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Danggui Niantong decoction ameliorates joint inflammation and cardiopulmonary injury in TNF-Tg mice

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Abstract

Objective: Rheumatoid arthritis (RA) is a common autoimmune disease characterized by multiple joint lesions and systemic complications. Danggui Niantong decoction (DGNTT) has been clinically used for RA treatment; however, its beneficial effect on cardiopulmonary complications has not been reported.

Methods: Female tumor necrosis factor-transgenic (TNF-Tg) mice were used to evaluate the therapeutic effects of DGNTT on arthritis and cardiopulmonary complications. Methotrexate (MTX) served as a positive control. Histopathological assessment of the joint sections was performed using hematoxylin and eosin (HE), Alcian Blue/Orange G, and tartrate-resistant acid phosphatase staining. Bone mass was assessed by micro-computed tomography, inflammatory infiltrates in the heart and lungs were evaluated by HE staining, cardiopulmonary fibrotic injury was identified by Masson's trichrome staining, and hypertrophy of mouse cardiomyocytes was measured by wheat germ agglutinin (WGA) staining.

Results: DGNTT mitigated the inflammation of the ankle joint synovium, decreased the number of osteoclasts, and increased the area of cartilage and bone mass in TNF-Tg mice. In addition, DGNTT decreased the infiltration of inflammatory cells into the lung and heart tissues, accompanied by a reduction in cardiopulmonary fibrosis and myocardial cell hypertrophy in TNF-Tg mice. As a positive control drug, MTX attenuated the pathological changes in joints, but had no beneficial effect on cardiopulmonary inflammation and fibrosis in TNF-Tg mice.

Conclusions: DGNTT improved joint lesions and alleviated cardiopulmonary complications in TNF-Tg mice.

Keywords: Cardiovascular system disease, Danggui Niantong decoction, Interstitial lung disease, Rheumatoid arthritis, TNF-Tg mice

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease associated with progressive disability and systemic complications that directly impair a patient's quality of life, and even shorten the patient's lifespan in severe cases[1]. Systemic complications of RA include interstitial lung disease (ILD), cardiovascular disease, sarcopenia, autoimmune liver disease, and cognitive dysfunction[2]. ILD and cardiovascular disease are the two leading causes of death in RA[3–5]. RA affects approximately 0.5% to 1% of the global population[6–7]. Approximately 30% of patients with RA develop RA-ILD, and the median survival time of patients with RA-ILD is approximately 2.6 to 3.5 years[8–9]. Mortality in patients with RA-ILD accounts for approximately 10% to 20% of RA-related mortality[10]. In addition, compared to healthy individuals, patients with RA have 48%, 68%, and 41% higher risks of cardiovascular disease, myocardial infarction, and stroke, respectively[11]. These cardiovascular complications result in a 50% higher risk of mortality in RA patients than that in the general population[12].

Cardiopulmonary complications contribute to high mortality rates in patients with RA. Therefore, it is equally important to alleviate joint symptoms and complications. However, drugs for treating cardiopulmonary complications in RA are still lacking. The drugs commonly used clinically to treat RA include nonsteroidal

anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids, and anti-tumor necrosis factor (TNF)-alpha agents[1]. Although these drugs have shown improved efficacy in the joint manifestations of RA, their role in alleviating cardiopulmonary complications is limited, and some may cause or exacerbate cardiopulmonary complications such as lung infection[5], hypertension[13], and myocardial infarction[14]. For example, methotrexate (MTX), the most widely used initial DMARD monotherapy or combination therapy for RA, causes pulmonary function limitations and toxicity[15]. TNF inhibitors have also been reported to increase the potential risk of ILD events and may aggravate the severity of preexisting ILD in patients with RA[16]. Currently, in addition to glucocorticoids with or without immunosuppressive therapy, drugs for the primary treatment of cardiopulmonary diseases are used to manage RA complications. For example, the anti-fibrotic drug nintedanib, which is used to treat RA-ILD, has been proven to slow down the rate of decline in forced vital capacity in patients with progressive fibrotic RA-ILD[17]. Similarly, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are primarily used in patients with RA hypertension[18]. However, the simultaneous use of these drugs and anti-rheumatic drugs increases the burden on the kidneys of patients and enhances adverse effects. Therefore, it is important to identify drugs that can simultaneously treat joint inflammation and cardiopulmonary complications.

In traditional Chinese medicine (TCM), Danggui Niantong decoction (DGNTT) was first recorded in Zhang Yuansu's *Medical Qi Yuan* and has been used for RA treatment since the Jin and Yuan Dynasties in China[19–20]. DGNTT was produced in China according to the quality control standards of the Chinese Pharmacopoeia. It was composed of *Notopterygium* incisum K.C. Ting ex H.T. Chang. (Qiang Huo), *Saposhnikovia divaricata* (Turcz.) Schischk. (Fang Feng), *Cimicifuga foetida* L. (Sheng Ma), *Pueraria montana* (Lour.) Merr. (Ge Gen), *Atractylodes macrocephala* Koidz. (Bai Zhu), *Atractylodes lancea* (Thunb.) DC. (Cang Zhu), *Angelica sinensis* (Oliv.) Diels. (Dang Gui), *Panax ginseng* C.A.Mey. (Ren Shen), *Glycyrrhiza uralensis* Fisch. (Gan Cao), *Sophora flavescens* Aiton. (Ku Shen), *Scutellaria baicalensis* Georgi. (Huang Qin), *Anemarrhena asphodeloides* Bunge. (Zhi Mu), *Artemisia capillaris* Thunb. (Yin Chen), *Polyporus umbellatus* Fries. (Zhu Ling), and *Alisma plantago-aquatica* L. (Zhe Xie). Previous studies have shown that DGNTT effectively alleviates joint inflammation, swelling, pain, and functional activity problems[21–22]. In addition, pharmacological studies have suggested that the beneficial effects of DGNTT on RA are related to its anti-inflammatory, analgesic, and anti-apoptotic effects[23–24]. However, whether DGNTT can reduce cardiopulmonary complications in RA is currently unknown. TNF-transgenic (TNF-Tg) mice express the human TNF-α gene on a C57BL/6 background and spontaneously develop erosive polyarthritis with several characteristics observed in patients with RA, as well as the pathological phenotypes of cardiopulmonary complications[25]. Female TNF-Tg mice are useful tools for dissecting the molecular mechanisms of the pathogenic process and evaluating the efficacy of novel therapeutic strategies for RA[26–28] and the cardiopulmonary complications of RA[29–31]. In the present study, we used TNF-Tg mice to evaluate the protective effect of DGNTT against the cardiopulmonary complications of RA.

