

Astragaloside IV Ameliorates Diabetic Cardiomyopathy by Suppressing the GNG2/MRAS-ERK Signaling Pathway

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Objective: This study aims to investigate the mechanism of AGS-IV in treating diabetic cardiomyopathy (DCM) by establishing animal and cellular models of the disease.

Methods: A DCM rat model was established by feeding a high-fat diet combined with streptozotocin (STZ) injection, and a DCM cell model was created through glucose induction. In model rats, the cardiac weight-to-body weight ratio, the left ventricular weight-to-heart weight ratio, and ventricular wall thickness were measured. ELISA was used to detect Collagen1 and MMP-2 levels in myocardial tissue, serum, and cultured cells. The mRNA levels of GNG2, MRAS, and ERK in myocardial tissue and cultured cells were measured using RT-PCR.

Results: In vivo, experiments demonstrated that AGS-IV effectively reduced the cardiac weight-to-body weight ratio, left ventricular weight-to-heart weight ratio, and ventricular wall thickness in DCM rat models. It also decreased Collagen I levels in myocardial tissue and MMP-2 levels in serum, accompanied by downregulated mRNA expression of GNG2, MRAS, and ERK in myocardial tissue. In vitro, AGS-IV significantly reduced Collagen I and MMP-2 levels in DCM cell models and downregulated GNG2, MRAS, and ERK mRNA expression.

Conclusion: AGS-IV exerts therapeutic effects on DCM by regulating the GNG2/MRAS-ERK signaling pathway.

Keywords: AGS-IV, diabetic cardiomyopathy, in vivo, in vitro

Background

Diabetes mellitus (DM) is a metabolic disease caused by the combined effects of genetic and environmental factors. Currently, the global prevalence of DM continues to rise, with epidemiological studies predicting that approximately 1.3 billion people worldwide will be affected by diabetes by 2050.¹ Heart failure represents one of the most severe complications in DM patients. Studies have demonstrated that compared with healthy individuals, diabetic patients face a 2–4 times higher risk of developing heart failure.² Among the causes of mortality in DM patients, cardiovascular disease-related deaths rank first, with up to 80% of diabetic patients developing cardiovascular complications. Diabetic cardiomyopathy (DCM) is a diabetes-specific cardiac disorder characterized by myocardial structural and functional abnormalities in the absence of other cardiac risk factors (such as coronary artery disease, hypertension, or severe valvular disease). Its primary pathological feature is systolic dysfunction, ultimately leading to heart failure. The management of DCM requires long-term glycemic control and continuous intervention of multiple risk factors. Each acute episode necessitates substantial medical expenditures while posing serious threats to patients' lives, making DCM

a significant burden on healthcare systems and patients' well-being. Therefore, conducting research on DCM treatment and identifying novel therapeutic targets are of paramount importance for reducing the prevalence and mortality of DCM in our country, as well as for improving patients' quality of life.

GNG2 is a key component of G protein heterotrimers. Its encoding gene is located on human chromosome 1. The protein is widely expressed in tissues such as myocardium and nerves. It is closely associated with cell proliferation, contraction, and regulation of calcium homeostasis. Studies have demonstrated that abnormal expression or function of GNG2 is closely related to pathological changes, including myocardial remodeling and decreased cardiac function, thereby identifying it as an important molecular target for cardiovascular diseases such as dilated cardiomyopathy.^{3,4} MRAS is a member of the Ras superfamily. Its encoding gene is located on human chromosome 3. It is expressed in tissues such as myocardium and vascular smooth muscle and participates in physiological processes including cell proliferation, differentiation, and cytoskeleton remodeling. In myocardial tissue, MRAS regulates cardiomyocyte growth and survival, as well as myocardial fibrosis, by activating downstream pathways such as Raf-MEK-ERK and PI3K-AKT. Moreover, its abnormal activation disrupts myocardial homeostasis and induces myocardial hypertrophy and ventricular dilation. This abnormal activation is closely associated with the occurrence and progression of dilated cardiomyopathy, making MRAS a crucial target in research on the molecular mechanisms of cardiovascular diseases.^{5,6} ERK is a core member of the mitogen-activated protein kinase (MAPK) family, mainly including two isoforms: ERK1 (MAPK3) and ERK2 (MAPK1). Their encoding genes are located on human chromosomes 16 and 22, respectively. ERK proteins are widely expressed in various tissues such as myocardium, liver, and kidney, and participate in regulating cardiomyocyte proliferation, differentiation, apoptosis, and myocardial fibrosis in myocardium. Under physiological conditions, moderate activation of ERK is essential for maintaining myocardial homeostasis. However, sustained ERK activation under pathological conditions disrupts the balance between cardiomyocyte growth and apoptosis, leading to myocardial remodeling and decreased cardiac function. This mechanism plays a key role in the development and progression of cardiovascular diseases such as dilated cardiomyopathy and myocardial infarction.^{7,8}

