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Gallic acid attenuates pulmonary fibrosis in a mouse model of transverse aortic contraction-induced heart failure

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ABSTRACT

Gallic acid, a trihydroxybenzoic acid found in tea and other plants, attenuates cardiac hypertrophy, fibrosis, and hypertension in animal models. However, the role of gallic acid in heart failure remains unknown. In this study, we show that gallic acid administration prevents heart failure-induced pulmonary fibrosis. Heart failure induced in mice, 8 weeks after transverse aortic constriction (TAC) surgery, was confirmed by echocardiography. Treatment for 2 weeks with gallic acid but not furosemide prevented cardiac dysfunction in mice. Gallic acid significantly inhibited TAC-induced pathological changes in the lungs, such as increased lung mass, pulmonary fibrosis, and damaged alveolar morphology. It also decreased the expression of fibrosis-related genes, including collagen types I and III, fibronectin, connective tissue growth factor (CTGF), and phosphorylated Smad3. Further, it inhibited the expression of epithelial-mesenchymal transition (EMT)-related genes, such as N-cadherin, vimentin, E-cadherin, SNAI1, and TWIST1. We suggest that gallic acid has therapeutic potential for the treatment of heart failure-induced pulmonary fibrosis.

1. Introduction

Heart failure is a fatal condition that leads to cardiac dysfunction. Transverse aortic constriction (TAC) is used to experimentally induce cardiac hypertrophy and heart failure in animals [1]. TAC induces left ventricular hypertrophy and cardiac dysfunction after 6 weeks [2]. Chronic TAC causes severe pulmonary fibrosis, inflammation, and alveolar remodeling [3]. The weight of the lungs increases in TAC-induced heart failure. These reports suggest that TAC induces severe pulmonary diseases. The TAC animal model mimics pulmonary hypertension due to left heart disease. Pulmonary hypertension is classified into five categories: pulmonary artery hypertension, pulmonary hypertension due to left heart disease, pulmonary hypertension due to lung diseases or hypoxia or both, chronic thromboembolic pulmonary hypertension, and pulmonary hypertension with unclear multifactorial mechanisms [4]. Several studies have reported that the prevalence of pulmonary hypertension caused by left heart diseases is approximately 70%, and it was measured by using echocardiography [5–7]. Pulmonary fibrosis is associated with pulmonary hypertension [8,9]. The phosphodiesterase 5 inhibitor sildenafil and the soluble guanylate cyclase stimulator riociguat ameliorated pulmonary hypertension due to left heart diseases in a TAC mouse model [2]. The antioxidant peptide SS-31 attenuated TAC-induced pulmonary arterial hypertension in mice [10].

Many drugs clinically used to treat or delay heart failure, such as angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), beta-blockers, aldosterone antagonists, and vasodilators [11], have been proven to be effective in regulating heart failure-induced remodeling [12].

Despite significant advancement in the development of drugs for heart failure, the mortality rate is relatively high. Recently, therapy by neurohormonal inhibition has been proven effective against chronic heart failure [13,14]. Additionally, dietary phytochemicals have been reported to protect against a variety of pathological diseases [15]. Resveratrol found in grape wine protects against pressure overload-induced heart failure [16]. Imperatorin, a dietary furanocoumarin,

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prevents cardiac hypertrophy and the transition to heart failure in a TAC mouse model [17]. The natural compound, curcumin has shown potential as a novel therapeutic agent against heart failure [18].

Gallic acid, a trihydroxybenzoic acid found in tea and other plants, attenuates vascular calcification [19], cardiac hypertrophy [20], cardiac fibrosis [20], and hypertension. However, the effect of gallic acid on heart failure is not determined. Furthermore, given that heart failure accompanies pulmonary edema, we also investigated the effect of furosemide (a diuretic) on TAC-induced pulmonary fibrosis.

The aim of this study was to evaluate the effect of gallic acid on pulmonary fibrosis and pathological remodeling in TAC-induced heart failure. In this study, we show for the first time that gallic acid inhibits pulmonary fibrosis, expression of related marker genes, and epithelial-mesenchymal transition (EMT). We observed that gallic acid attenuated heart failure-induced pathological changes in the pulmonary tissues and heart dysfunction. Our findings suggest that gallic acid could be used as a potential therapeutic agent for the treatment of pathological pulmonary fibrosis.

2. Materials and methods

2.1. TAC and administration of gallic acid or furosemide

All animal procedures were approved by the Animal Experimental Committee of Chonnam National University Medical School (CNU IACUC-H-2015-52) and carried out according to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publications, 8th edition, 2011). CD-1 male mice (8-week-old, 30–35 g) were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (14 mg/kg). The surgical procedure was performed as reported previously [21]. After endotracheal intubation, the transverse aortic arch was ligated (7–0 silk suture) between the brachiocephalic and left common carotid arteries with an overlaying 27 G needle. Mice in the sham group underwent the same procedure except for TAC. Gallic acid (100 mg/kg/day), furosemide (3 mg/kg/day), or vehicle (DMSO) was intraperitoneally administered daily to TAC mice for 2 weeks. The induction of TAC-induced heart failure after 8 weeks was confirmed by echocardiography that was carried out every 2 weeks. After induction, we evaluated heart function every week using echocardiography.