Materials and methods

Experimental animals

The TNF-Tg mice with a C57BL/6 background (line 3,647) were a gift from Prof. Lian Pingxing (University of Rochester). This line of TNF-Tg mice carries one copy of the human TNF transgene and spontaneously develops inflammatory arthritis similar to RA[32]. The mice were bred and raised at the Shanghai Southern Model Animal Center in (specified pathogen-free-grade rearing environment). The temperature and humidity of the animal room were maintained at 25°C and 70%, respectively, with a light/dark cycle of 12 h. All animal manipulations followed the "Regulations on the Management of Laboratory Animals" approved by the Animal Regulations of the National Science and Technology Commission of China. All the experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Research Center of the Southern Model (ethical approval code: 2022-0031).

Preparation of DGNTT

DGNTT consists of 15 Chinese herbs (Table 1). All Chinese herbs were purchased from Long Hua Hospital, which is affiliated with the Shanghai University of TCM. The active ingredients of DGNTT were validated using high-performance liquid chromatography for quality control [Supplementary Materials, http://links.lww.com/AHM/A73]. The decoction was prepared by adding water 12 times the amount of 15 herbs, decocting for 30 min, and filtering to obtain filtrate 1. The residuce was boiled in the water eight times of its volume for 30 min and filtered to obtain filtrate 2. Filtrates 1 and 2 were mixed and concentrated to obtain DGNTT at a final concentration of 1.89 g/mL, which was packaged and stored in a refrigerator at –80°C for further use.

Table 1

Drug composition of Danggui Niantong decoction

Drug name	Amount (g)
Notopterygium incisum K.C. Ting ex H.T. Chang (Qiang Huo)	15
Saposhnikovia divaricata (Turcz.) Schischk (Fangfeng)	9
Cimicifuga foetida L. (Shengma)	3
Pueraria montana (Lour.) Merr. (Gegen)	6
Atractylodes macrocephala Koidz. (Baizhu)	3
Atractylodes lancea (Thunb.) DC. (Cangzhu)	9
Angelica sinensis (Oliv.) Diels. (Danggui)	9
Panax ginseng C.A.Mey. (Renshen)	6
Glycyrrhiza uralensis Fisch. (Gancao)	15
Sophora flavescens Aiton. (Kushen)	6
Scutellaria baicalensis Georgi. (Huangqin)	3
Anemarrhena asphodeloides Bunge. (Zhimu)	9
Artemisia capillaris Thunb. (Yinchen)	15
Polyporus umbellatus Fries. (Zhuling)	9
Alisma plantago-aquatica L. (Zhexie)	9

Other reagents

Formalin (10%) was purchased from Guangzhou Vigas Biotechnology Co., Ltd.; absolute ethanol was purchased from Shanghai Ling feng Chemical Reagent Co., Ltd.; xylene, hydrochloric acid, ammonia water, anhydrous sodium acetate, and fuchsin basic were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai); hematoxylin-Eosin (HE) were purchased from Nanjing Jian Cheng Technology Co., Ltd.; Masson's trichrome staining kit was purchased from Solarbio Technology Co., Ltd. (Beijing); Alcian Blue/Orange G (ABOG) solution, phloxin B, L-(+)-tartaric acid, and naphthol AS-BI phosphate were obtained from Sigma Co., Ltd. (American); phosphate-buffered saline was purchased from Medicago Co., Ltd. (Canadian); bovine serum albumin was purchased from Roche Pharmaceutical Co., Ltd. (Shanghai); Wheat germ agglutinin (WGA) antibody was purchased from Vector Laboratories Co., Ltd. (Shanghai); and anti-fluorescence quenching mounting solution (containing 4′, 6-diamidino-2-phenylindole [DAPI]) was purchased from Bi Yun Tian Biotechnology Co., Ltd. (Shanghai).

Experiment grouping and processing

Fifteen 3-month-old female TNF-Tg mice were randomly divided into Saline, DGNTT, and MTX groups. Non-transgenic littermates were considered the wild-type (WT) group, with five mice in each group. In the DGNTT group, mice were orally administrated 0.2 mL DGNTT solution (1.89 g/mL) once daily for 84 d. In the MTX group, 0.2 mL MTX (0.1 mg/mL) was administered twice weekly for 84 d. In the WT and saline groups, mice were intragastrically administered 0.2 mL of normal saline once daily for 84 d.

Detection indicators and methods

Micro- computed tomography bone microstructure analysis

All mice were sacrificed after 12 weeks of treatment and the ankle joints were isolated, fixed with 70% ethanol for 1 week, and soaked in 0.9% NaCl solution overnight. Micro-CT scanning was performed with a resolution of 15.6 μm at a voltage of 55 kV and a current of 72 μm. Scanco uses its own software. Three-dimensional structural and morphological measurements were reconstructed and analyzed systematically using CTAn and CTVox software (Bruker Instruments Ltd, Bruker).

Histopathological evaluation

After the mice were sacrificed, ankle joint, heart, and lung specimens were collected. The ankle joints were fixed in 10% formalin for 24 h at 25°C room temperature and decalcified with 10% ethylenediaminetetraacetic acid for 4 weeks; the decalcification solution was changed every 72 h. Decalcified tissues were dehydrated and embedded in paraffin. The hearts and lungs were fixed in 10% formalin for 24 h, washed with running water for 2 h, dehydrated, and embedded in paraffin. The embedded joints, heart, and lungs were serially sectioned at 3 to 5 μm sagittal and stained with HE. In addition, the ankle joints were stained with ABOG and tartrate-resistant acid phosphatase (TRAP). The heart and lungs were stained with Masson trichrome. All stained tissue sections were photographed using a VS120, and the images were analyzed using ImageJ software (National Institutes of Health, USA).

