$ EGCG for 0, 6, 12 and 24 h. Cell counting kit-8 (CKK-8) assay was used to analyze the cell viability at these time points. (B–H) RASMCs were pre-treated with 20, 40 or 80 $\mu\text{g/mL}$ EGCG for 2 h and exposed to Ang II for 12 h. Western blotting was subsequently performed to determine the protein levels of phosphorylated (p)-JAK2, JAK2, p-STAT3, STAT3, MMP9, Bax and Bcl2 in RASMCs, and (I) the expression of MMP9 was determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). GAPDH served as the internal control. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). *** $p < 0.001$, vs. Con; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, vs. Mod.

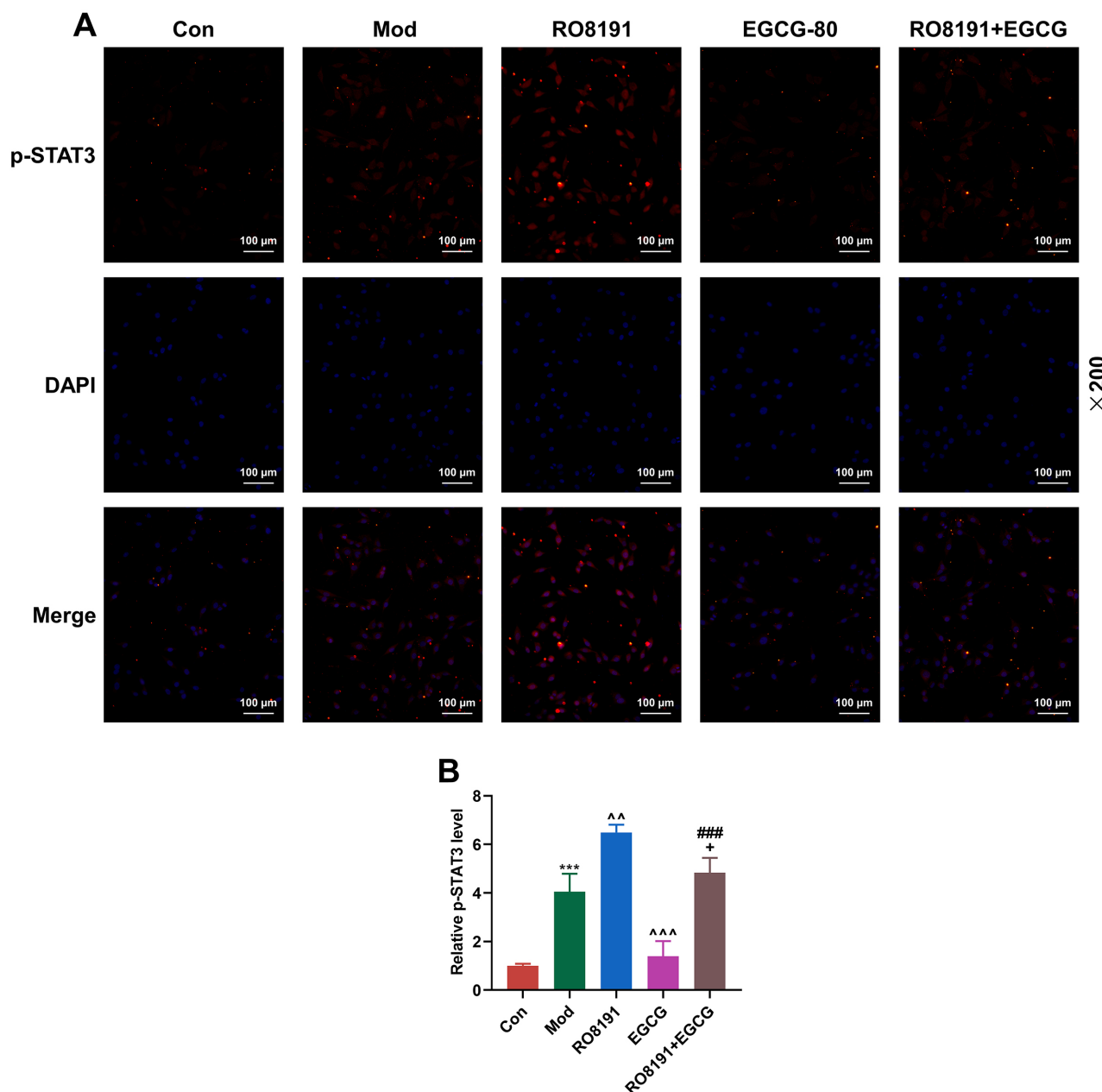
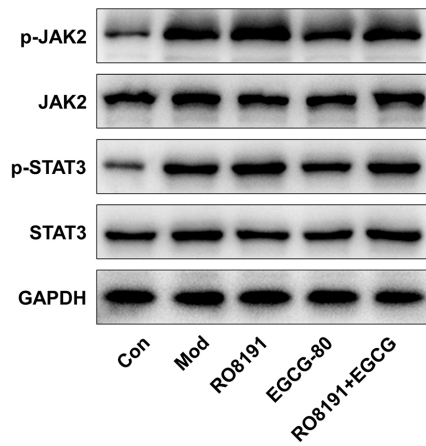