2.2. Histology and Masson's trichrome staining

The paraffin-embedded tissues were sectioned 3 μm thick, deparaffinized with xylene, and then rehydrated with different grades of alcohol. Hematoxylin and eosin (H & E) staining was performed as described [22]. The arterial wall thickness was measured using NIS Elements Software (Nikon, Japan). Masson's trichrome staining was performed as described previously [23].

2.3. Echocardiography

Echocardiography was performed using a Vivid S5 echocardiography system (Vivid S5, GE Healthcare, USA) with a 13 MHz linear array transducer. The procedures were carried out on mice after anesthetizing them with tribromoethanol (Avertin, 114 mg/kg intraperitoneal injection). M-mode (2-D guided) images and recordings were acquired from the long axis view of the left ventricle at the level of the papillary muscles. The thickness of the anterior and posterior wall was measured from the images whereas the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured from the M-mode recordings. Fractional shortening (FS) was calculated as FS (%) = (LVEDD − LVESD) × 100/LVEDD.

Table 1
Primers for reverse transcription-polymerase chain reaction (RT-PCR).

Gene (mouse)	Primer sequence (5′ to 3′)
GAPDH	F: GCATGGCCTTCCGTGTTCCT
	R: CCCTGTTGCTGTAGCCGTATTCAT
Collagen type I	F: GAGCGGAGAGTACTGGATCG
	R: GCTTCTTTTCCTTGGGGTTC
Collagen type III	F: TGATGGAAAACCAGGACCTC
	R: CAGTCTCCCCATTCTTTCCA
Fibronectin	F: GATGCACCGATTGTCAACAG
	R: TGATCAGCATGGACCACTTC
CTGF	F: CAAAGCAGCTGCAAATACCA
	R: GGCCAAATGTGTCTTCCAGT
Smad3	F: CACAGCCACCATGAATTACG
	R: GTGTTCTCGGGAATGGAATG
N-cadherin	F: AGTTTCTGCACCAGGTTTGG
	R: TGATGATGTCCCCAGTCTCA
Vimentin	F: AAGGAAGAGATGGCTCGTCA
	R: TTGAGTGGGTGTCAACCAGA
E-cadherin	F: CGGAGAGGAGAGTCGAAGTG
	R: CATGCTCAGCGTCTTCTCTG
SNAI1	F: GAGGACAGTGGCAAAAGCTC
	R: CCAGGCTGAGGTACTCCTTG
TWIST1	F: CAGCGGGTCATGGCTAAC
	R: CAGCTTGCCATCTTGGAGTC

2.4. Reagents

Gallic acid (G7384) and furosemide (S1603) were purchased from Sigma (Billerica, MA, USA) and Selleckchem (Houston, TX, USA), respectively.

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the aortic tissue was isolated with the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA), and 1 μg of RNA was used for the reverse transcription reaction with TOPscript RT DryMIX (Enzynomics, South Korea). mRNA levels were quantified with the SYBR Green PCR kit (Enzynomics). The PCR primers used in this study are shown in Table 1.

2.6. Western blot

Total protein from lung tissues was extracted in a lysis buffer (RIPA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 mM Na_3VO_4, and 5 mM NaF) containing a protease inhibitor cocktail (Calbiochem, EMD Millipore, MA, USA). Proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST buffer (20 mM Tris, 200 mM NaCl, and 0.04% Tween 20) for 1 h at 25 °C. The membranes were incubated overnight at 4 °C with primary antibodies against collagen types I and III, fibronectin, connective tissue growth factor (CTGF), p-Smad3, Smad3, N-cadherin, E-cadherin, SNAI1, and β-actin. They were then incubated with the anti-rabbit or anti-mouse horseradish-peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Protein bands were visualized using Immobilon Western detection reagents (EMD Millipore, Billerica, MA, USA). The Bio-ID software was used to quantify protein expression (Vilber Lourmat, Eberhardzell, Germany).

2.7. Statistical analysis

All data are expressed as means ± standard errors (SE). Differences between data were analyzed by one-way analysis of variance (ANOVA) with the Bonferroni post hoc test using GraphPad Prism, version 5 and a value of $P < 0.05$ was considered significant.