Immunohistochemistry

Heart paraffin sections were deparaffinized, and WGA staining was performed to evaluate the cardiomyocyte area. Mouse myocardial tissue slices were placed in 0.3% H_2O_2/MeOH solution for approximately 10 min, then in lemon salt buffer (0.01 mol/L), and boiled in a water bath at 95 to 98°C for 40 min. Prediluted WGA staining solution was added to each tissue and incubated at 37°C for 1 h in the dark. After incubation, the tissue was washed thrice for 5 min each. The slices were mounted with an anti-fluorescence quenching mounting solution (containing DAPI) and cardiomyocytes were observed under a fluorescence microscope. Finally, the cross-sectional area of the mouse cardiomyocytes was calculated using the ImageJ software.

Pulmonary fibrosis Ashcroft score

The standard Ashcroft scores[33] for pulmonary fibrosis are presented in Table 2. After HE staining of paraffin sections of the lung tissue using a VS120 scan to observe the whole film of each sample, each sample was evaluated for fibrosis severity. The average score was calculated after a comprehensive evaluation of the entire film. The predominant degree of fibrosis (lesions occupying more than half the field area) was recorded for each field.

Statistical analysis

Data were presented as mean ± standard deviation (mean ± SD), and statistical analysis was performed using SPSS 25 (International Business Machines Corporation, Armonk, NY). Data normality was tested using the Shapiro-Wilk normality test. If the observed values obeyed a normal distribution and the variances of each group were equal, the data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test or Tukey's correction for multiple comparisons. If the variances were not equal, a non-parametric

Table 2

Ashcroft score

Histopathological features	Degree of fibrosis
Normal lung tissue	0
Mild fibrous thickening of alveolar or bronchiolar walls	2
Moderate thickening of alveolar or bronchiolar walls without significant structural damage to the lungs	4
Increased fibrosis with the destruction of lung structure and formation of fibrous bands or masses	6
Severe structural damage and large areas of fibrosis; honeycomb lung is classified in this category	7
The entire area is filled with fibrous tissue	8

Kruskal-Wallis one-way ANOVA followed by the Holm Stepdown Bonferroni procedure was used. Statistical significance was set at $P < 0.05$ significant.

Results

DGNTT alleviated inflammation of synovial membrane and cartilage damage in the ankle joints of TNF-Tg mice

To investigate the effect of DGNTT on the ankle joints of TNF-Tg mice, we observed and quantified synovial inflammation, cartilage, and bone area in HE and ABOG-stained sections (Figure 1A and B). The ankle joints of the WT mice were structurally intact without inflammation of the synovial membrane or cartilage destruction. In TNF-Tg mice, we observed inflammatory cell infiltration, synovial hyperplasia, and reduced cartilage and bone areas in the ankle joints. Compared to the saline group, the DGNTT group showed significantly reduced synovial inflammation and increased cartilage and bone areas in the ankle joints. In the MTX group, the synovial area of the ankle joints was reduced and the bone area was restored; however, cartilage destruction did not improve. These results indicated that DGNTT effectively inhibited the development of joint inflammation and destruction of cartilage and bone in TNF-Tg mice.

DGNTT reduced the number of osteoclasts and bone loss in the ankle joints of TNF-Tg mice

To further explore the role of DGNTT in ankle bone mass in TNF-Tg mice, we used TRAP staining and micro-CT to determine and quantify osteoclast number and bone volume (Figure 2A and B). We found a low number of osteoclasts and intact bone mass in the ankle joints of WT mice. In the Saline group, there was a significant increase in the number of osteoclasts and bone loss in the ankle joints of TNF-Tg mice. Compared with the saline group, DGNTT reduced the number of osteoclasts in the ankle joints and restored the volume of the talus. MTX also reduced osteoclast numbers and improved talar volume; however, there was no statistically significant difference between the MTX and DGNTT groups. These results suggest that DGNTT inhibits bone loss by reducing the number of osteoclasts.

DGNTT mitigated lung inflammation and improved lung tissue structure in TNF-Tg mice

HE staining was performed on paraffin-embedded sections of lung tissues to study the effects of DGNTT on lung inflammation in TNF-Tg mice. The results of