Fig. 5. Activation of p-STAT3 by RO8191 in Ang II-induced RASMCs reversed the effects of EGCG. (A) RASMCs were pre-treated with or without EGCG or/and RO8191 for 2 h before Ang II induction. Immunofluorescence assay was subsequently performed to detect p-STAT3 positive staining (red) in the cells ($\times 200$ magnification, scale bar: 100 μm). DAPI (blue) staining of nuclei. (B) Quantitative analysis of p-STAT3 fluorescence intensity. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). *** $p < 0.001$, vs. Con; ^^ $p < 0.01$, ^^ $p < 0.001$, vs. Mod. + $p < 0.05$, vs. RO8191; ### $p < 0.001$, vs. EGCG.

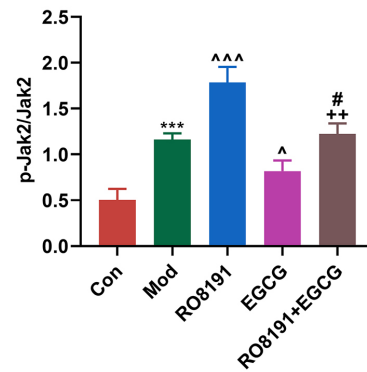
in AAA, among which MMP9 can degrade elastin, Collagen I, Collagen IV and fibrinogen [8,9]. Interestingly, a recent study by Yao et al. [13] reported that MMP9 transcription in AAA tissues was associated with STAT3 phosphorylation. Based on TUNEL assay and western blotting, we found that EGCG not only reduced cell apoptosis by down-regulating the pro-apoptotic protein Bax and up-regulating the anti-apoptotic protein Bcl2, but also increased the Collagen I expression and decreased the ex-

pression of ICAM-1, VCAM-1 and MMP9 in the abdominal aorta of AAA rats. Inflammation-induced cell adhesion and subsequent thinning of the aneurysmal wall are important contributors to the increased risk of AAA rupture [35]. ICAM-1 and VCAM-1 both belong to the immunoglobulin superfamily and are expressed in vascular endothelial cells, where they mediate cell-cell adhesion and promote the migration and chemotaxis of inflammatory cells [36]. Taken together, these findings suggest that EGCG may al-

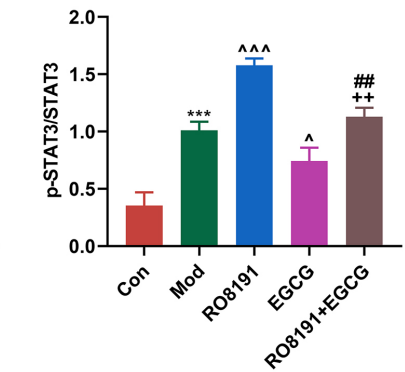
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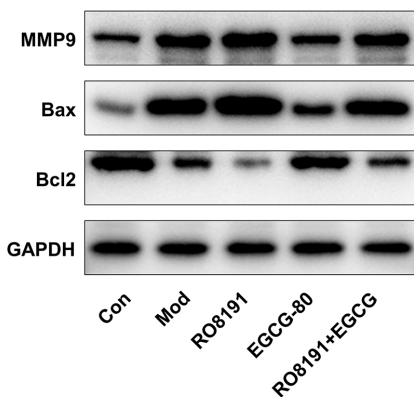
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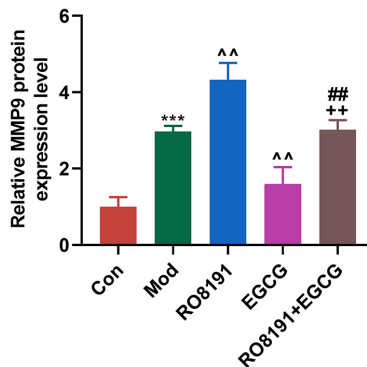
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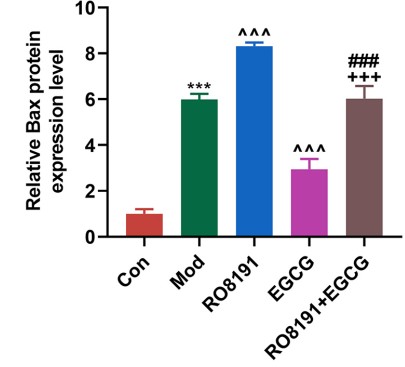
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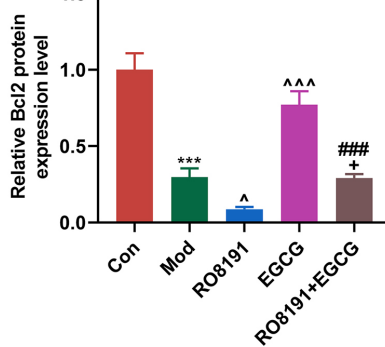
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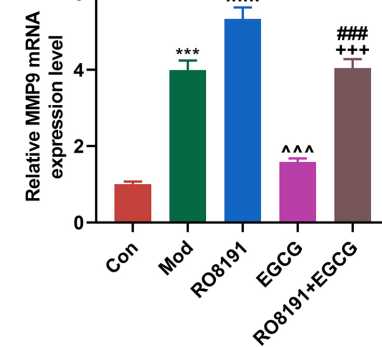