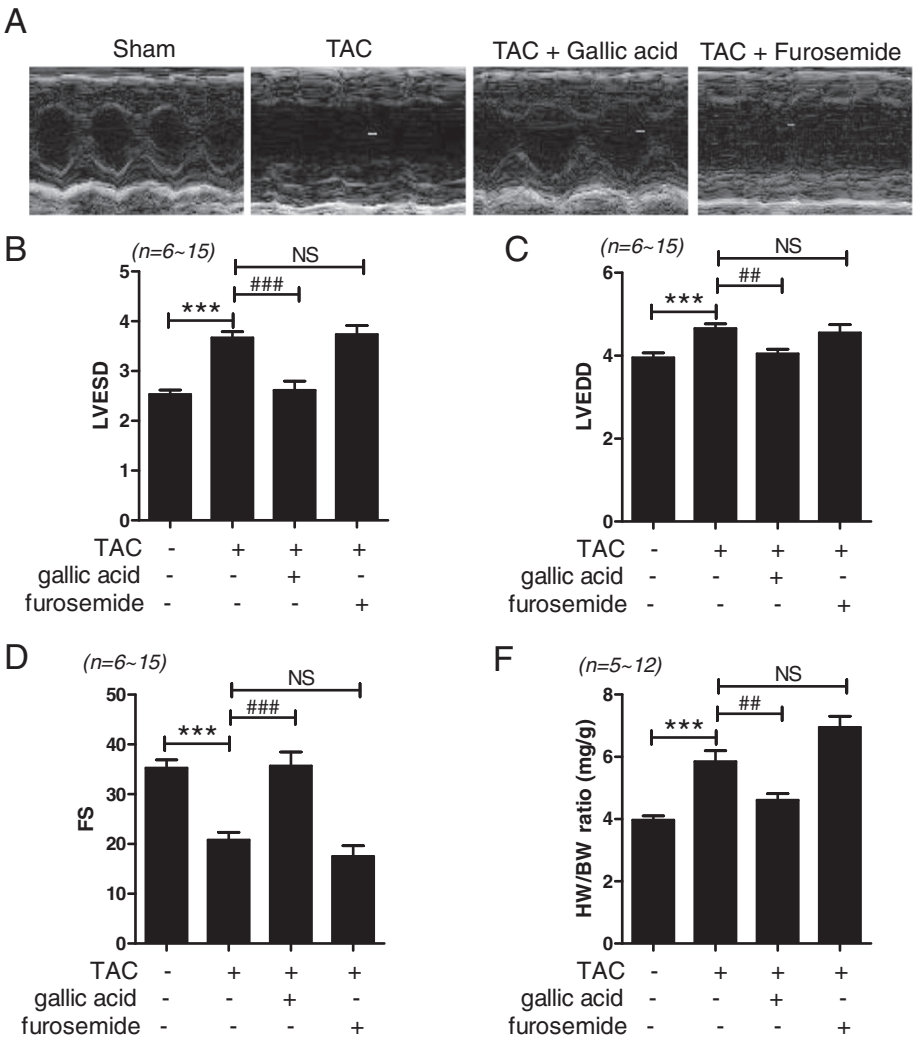

Fig. 1. Gallic acid attenuates heart dysfunction in the transverse aortic constriction (TAC)-induced heart failure model.

3. Results

3.1. Gallic acid attenuates heart dysfunction in the TAC-induced heart failure model

To identify whether gallic acid protects against TAC-induced heart failure, we evaluated the heart function by echocardiography. Increased left ventricular dimensions characterize heart failure. Echocardiography revealed that gallic acid treatment for 2 weeks inhibited the TAC-induced increase in the LVESD and LVEDD in the TAC + Gallic acid group; however, treatment with furosemide, a well-known diuretic, failed to do so (Fig. 1A–C). Fractional shortening (FS, %) in the TAC group was reduced compared to that in the sham group. Gallic acid administration restored the reduced FS in the TAC + Gallic acid group; however, furosemide did not affect FS (Fig. 1D). After 10 weeks of undergoing the TAC surgery, the heart weight to body weight (HW/BW) ratio was found to increase in the TAC group whereas gallic acid treatment significantly reduced this ratio. However, treatment with furosemide did not reduce the HW/BW ratio (Fig. 1F).

3.2. Gallic acid reduces TAC-induced increase in lung weight

To determine whether gallic acid suppresses TAC-induced lung disease, we determined the ratio of lung weight to body weight (LW/BW) and lung weight to tibia length (LW/TL). Increased LW/BW and LW/TL ratios were observed in the TAC group compared to that in the sham group (Fig. 2A–C). Gallic acid treatment significantly reduced these ratios; however, furosemide did not prevent the TAC-induced increase in these ratios (Fig. 2A–C).

3.3. Gallic acid prevents TAC-induced pathological alveolar remodeling and reduces collagen deposition in lung tissues

To investigate whether gallic acid affects TAC-induced morphological changes in lung tissues, we performed H & E staining. As shown in Fig. 3A, lung tissues of the sham group showed normal open alveolar spaces. However, those in the TAC group revealed collapsed alveolar spaces, an increase in type II pneumocytes (alveolar epithelium), and widening of the interalveolar septum. Treatment with gallic acid but not furosemide prevented these TAC-induced pathological changes in the lung tissues. Further, we examined collagen deposition that was stained blue by Masson's trichrome staining in the pulmonary interstitium. Pulmonary fibrosis was observed in the TAC group that exhibited greater collagen deposition than the sham group. Treatment with gallic acid and furosemide decreased pulmonary fibrosis (Fig. 3B).

3.4. Gallic acid suppresses pulmonary fibrosis in the TAC-induced heart failure model

To further confirm whether gallic acid inhibits pulmonary fibrosis in the TAC-induced heart failure model, we examined the expression of fibrosis-related marker genes by RT-PCR and western blot analysis. We