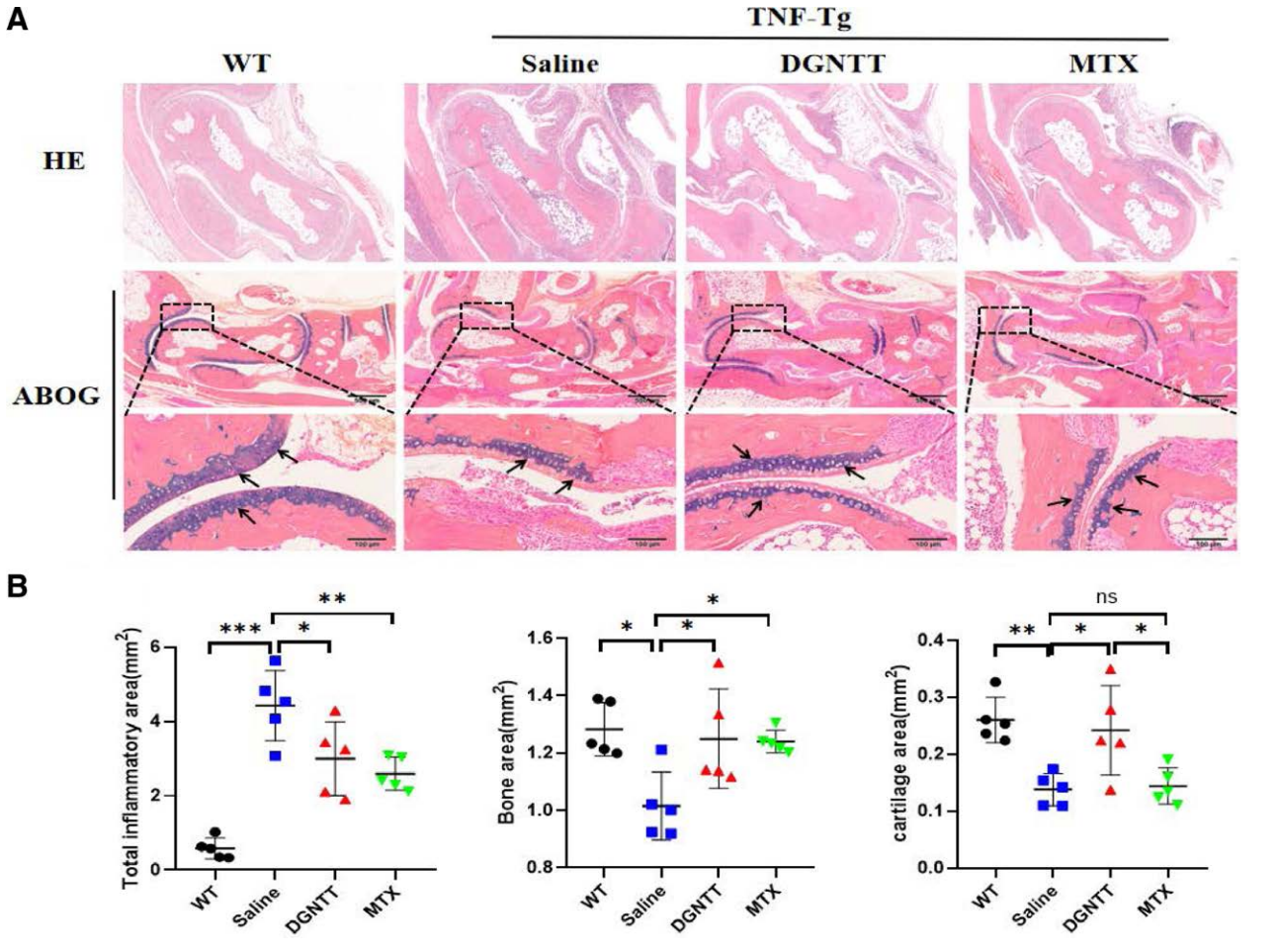

Figure 1. DGNTT reduced the area of synovial inflammation and alleviated cartilage destruction in TNF-Tg mice. (A) HE and ABOG staining were used to evaluate the effect of Saline, DGNTT, and methotrexate on TNF-Tg mice. AB-positive staining (black arrow) shows ankle cartilage. Scale bar, 500/100 μm. (B) Quantification of inflammation, bone, and cartilage areas. Results are shown as mean ± SD for five legs per group. *$P < 0.05$ *vs*. TNF-Tg + saline, **$P < 0.01$ *vs*. TNF-Tg + saline, ***$P < 0.001$ *vs*. TNF-Tg + saline. ABOG: Alcian Blue/Orange G; DGNTT: Danggui Niantong decoction; HE: Hematoxylin and eosin; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; WT: wild type.

HE staining and the Ashcroft score (Figure 3A and B) showed no obvious structural abnormalities, such as congestion, edema, or inflammatory cell infiltration, in the lung tissue of the normal group of mice. The lung tissue in the saline group showed marked thickening of the alveolar wall, reduction in the alveolar septum, and inflammatory cell infiltration around the blood vessels and bronchioles. The normal structure of lung tissue was destroyed. Compared to the saline group, inflammatory cell infiltration of the lung tissue in the DGNTT group was significantly reduced, the alveolar septum was enlarged, and the alveolar structure was relatively clear. In the MTX group, inflammatory cell infiltration, thickening of the alveolar wall, and shrinkage of the alveolar space in lung tissues did not improve. These results show that DGNTT has a better therapeutic effect on the pathological changes in RA-ILD, whereas MTX has a limited effect on lung inflammation.

DGNTT reduced the perivascular fibrosis in the lungs of TNF-Tg mice

To investigate the effect of DGNTT on lung fibrosis in TNF-Tg mice, Masson's trichrome staining was used to observe and quantify changes in collagen fibers in the lungs. The results (Figure 4A and B) revealed that WT mice had only mild collagen fibrils around the blood vessels in the lungs, whereas there were a large number of blue collagen fibers around the blood vessels in the lungs of TNF-Tg mice, indicating substantial changes in the structure of the lung. Compared to mice in the saline group, perivascular blue collagen fibrous tissue was significantly reduced, and pulmonary fibrosis was relieved in the DGNTT group. However, in the MTX group, blue collagen fibers around the lung blood vessels were still abundant and tissue fibrosis remained severe. These results showed that DGNTT could more effectively reduce perivascular fibrosis in the lungs of TNF-Tg mice than MTX.

DGNTT decreased myocardial inflammation and myocardial fiber disorders in TNF-Tg mice

To evaluate the effect of DGNTT on the hearts of TNF-Tg mice, HE staining was used to evaluate cardiac inflammation and myocardial alignment. The results (Figure 5) showed that compared to WT mice, inflammatory cells infiltrated the heart tissue of TNF-Tg mice, and the myocardial fibers were disordered and disorganized. In the DGNTT group, cardiac inflammatory cells were reduced, and myocardial fibers were ordered and continuous. Cardiac inflammation and myocardial fiber disorders did not change in the MTX group. These results indicated that DGNTT may have a better therapeutic effect on RA cardiac complication inflammation, whereas the effect of MTX was insignificant.