Astragalus membranaceus (Huangqi) was first documented in *Shennong Bencao Jing* (The Divine Farmer's Materia Medica Classic), where it was described as "mildly warm in nature, sweet in taste, and attributive to the lung and spleen meridians, classified as a superior-grade herb." In traditional Chinese medicine (TCM), *Astragalus* is considered a vital tonic with multiple therapeutic effects, including replenishing qi, strengthening the spleen, elevating yang, consolidating the defensive qi, promoting diuresis, draining pus, and regenerating tissue. It is commonly used to treat spleen qi deficiency, lung qi deficiency, spontaneous sweating due to qi deficiency, as well as chronic non-healing ulcers caused by qi and blood deficiency. Modern pharmacological studies have revealed that *Astragalus* exhibits immunomodulatory, hypoglycemic, anti-aging, anti-inflammatory, lipid-regulating, anti-fibrotic, and cardioprotective properties. Its mechanisms include promoting insulin secretion, enhancing insulin sensitivity, upregulating antioxidant factors, and modulating apoptosis-related signaling pathways. These actions collectively contribute to glycemic control, reduction of cardiac inflammation, inhibition of cardiomyocyte apoptosis, and suppression of myocardial fibrosis, thereby potentially delaying the progression of DCM. Astragaloside IV (AGS-IV), one of the primary bioactive constituents of *Astragalus*, represents the most pharmacologically active component among astragalosides. Numerous studies have confirmed that AGS-IV can effectively improve both systolic and diastolic cardiac functions, demonstrating significant therapeutic effects against DCM.^{9,10} To further elucidate the mechanistic basis of AGS-IV in DCM treatment, this study aims to validate its efficacy and mechanisms through establishing both in vivo and in vitro DCM models. The findings are expected to provide experimental evidence supporting further research and clinical application of AGS-IV.

Materials and Methods

Animals and Cells

Twenty clean-grade healthy male Sprague-Dawley (SD) rats weighing 200±20 g were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Animal License No.: SCXK(Liao)2020-0001). The rats were housed in the animal laboratory of Harbin Medical University under controlled conditions: temperature maintained at 20–26°C, humidity at

40–70%, with a 12/12-hour light-dark cycle. This study was conducted in compliance with the ethical requirements approved by the Institutional Animal Care and Use Committee.

The H9C2 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences Committee on Type Culture Collection. The cells were cultured in medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Model Construction and Grouping

After one week of acclimatization feeding, the rats were randomly divided into a control group (n=6) and a model group (n=14). The animal model was established through high-fat diet feeding combined with intraperitoneal injection of streptozotocin (STZ) at a dosage of 50 mg/kg once daily for two consecutive days. Meanwhile, control group rats were fed with standard chow and received equivalent volumes of physiological saline via intraperitoneal injection during the same period. After 8 weeks of model establishment, fasting blood glucose levels were measured, with levels ≥ 11.1 mmol/L considered indicative of successful model induction. Following successful modeling, the model group rats were further randomly subdivided into a model subgroup (n=6) and a treatment subgroup (n=6). The treatment subgroup received Astragaloside IV (AGS-IV) via intragastric administration at a dose of 50 mg/kg/day for 8 weeks, while continuing the high-fat diet regimen.