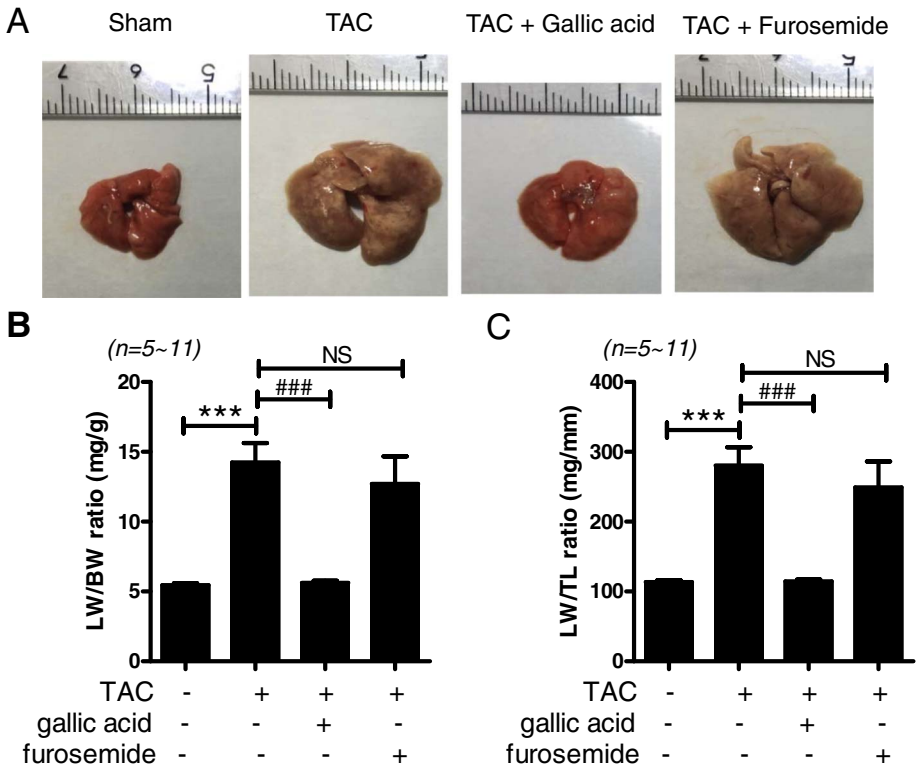

Fig. 2. Gallic acid inhibits the increase in lung size in the TAC-induced heart failure model.

found that gallic acid treatment reduced the mRNA levels of collagen types I and III, fibronectin, and CTGF (Fig. 4A–D). Smad3 is also reported to be involved in the development of pulmonary fibrosis [24]. However, no significant changes in Smad3 mRNA levels among the different groups were observed (Fig. 4E). Similar results were obtained by western blot analysis (Fig. 5A–D). Collagen type I, fibronectin, and CTGF protein levels increased in the lung tissues of the TAC group compared to those in the sham group. The protein levels of collagen type I and fibronectin were significantly reduced by treatment with gallic acid but not furosemide. However, the CTGF protein level was significantly reduced by treatment with both gallic acid and furosemide (Fig. 5D). Moreover, the phosphorylated Smad3 protein level increased in the lung tissues of the TAC group compared to that in the sham group; both gallic acid and furosemide decreased this level. However, the Smad3 protein level remained unaltered irrespective of the treatments (Fig. 5A).

3.5. Gallic acid suppresses TAC-induced EMT in the lung tissues

It is reported that EMT contributes to tissue fibrosis [25,26]. To investigate whether gallic acid affects EMT in pulmonary fibrosis, we evaluated EMT markers and related transcription factors by RT-PCR and western blot analysis. N-cadherin and vimentin mRNA levels increased in the TAC group compared to the those in the sham group. These levels were significantly reduced by gallic acid but not by furosemide treatment (Fig. 6A and B). Conversely, the E-cadherin mRNA level decreased in the TAC group compared to that in the sham group. However, gallic acid but not furosemide completely restored its expression (Fig. 6C). The transcription factor, SNAI1 is known to suppress E-cadherin expression [27,28]. Consequently, the expression of SNAI1 mRNA was upregulated in the TAC group compared to that in the sham group, as expected (Fig. 6D). In addition, the mRNA expression of TWIST1 increased in the TAC group; however, gallic acid reduced its mRNA expression (Fig. 6D and E). We assessed the protein levels of EMT markers by western blot analysis. As shown in Fig. 6F, protein levels of N-cadherin and SNAI1 increased due to TAC whereas they were reduced by gallic acid treatment. Further, the level of E-cadherin decreased in the TAC group, which was restored by gallic acid but not furosemide.

4. Discussion

In this study, we demonstrated that gallic acid could be used as a novel potential therapy for heart failure-induced pulmonary fibrosis. We observed that gallic acid attenuated heart failure-induced pulmonary fibrosis and structural remodeling as well as cardiac dysfunction in a mouse model of TAC-induced heart failure. Further, gallic acid significantly prevented the increase in lung weight.

Gallic acid has beneficial effects on isoproterenol-induced cardiac hypertrophy and fibrosis in mice [20]. It suppresses high blood pressure, cardiac remodeling, and cardiac fibrosis in N^G-nitro-L-arginine methyl ester-induced hypertension in mice [23]. Therefore, we hypothesized that gallic acid could treat heart failure and heart failure-induced pulmonary fibrosis. In this study, we demonstrated via H & E and Masson's trichrome staining that gallic acid administration prevents pulmonary fibrosis and pathological changes in the lungs. Heart failure is characterized by the increase in ventricular lumen and decrease in FS. We observed that TAC-induced heart failure increased the LVESD and LVEDD; however, they were reduced by gallic acid treatment.

Furosemide is used to treat edema and swelling caused by heart failure [29]. In the present study, we administered furosemide (3 mg/kg/day) daily for 2 weeks to mice that had undergone TAC surgery 8 weeks prior. In our experiment, furosemide did not prevent cardiac dysfunction and alveolar remodeling. However, it has been reported to prevent thiazolidinedione-induced cardiac hypertrophy in mice [30]. In combination with captopril, it improves cardiac remodeling in a rat model of heart failure induced by coronary ligation [31]. Contrastingly, furosemide did not act on heart failure in our experiment probably due to the difference in the animal model, dosage, and treatment duration.