DGNTT alleviated cardiac perivascular fibrosis and cardiomyocyte hypertrophy and improved cardiac architecture in TNF-Tg mice

To further study the effect of DGNTT on the hearts of TNF-Tg mice, we evaluated and quantified cardiac

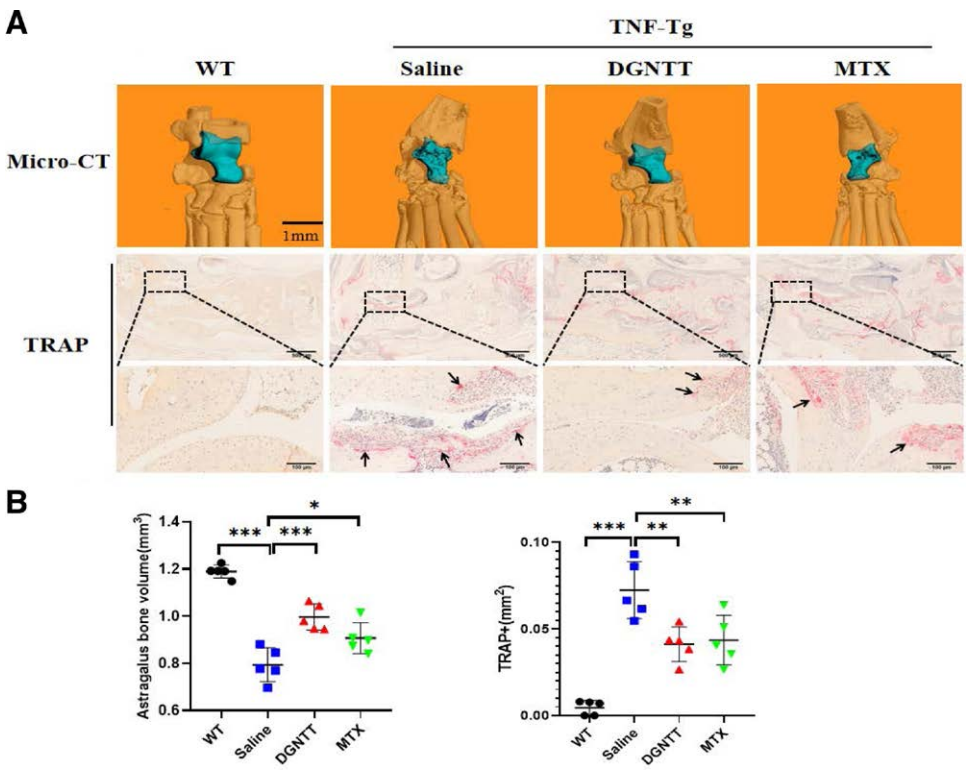

Figure 2. DGNTT decreased bone destruction in ankle joints of TNF-Tg mice. (A) Micro-CT and TRAP staining were used to evaluate the effect of saline, DGNTT, and methotrexate on ankle joints. The black arrow indicates TRAP + osteoclasts. Scale bar, 500/100 μm. (B) Quantification of astragalus bone volume and TRAP + area. Results are shown as mean ± SD for five legs per group. *$P < 0.05$ *vs*. TNF-Tg + saline, **$P < 0.01$ *vs*. TNF-Tg + saline, ***$P < 0.001$ *vs*. TNF-Tg + saline. CT: Computed tomography; DGNTT: Danggui Niantong decoction; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; TRAP: Tartrate-resistant acid phosphatase; WT: wild type.

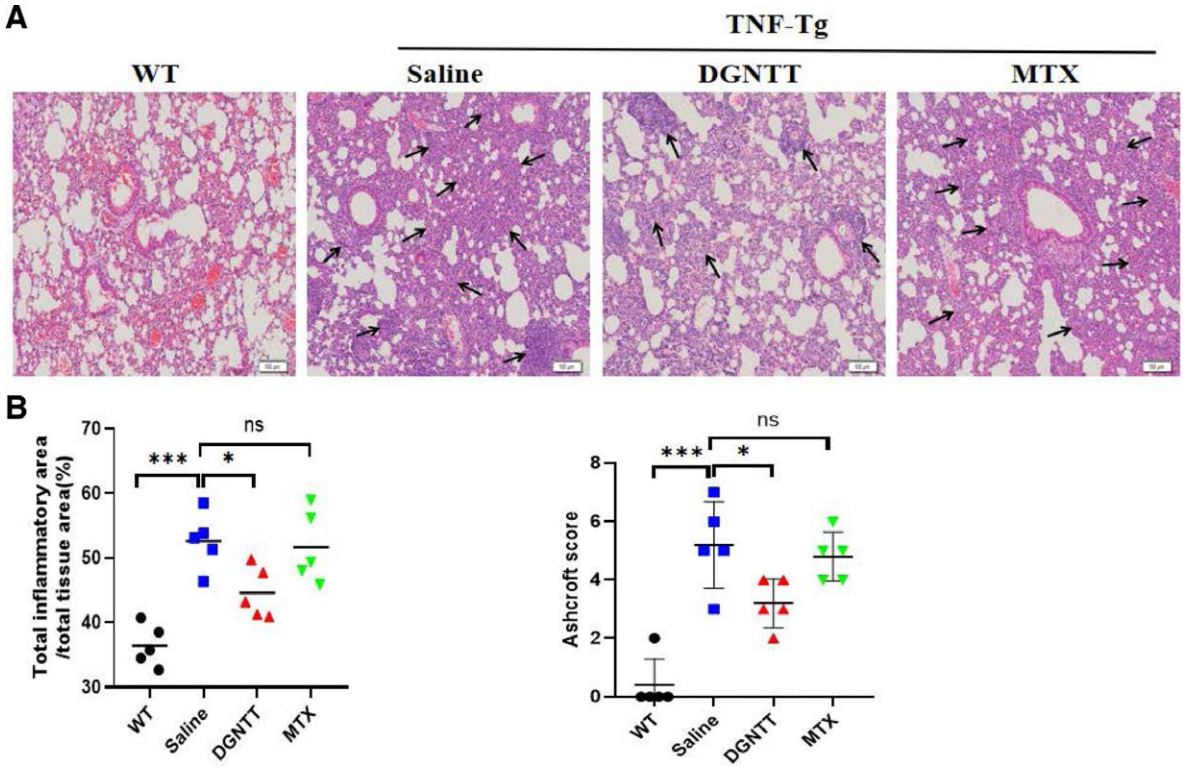

Figure 3. DGNTT reduced lung inflammation area and improved lung tissue structure (A) HE staining was used to show the effects of saline, DGNTT, and methotrexate on lung inflammation in TNF-Tg mice. The black arrow represents inflammation. Scale bar, 100 μm. (B) Quantification of inflammation area of lung tissue and statistics of pulmonary Ashcroft score. Results are shown as mean ± SD for five lungs per group.*$P < 0.05$ *vs*. TNF-Tg + saline, **$P < 0.01$ *vs*. TNF-Tg + saline, ***$P < 0.001$ *vs*. TNF-Tg + saline. DGNTT: Danggui Niantong decoction; HE: Hematoxylin and eosin; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; WT: wild type.