When the cells entered the logarithmic growth phase, they were divided into three groups: control group, model group, and treatment group. The model group and treatment group were exposed to 22.0 mmol/L glucose for 72 hours to establish the diabetic cardiomyopathy cell model.¹¹

Materials

MMP-2 ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Cat# H146-1-2); RNeasy Mini Kit (Takara Beijing, Cat# RR037A); RNeasy Mini Kit (QIAGEN Germany, Cat# 74104); TBGreen Advantage qPCR Premix (Takara Beijing, Cat# 639676).

Instruments

M200 Multifunctional Microplate Reader (BioTek, USA); Sorvall ST 8R High-Speed Refrigerated Centrifuge (Thermo Fisher Scientific, USA); Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific, USA).

Detection of Collagen I and MMP-2 Content

Following body weight measurement, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Blood samples were collected via abdominal aorta puncture using aseptic techniques. The collected blood was allowed to clot at room temperature for 3 hours, followed by centrifugation at $3,000 \times g$ for 20 minutes at 4°C to obtain serum (supernatant), which was aliquoted and stored at -80°C until analysis. Cardiac tissues were rapidly excised, thoroughly rinsed with ice-cold physiological saline (0.9% NaCl), and immediately snap-frozen in liquid nitrogen for subsequent protein extraction. The concentrations of Collagen 1 in myocardial tissue homogenates and MMP-2 in serum were quantitatively determined using commercially available ELISA kits according to the manufacturers' protocols. All assays were performed in duplicate, with appropriate quality controls included in each run.

Following model establishment and drug treatment, cells from each experimental group were harvested by gentle scraping. Cell suspensions were centrifuged at $1,000 \times g$ for 5 minutes at 4°C, washed twice with phosphate-buffered saline (PBS, pH 7.4), and processed for protein extraction. Collagen 1 and MMP-2 levels in cell lysates were measured using standardized ELISA procedures as specified in the respective kit protocols.

Measurement of Cardiac Weight and Left Ventricular Hypertrophy Index

Following euthanasia, rat hearts were excised and immediately rinsed with ice-cold physiological saline (0.9% NaCl). Residual blood was carefully removed using filter paper. After dissecting away the great vessels and connective tissues, the whole heart weight (WHW) was measured using an analytical balance (precision: 0.1 mg). Next, the heart was placed on sterile filter paper on ice and fixed with the left ventricle facing upward to distinguish the structural differences between the left ventricle (with the largest volume and thickest ventricular wall) and the right ventricle and atrium. The

atrium was clamped and cut along the atrioventricular groove to remove it, and then the left ventricle and right ventricle were separated along the edge of the interventricular septum. The residual tissue was trimmed to obtain a pure left ventricle. The following indices were calculated: Whole heart weight index (WHWI): WHW (mg) / body weight (g); Left ventricular hypertrophy index (LVHI): LV weight (mg) / body weight (g). All measurements were performed by two independent investigators blinded to the experimental groups, with the mean values used for statistical analysis. The dissection procedure was conducted on a chilled platform (4°C) to minimize protein degradation.

Measurement of Ventricular Anterior Wall Thickness

After weighing the myocardial tissue, left ventricular samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 µm thickness along the equatorial plane. The sections were then photographed under a microscope, and the thickness of the left ventricular anterior wall was measured using ImageJ software.

Rt-Pcr

The cardiac tissues of the mice were removed, rinsed with normal saline, and then quickly frozen in liquid nitrogen for later use. The cardiac tissues of the mice were taken, and the total RNA of the cardiac tissues was extracted using the RNeasy Mini Kit. The cDNA was synthesized using the Prime-Script™ RT Reagent Kit. Real-time PCR was established using the TBGreen Advantage qPCR Premix. With the primers of GNG2, MRAS, and ERK genes, the real-time PCR operation was carried out in a rapid real-time PCR system. We used GAPDH as the housekeeping gene. The relative expression level of the target gene was calculated by the 2-ΔΔCt method. The primer sequences are listed in Table 1.

After the cell model establishment and administration, the cells were collected by centrifugation and washed with PBS. After washing, the PCR detection method was the same as that for the cardiac tissues. Primer design and synthesis were performed by Sangon Biotech (Shanghai) Co., Ltd.

Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism 8.0.1 software. All data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences among multiple groups, and Student's *t*-test was applied for comparisons between two groups. For comparisons between two specific groups, *t*-tests were applied. A *p*-value < 0.05 was considered statistically significant.

Results

In vivo Validation of the Therapeutic Effects and Mechanisms of AGS-IV on DCM

The detection of serum and cardiac tissue in model rats showed that compared with the blank group, the heart weight/body weight ratio and left ventricle/heart weight ratio of model rats were significantly increased, the ventricular wall was significantly thickened, and the levels of Collagen1 in myocardial tissue and MMP-2 in serum were significantly elevated. Compared with the model group, the heart weight/body weight ratio, left ventricle/heart weight ratio, and ventricular wall thickness were significantly decreased in the AGS-IV treatment group, and the levels of Collagen1 in myocardial tissue and MMP-2 in serum

Table 1 Primer Sequence

Gene	Primer Sequence	Length of Fragment
GNG2	5' TACATCGCATCCAT 3'	262bp
NM_001257349.1	5' TCCCAAGCCACCA 3'	
MRAS	5' GTTGATTGAGCCCAGTT 3'	270bp
NM_012981.3	5' CAGACATTTATCGGAGG 3'	
ERK	5' TGAAGCCCTCCAATC 3'	116bp
M61177.1	5' CACATACTCGGTCTAG 3'	
GAPDH	5' CCACGGCAAGTTCAA 3'	144bp
NM_017008.4	5' CCAGTAGACTCCACG 3'	

were significantly reduced (Figure 1A–D). Compared with the blank group, the mRNA expression levels of GNG2, MRAS, and ERK in the cardiac tissue of model rats were significantly increased. Compared with the model group, the mRNA expression levels of GNG2, MRAS, and ERK in the cardiac tissue of AGS-IV treatment group were significantly decreased (Figure 1E).