Although the TAC model was used to induce heart failure, cardiac hypertrophy was observed after 10 weeks of the TAC surgery. TAC-induced increase in the HW/BW ratio was prevented by gallic acid but not

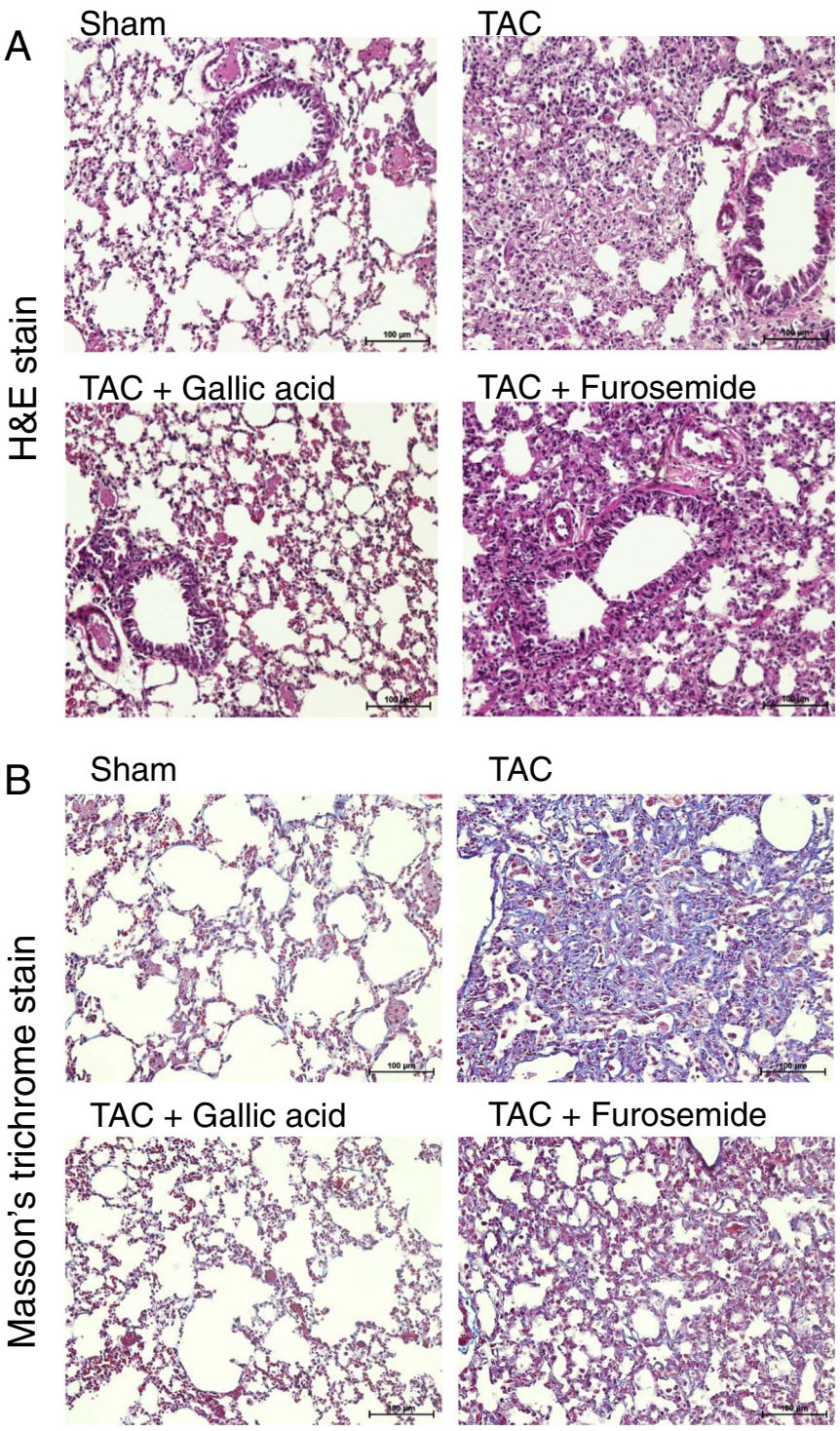

Fig. 3. Gallic acid reduces TAC-induced pathological alveolar remodeling and collagen deposition in the lung tissues.

furosemide, indicating that gallic acid is a better anti-hypertrophic agent than furosemide.

Heart failure induces profound alveolar remodeling and severe pulmonary fibrosis in mice [3]. In our experimental model, pulmonary diseases appeared after 10 weeks of TAC surgery. TAC increased the mass of the lungs, which was indicated by the increase in the LW/BW and LW/TL ratios. This increase was completely inhibited by gallic acid but not furosemide. Moreover, treatment with gallic acid but not with furosemide reduced TAC-induced morphological abnormalities in the lungs. The lung tissues in the TAC group revealed damaged alveolar spaces, abnormal growth of type II pneumocytes, and widening of the interalveolar septum. The beneficial effect of gallic acid in preventing pathological changes in the lung tissues suggests that it could be used as a therapeutic agent for pulmonary diseases secondary to left ventricular diseases. Clinically, pulmonary hypertension is a common complication in patients with heart failure [32]. Furthermore, TAC caused pulmonary fibrosis due to increased collagen deposition in the lung tissues as determined by Masson's trichrome staining; gallic acid reduced the collagen deposition. In addition, gallic acid treatment reduced the expressions of fibrosis-related marker genes, including collagen types I and III, fibronectin, CTGF, and vimentin. However, furosemide only reduced the expression of CTGF. Recently, we reported that gallic acid