fibrosis and cardiomyocyte cross-sections using Masson trichrome and WGA staining of cardiac sections. Experimental results (Figure 6A and B) showed that, in contrast to WT mice, TNF-Tg mice exhibited an increased area of collagen fibers around the cardiac vessels and an enlarged cardiomyocyte cross-section of the heart. In the DGNTT group, the area of perivascular blue collagen fibers was reduced, and the enlarged cardiomyocyte cross-section was improved. There were still numerous blue collagen fibers around the heart vessels and the cardiomyocyte cross-section remained enlarged in the MTX group. These results indicate that TNF-Tg mice have perivascular fibrosis and myocardial cell hypertrophy in the heart, and DGNTT could better inhibit perivascular myocardial tissue fibrosis and improve myocardial cell hypertrophy in TNF-Tg mice than MTX.

Discussion

In this study, we found that DGNTT reduced ankle synovial inflammation and bone destruction, and alleviated inflammation and fibrosis in the heart and lungs of TNF-Tg mice. In addition, DGNTT ameliorated cardiomyocyte hypertrophy and improved the lung tissue structure in TNF-Tg mice. Therefore, our study indicated that DGNTT effectively treats cardiopulmonary complications in TNF-Tg mice. These findings reflect the advantages of using multiple targets and the minimal adverse effects of TCM for the treatment of RA complications.

TNF-Tg mice carry one copy of the full-length human TNF gene with a modified 3′-untranslated region (3′-UTR) exchanged with the β-bead protein 3′-UTR, which presents spontaneous inflammatory arthritis at 3 months of age and complicated with inflammatory cell infiltration into pulmonary vascular and peribronchial[25]. In this study, TNF-Tg mice exhibited pathological changes in the lungs and heart that mimicked the clinical features of RA and cardiopulmonary complications, including increased collagen fibers around the lung and heart vessels, disorganized myocardial fibers, and cardiomyocyte hypertrophy. Chronic inflammation may contribute to the development of cardiopulmonary complications in RA. Overexpression of the TNF gene in mice results in systemic inflammation that affects multiple organs, including the lungs and heart, resembling the systemic inflammation induced by a persistent autoimmune response in patients with RA. Therefore, TNF-Tg mice are a powerful model for testing the effects of drugs on cardiopulmonary complications in RA. According to statistics, women are three times more likely to develop RA than men[34], and the onset of symptoms is earlier in females than in males in TNF-Tg mice; therefore, we used female TNF-Tg mice as a model of RA and RA cardiopulmonary complications.

MTX is the first-line DMARD for RA[35], but its role in patients with RA-ILD remains controversial. The use of MTX has long been associated with lung injury, including acute[36] and chronic pneumonia[37–38]. Interleukin (IL)-8 is involved in the pathogenesis of acute or chronic lung disease[39–40]. It has been reported that MTX promotes IL-8 secretion by human bronchial and airway epithelial cells[41], and the levels of serum IL-8 are elevated in bronchoalveolar lavage fluid of patients with MTX-associated pneumonia[42]. However, it has recently