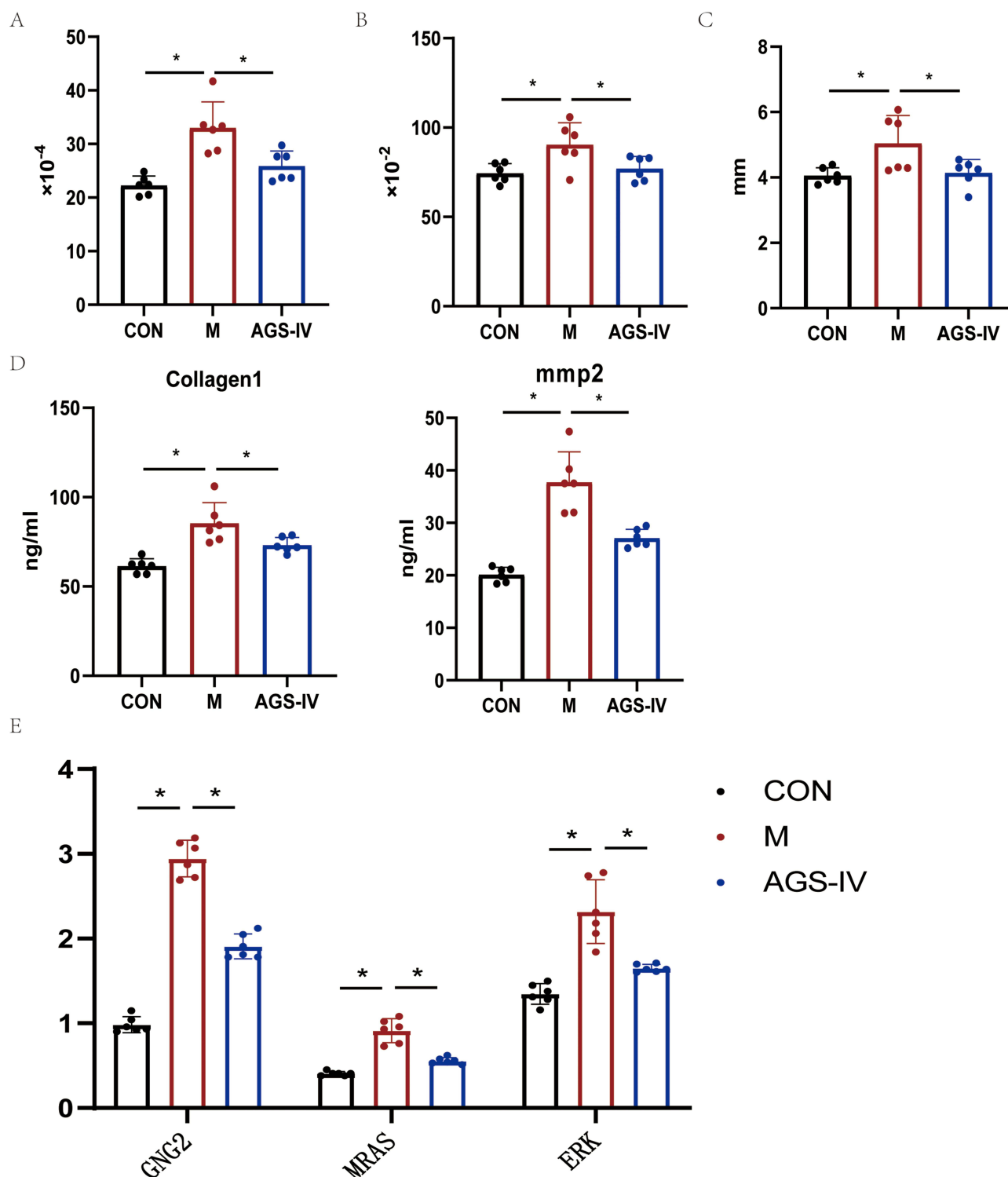


Figure 1 (A) Heart weight/body weight ratio of rats in each group ($p=0.004$, $p=0.0105$); (B) Left ventricle/heart weight ratio of rats in each group ($p=0.0149$, $p=0.0407$); (C) Ventricular wall thickness of rats in each group ($p=0.0136$, $p=0.0109$); (D) Expression levels of Collagen1 in myocardial tissue ($p=0.007$, $p=0.0355$) and MMP-2 in serum of rats in each group ($p=0.0069$, $p=0.015$); (E) mRNA expression of GNG2 ($p<0.001$, $p=0.0002$), MRAS ($p=0.0006$, $p=0.0013$) and ERK ($p=0.0013$, $p=0.0015$) in cardiac tissue of rats in each group. (* $p<0.05$).