Fig. 6. Involvement of JAK2/STAT3 signaling pathway in the mechanism of EGCG in Ang II-induced RASMCs. (A–G) RASMCs were pre-treated with or without EGCG or/and RO8191 for 2 h before Ang II induction. Then, protein levels of p-JAK2, JAK2, p-STAT3, STAT3, MMP9, Bax and Bcl2 were examined by western blot, and (H) the expression of MMP9 was determined by qRT-PCR. GAPDH served as the internal control. Data are presented as mean $\pm$ SD (n = 3 independent experiments). *** $p < 0.001$, vs. Con; ^ $p < 0.05$, ^ $p < 0.01$, ^ $p < 0.001$, vs. Mod. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, vs. RO8191; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EGCG.

leviate aortic tissue damage, reduce MMP9-mediated ECM degradation and tissue adhesion in AAA, possibly through inhibition of the JAK/STAT3 signaling pathway.

ASMCs are the main cellular components of the medial layer of the aortic wall and contribute to ECM synthesis, which is essential for maintaining normal vascular

elasticity [37]. Apoptosis of ASMCs induced by proteolytic degradation and oxidative stress in the aortic wall has been implicated in AAA formation [38]. To further validate the mechanism of EGCG *in vitro*, we used Ang II induced RASMCs. We found that EGCG did not impair RASMCs viability and decreased the p-JAK2/JAK2 and p-

STAT3/STAT3 ratios as well as MMP9 and Bax expression, while increasing Bcl2 expression in Ang II-induced RASMCs. These findings suggest that EGCG may protect ASMCs against apoptosis in AAA by inhibiting the JAK/STAT3 signaling pathway. Moreover, RO8191, an activator of the JAK/STAT3 signaling pathway, was subsequently employed to further verify the involvement of this pathway in the effects of EGCG. Notably, RO8191-induced upregulation of MMP9 and enhancement of apoptosis in Ang II-induced RASMCs were counteracted by EGCG.

This study provides initial evidence that EGCG mitigates experimental AAA progression by inhibiting the JAK/STAT3/MMP9 pathway, suggesting a potential molecular basis for its use in AAA prevention. However, findings from rodent models and *in vitro* cell experiments cannot be directly extrapolated to humans because of species-specific differences, and the optimal EGCG administration strategy for AAA remains unclear. Future studies should validate these findings in large animal models and clinical trials and further explore the potential synergistic effects of EGCG with current AAA therapies for clinical translation. In addition, the *in vitro* rescue experiments with RO8191 further supported the involvement of the JAK/STAT3 pathway in the effects of EGCG, although the specificity of this mechanism requires further confirmation.

Conclusion

This study suggests that EGCG attenuates experimental AAA progression, potentially through inhibition of the JAK/STAT3 signaling pathway and the associated downregulation of MMP9 expression. Our findings provide preliminary molecular evidence supporting EGCG as a candidate for further investigation in AAA. Future studies are warranted to confirm the direct regulatory relationship between STAT3 and MMP9 using genetic approaches, evaluate therapeutic EGCG administration regimens in established AAA, and validate these findings in large animal models and clinical trials.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

JHN and JGH designed the research study; all authors performed the research; JHN, JGH and ZY collected and analyzed the data. XYZ contributed to conceptualization, investigation, and visualization. ZY and XYZ have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the

work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All animal-related experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the Zhejiang Center of Laboratory Animals (Approval No. ZJCLA-IACUC-20020226) and performed according to the guidelines of the China Council on Animal Care and Use. This study was reported in accordance with the ARRIVE Guidelines 2.0.

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Conflict of Interest

The authors declare no conflict of interest.

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