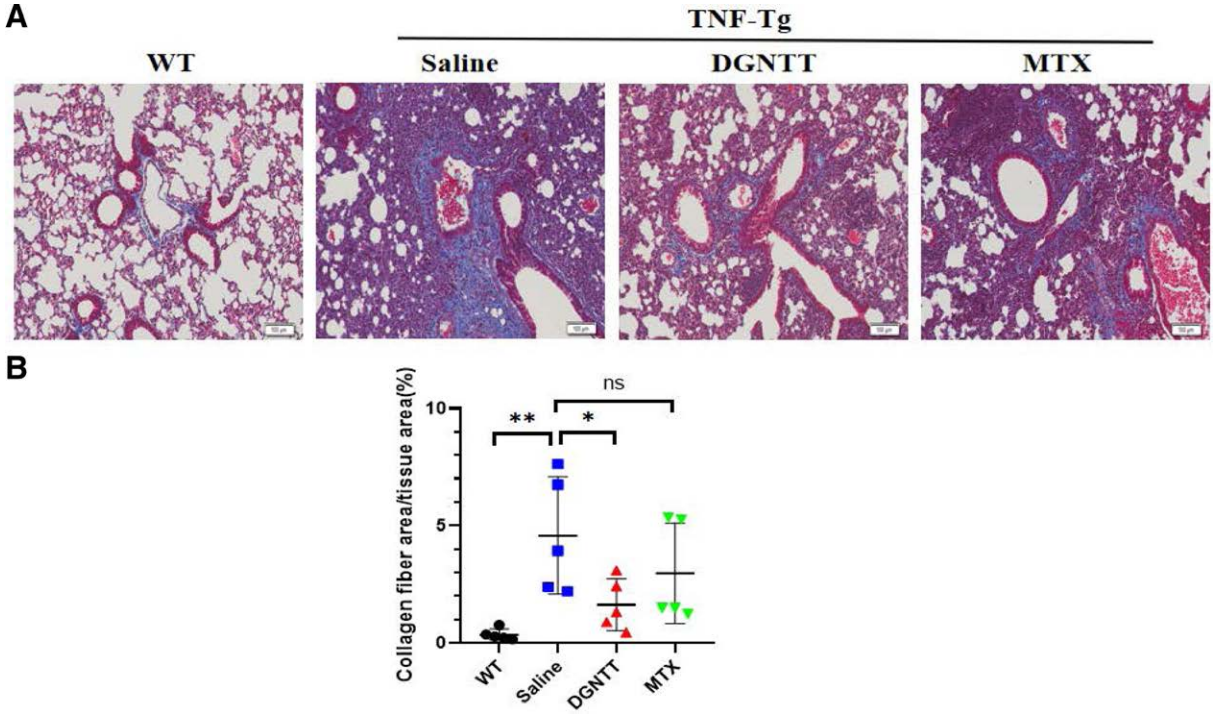

Figure 4. DGNTT attenuated the area of collagen fibers in the lung tissue of TNF-Tg mice. (A) Masson trichrome staining was used to show the therapeutic effect of saline, DGNTT, and methotrexate on pulmonary fibrosis. Blue-positive staining shows collagen fibers. Scale bar, 100 μm. (B) Quantification of collagen fiber area. Results are shown as mean ± SD for five lungs per group. *$P < 0.05$ *vs*. TNF-Tg + saline, **$P < 0.01$ *vs*. TNF-Tg + saline. DGNTT: Danggui Niantong decoction; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; WT: wild type.

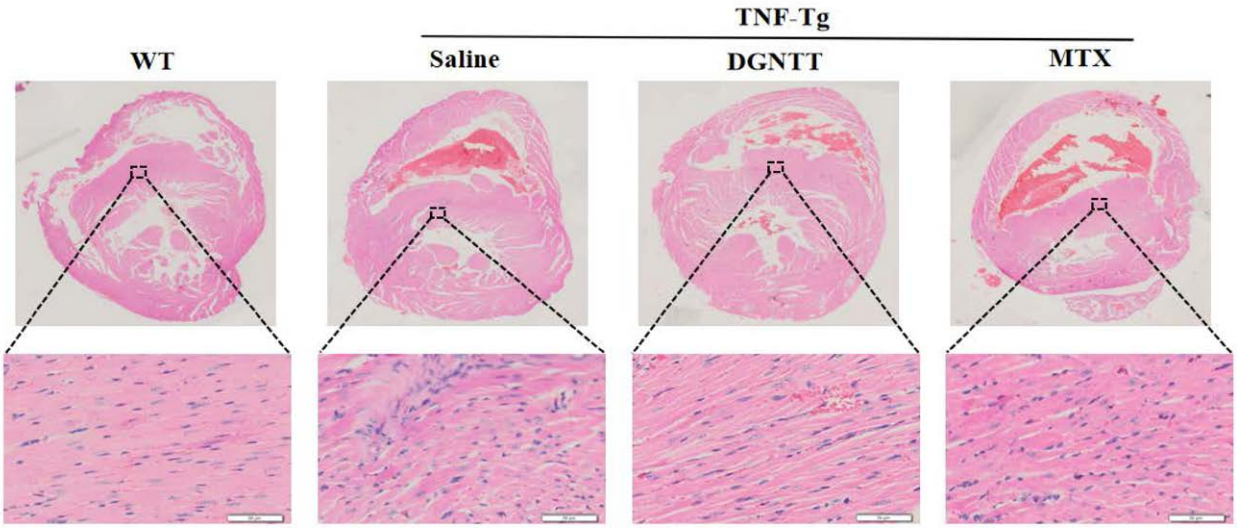

Figure 5. DGNTT attenuated cardiac inflammation and improved myocardial fiber disorder in TNF-Tg mice. HE staining was used to evaluate the effect of saline, DGNTT, and methotrexate on cardiac inflammation in TNF-Tg mice. Results are shown for five hearts per group. Scale bar, 50 μm. DGNTT: Danggui Niantong decoction; HE: Hematoxylin and eosin; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; WT: wild type.

been reported that MTX was not associated with an increased risk of RA-ILD but even delayed RA-ILD onset in patients with RA[43–45]. Collectively, evidence for the relationship between MTX and chronic fibrotic ILD in patients with RA is inadequate. Herein, we assessed the role of MTX in RA cardiopulmonary complications using TNF-Tg mice. The results indicated that MTX had no therapeutic effect on inflammation and fibrosis in the lungs and heart but only alleviated joint damage. Our results suggest that MTX may not result in cardiopulmonary complications in patients with RA.

In contrast to MTX, DGNTT exhibited a profound effect in alleviating pathological damage to the lungs and heart of TNF-Tg mice. The beneficial effects of DGNNT on cardiopulmonary complications in RA may be related to the bioactivities of their chemical constituents. It has been reported that polyacetylenes in *N. incisum* K.C. Ting ex H.T. Chang exhibited anti-inflammatory activity by inhibiting nitric oxide secretion[46], and scoparone in *A. capillaris* Thunb. exerts anti-osteoarthritic effects by inhibiting the expression of proinflammatory factors through the p38-MAPK and PI3K/Akt/NF-κB