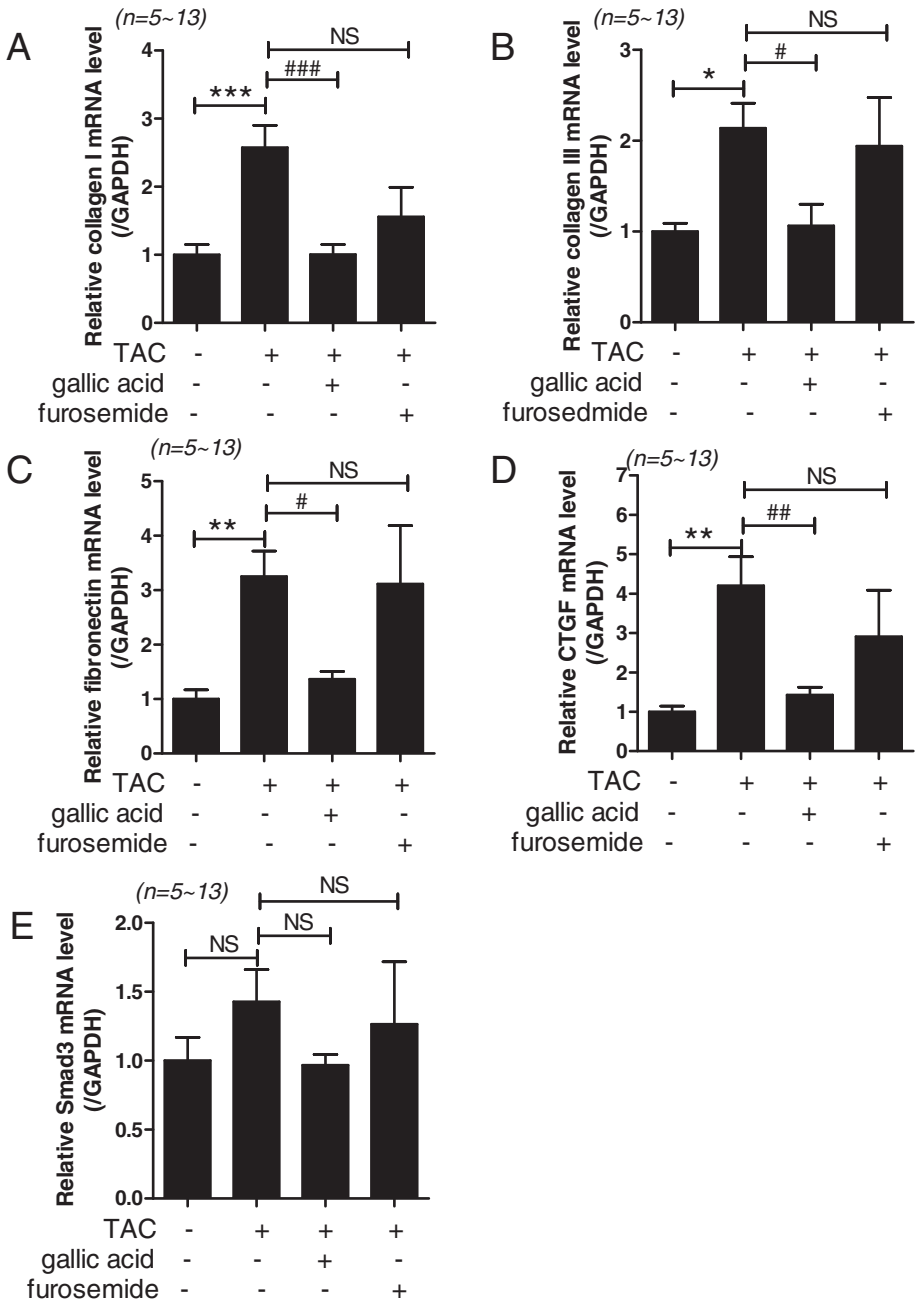

Fig. 4. Gallic acid reduces mRNA levels of fibrosis-related genes in the TAC-induced heart failure model.

suppresses cardiac fibrosis in an isoproterenol-induced hypertrophy mouse model, in which gallic acid decreased the phosphorylated Smad3 binding activity in the collagen type I promoter [20]. The transforming growth factor beta 1/Smad signaling pathway is crucial for the development of renal [33,34] and pulmonary fibrosis [35,36], and myofibroblast differentiation [37]. Gallic acid treatment decreased TAC-induced phosphorylated Smad3 protein levels, indicating that Smad signaling regulates pulmonary fibrosis. However, EMT may also cause pulmonary fibrosis, and we found that TAC reduced the mRNA and protein levels of E-cadherin, an epithelial cell marker, in the lung tissues; these levels were restored by gallic acid administration. Gallic acid also inhibited the TAC-induced N-cadherin expression, which is a mesenchymal cell marker. Vimentin and collagen type I are also mesenchymal cell markers. In addition, gallic acid treatment reduced TAC-induced SNAI1 and TWIST1 mRNA expressions. SNAI1 suppresses E-cadherin expression [38]. Consequently, EMT was inhibited by treatment with gallic acid but not furosemide, implying that gallic acid regulates EMT in pulmonary fibrosis caused by left ventricular disease. Specifically, gallic acid has the potential for treating pulmonary fibrosis induced by heart failure. Gallic acid has been reported to protect against bleomycin-induced pulmonary fibrosis in rats [39]. However, TAC-induced pulmonary fibrosis is quite different from bleomycin-induced pulmonary fibrosis. Bleomycin, a chemotherapeutic antibiotic, is commonly used to induce lung fibrosis in animal models [40]. Bleomycin directly causes fibrosis of the lung tissue, whereas TAC induces pulmonary fibrosis through left ventricular dysfunction. We need to further investigate the effect of gallic acid in a bleomycin-treated animal model that simulates the condition of patients with idiopathic pulmonary fibrosis.