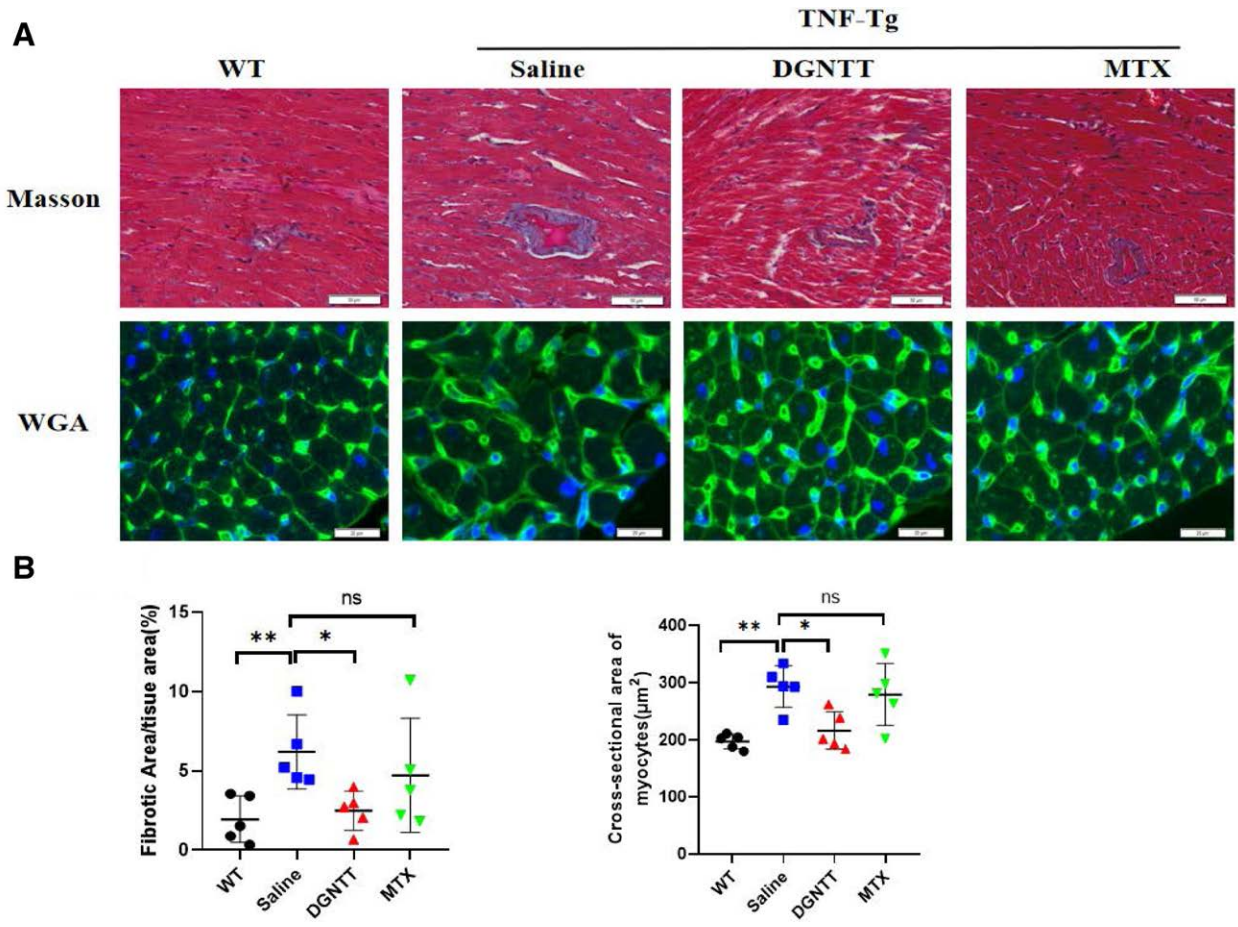

Figure 6. DGNTT reduced cardiac perivascular collagen fibers and alleviated cardiac hypertrophy in TNF-Tg mice. (A) Masson trichrome and wheat germ agglutinin staining were used to evaluate the therapeutic effects of saline, DGNTT, and methotrexate on cardiac fibrosis and cardiomyocyte hypertrophy in TNF-Tg mice. Scale bar, 50/20 μm. (B) Quantification of collagen fiber area and cross-sectional area of myocytes. Results are shown as mean ± SD for five hearts per group. *$P < 0.05$ *vs*. TNF-Tg + saline, **$P < 0.01$ *vs*. TNF-Tg + saline. DGNTT: Danggui Niantong decoction; HE: Hematoxylin and eosin; MTX: methotrexate; TNF-Tg: Tumor necrosis factor-transgenic; WT: wild type.

pathways[47–48]. In addition, the volatile oil of *A. macrocephala* Koidz exerts a significant anti-inflammatory effect by inhibiting prostaglandin E2 production[49]. Moreover, extracts from *N. incisum* K.C. Ting ex H.T. Chang reduced the concentration of inflammatory cytokines such as IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid of asthmatic mice[50]. Puerarin from *P. montana* (Lour.) Merr reduces angiotensin II-induced myocardial hypertrophy by suppressing the activation of extracellular regulated protein kinase (ERK1/2), p38, and nuclear factor kappa B signaling pathways[51]. Collectively, these results suggest that the beneficial effects of DGNTT on joint and cardiopulmonary injuries in TNF-Tg mice may be due to compounds with anti-inflammatory and cardiopulmonary systemic protective effects.

However, this study has several limitations. First, we did not analyze the main constituents absorbed in the blood after oral administration of DGNTT to TNF-Tg mice. Second, the effective components of DGNTT and the types of targeted immune cells were not confirmed. Third, the signaling pathways through which DGNTT inhibits systemic inflammation remain unclear. Fourth, because long-term chronic inflammation in female TNF-tg mice leads to lower fertility, it is difficult to obtain enough positive mice of the same age to meet the grouping of different doses of DGNTT. Therefore, our experiment only included a single-dose DGNTT group and a positive control group to study DGNTT interventions for RA and RA complications. In the future, we will combine network pharmacology and ultra-performance liquid chromatography-mass spectrometry to clarify the active ingredients of DGNTT and further analyze the transcriptomic and proteomic changes in the lung and heart tissues of TNF-Tg mice after DGNTT treatment to discover the possible molecular mechanisms underlying its pharmacological effects on cardiopulmonary complications. In addition, we plan to establish DGNTT with high, medium, and low concentration gradients for further studies.

Conclusions

MTX alleviated joint destruction in TNF-Tg mice with no beneficial effects on pathological lung and heart changes. In contrast, DGNTT improved joint lesions and had a good therapeutic effect on cardiac and pulmonary complications in TNF-Tg mice. It is expected to be a potential treatment for cardiopulmonary complications associated with RA.

Conflict of interest statement

The author declares no conflict of interest.

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Author contributions

Can Yang, Tao Chen, and Mengjiao Ma performed most of the experiments, analyzed the data, and drafted the manuscript. Qiang Li and Zhichao Liang were involved in the experimental operation and data analysis. Ning Li, Hao Xu, and Youhua Wang assisted with manuscript editing. Ning Li and Qianqian Liang designed the study and drafted and finalized the manuscript. All the authors have read and approved the final manuscript.

Ethical approval of studies and informed consent

Not applicable.

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None.

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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