Further, gallic acid induces apoptosis in primary cultured mouse lung fibroblasts [41]. Epigallocatechin-3-gallate in the green tea extract inhibited irradiation-induced pulmonary fibrosis in adult rats [42]. In addition, it protects against oxalate-induced EMT in renal tubular cells [43].

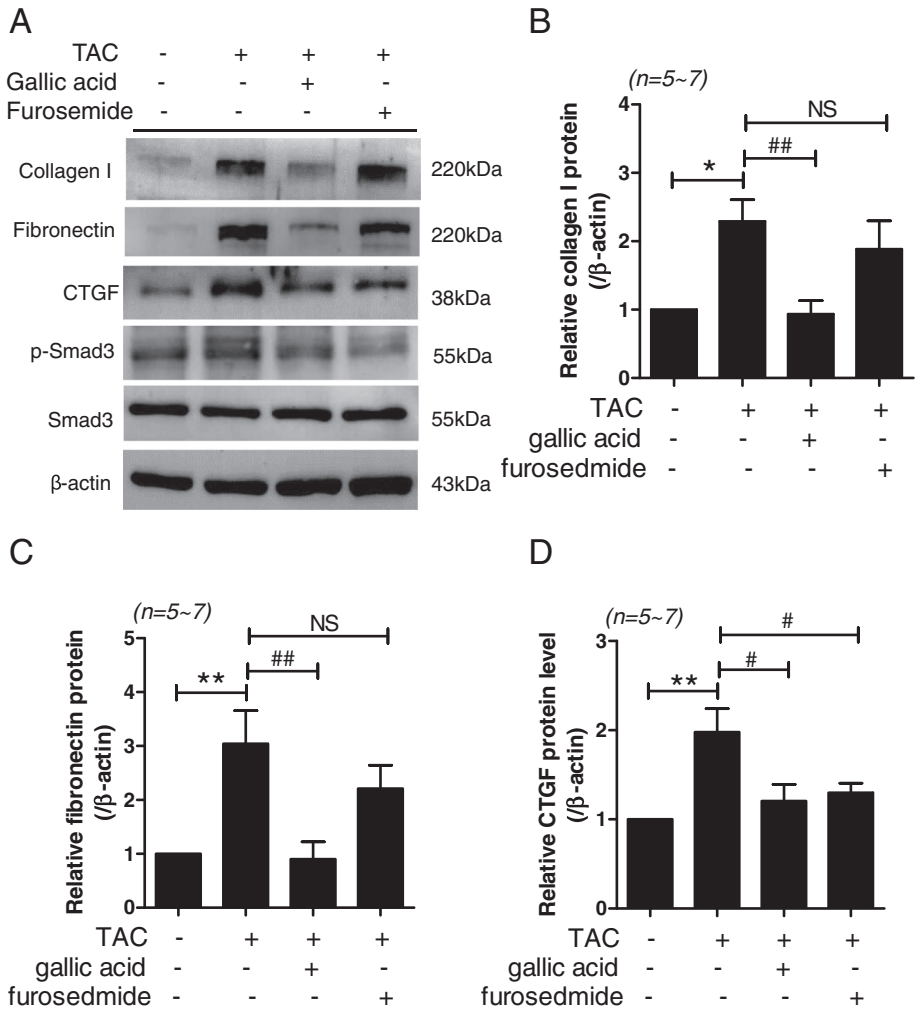

Fig. 5. Gallic acid reduces protein levels of fibrosis-related genes in the TAC-induced heart failure model.

In summary, our results demonstrate that gallic acid treatment attenuates cardiac dysfunction and hypertrophy as well as pulmonary fibrosis and remodeling in a mouse model of TAC-induced heart failure. Gallic acid showed better protection against pulmonary fibrosis than the diuretic, furosemide. We suggest that gallic acid could be used as a therapeutic agent for treating severe pulmonary diseases and heart dysfunction caused due to heart failure.

Disclosures

None.

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Heart failure was induced 8 weeks post TAC surgery in mice, after which they were administered either gallic acid or furosemide for 2 weeks. (A) Representative M-mode echocardiograms 10 weeks after TAC surgery (B) Left ventricular end-systolic diameter (LVESD) (C) Left ventricular end-diastolic diameter (LVEDD) (D) Fractional shortening (FS, %) (F) Heart weight to body weight ratio (HW/BW). $^{***}P < 0.001$ versus the sham group. $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the TAC group. NS indicates not significant.

(A) Representative photographs of the isolated lungs. The mice were randomized into 4 groups: Sham, TAC, TAC + Gallic acid, and TAC + Furosemide. A single division on the ruler indicates 1 mm (B) Lung weight to body weight ratio (LW/BW) (C) Lung weight to tibia length ratio (LW/TL) $^{***}P < 0.001$ versus the sham group. $^{\#\#\#}P < 0.001$ versus the TAC group. NS indicates not significant.

Heart failure was observed 8 weeks post TAC surgery in mice, after which they were administered either gallic acid or furosemide for 2 weeks. (A) Representative H & E stained images from the sham, TAC, TAC + Gallic acid, and TAC + Furosemide groups. Scale bar = 100 μm. (B) Representative Masson's trichrome staining of lung tissue sections from the sham, TAC, TAC + Gallic acid, and TAC + Furosemide groups. Blue staining indicates collagen deposition. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(A–E) Transcript levels for collagen types I and III, fibronectin, CTGF, and Smad3 in the 4 different groups are shown normalized to that of GAPDH. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the TAC group. NS indicates not significant.

Mice in the sham group were administered the vehicle, whereas those in the TAC group were administered either gallic acid or furosemide for 2 weeks. (A) Representative western blot images of protein abundance in the lungs tissues. Antibodies against collagen types I and III, fibronectin, p-Smad3, Smad3, CTGF, and β-actin were used. (B–D)

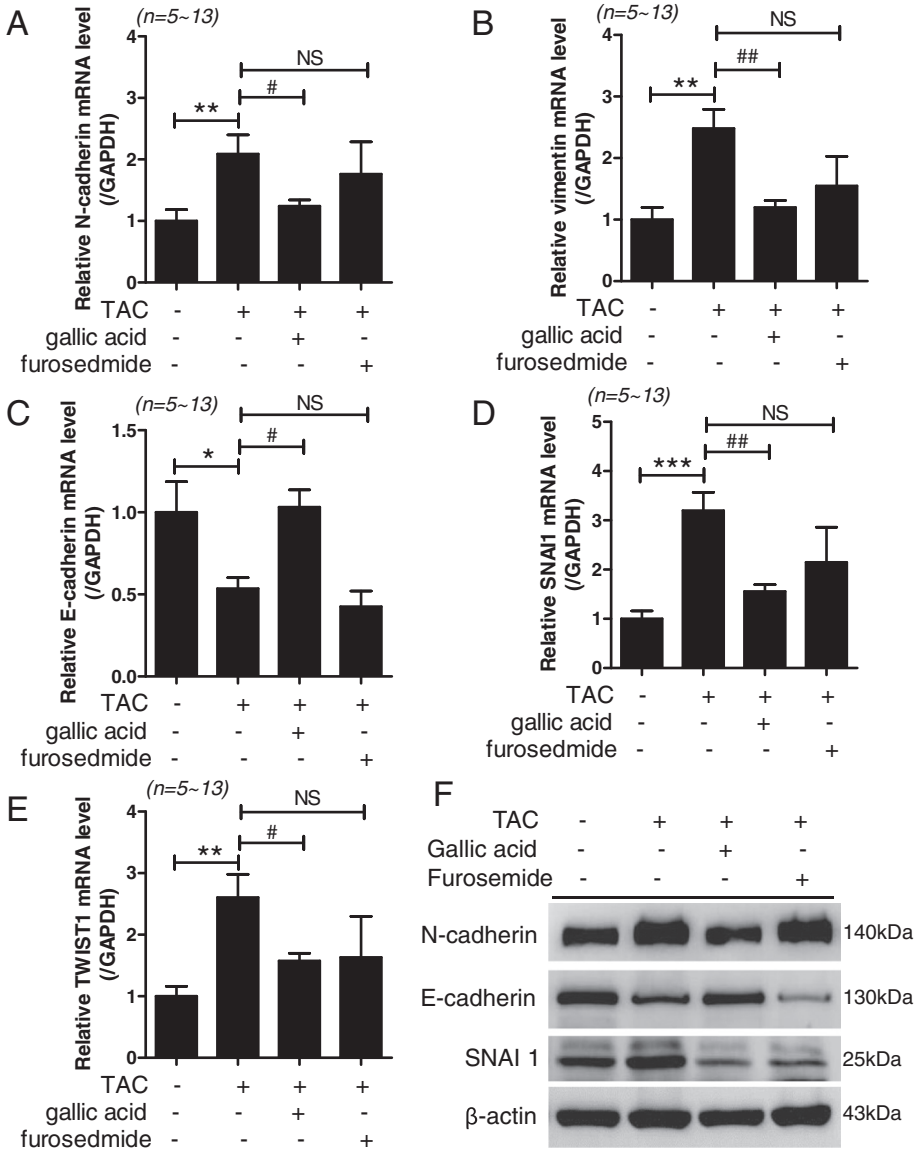

Fig. 6. Gallic acid inhibits epithelial-mesenchymal transition (EMT) in the TAC-induced heart failure model.

The bands were quantified by densitometry. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the TAC group. NS indicates not significant.

(A–E) Heart failure was observed after 8 weeks post TAC surgery in mice, after which they were administered either gallic acid or furosemide for 2 weeks. Transcript levels for N-cadherin, vimentin, E-cadherin, SNAI1, and TWIST1 in the 4 different groups are shown normalized to that of GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the TAC group. NS indicates not significant. (F) Representative immunoblots. Antibodies for N-cadherin, E-cadherin, SNAI1, and β-actin were used.

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