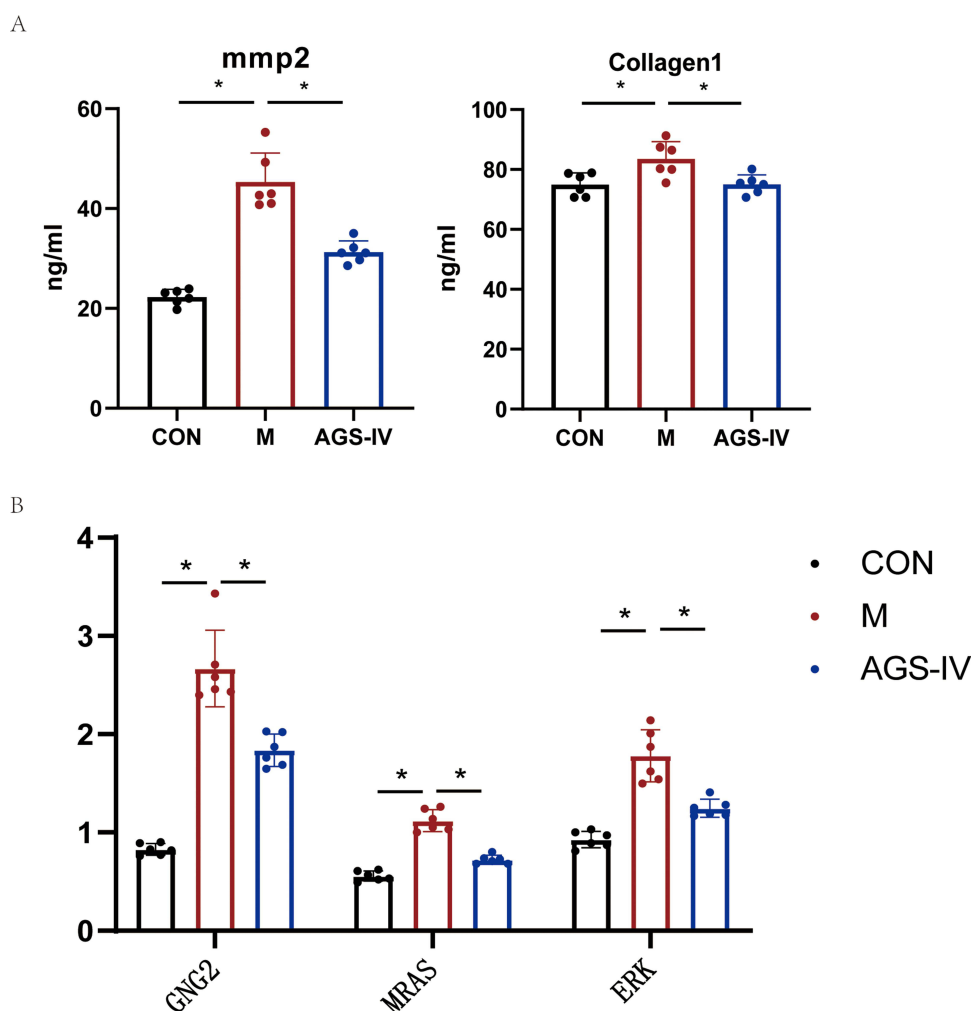


Figure 2 (A) Expression levels of Collagen1 ($p=0.0136$, $p=0.0109$) and MMP-2 ($p<0.001$, $p=0.0002$) in cells of each group; **(B)** mRNA expression of GNG2 ($p<0.001$, $p=0.0007$), MRAS ($p<0.001$, $p=0.0001$) and ERK ($p=0.0002$, $p=0.0009$) in cells of each group. (* $p<0.05$).

In vitro Validation of the Therapeutic Effects and Mechanisms of AGS-IV on DCM

The detection of model cells showed that compared with the blank group, the levels of Collagen1 and MMP-2 in the model group were significantly increased. In contrast, the levels of Collagen1 and MMP-2 in the AGS-IV treatment group were significantly lower than those in the model group (Figure 2A). Additionally, compared with the blank group, the mRNA expression levels of GNG2, MRAS, and ERK in the model group were significantly upregulated. Conversely, the mRNA expression levels of GNG2, MRAS, and ERK in the AGS-IV treatment group were significantly down-regulated compared with the model group (Figure 2B).

Discussion

In the composition of causes of death in DM patients, cardiovascular disease-related death ranks first. However, the early symptoms of DCM patients are not obvious, making it extremely easy to miss the optimal timing for diagnosis and treatment, so most cases continue to progress to severe conditions such as congestive heart failure and arrhythmia. Existing studies have shown that insulin resistance, abnormal insulin signaling transduction, excessive oxidative stress, inflammation, endothelial dysfunction, myocardial cell autophagy and apoptosis, myocardial fibrosis, etc., may all be involved in the pathophysiological process of DCM. Cardiac structural remodeling is the main cause of functional disorders. Subendocardial longitudinal fibers in the myocardium are greatly affected by myocardial ischemia, fibrosis, and hypertrophy factors. For example, decreased ventricular long-axis systolic function in diabetic patients triggers

compensatory increases in ventricular radial thickness and weight to maintain left ventricular ejection fraction, thereby leading to left ventricular hypertrophy. Patients exhibit significant increases in ventricular wall thickness and left ventricular weight index. At present, the treatment of dilated cardiomyopathy (DCM) is still centered on the strategy of “pharmacotherapy as the foundation, non-pharmacological intervention as a supplement, and etiology-stratified management as the guide”. Although a variety of pharmacological agents are available for DCM treatment, including angiotensin-converting enzyme inhibitors (ACEIs)/angiotensin II receptor blockers (ARBs), β -blockers, angiotensin receptor-neprilysin inhibitors (ARNIs), ivabradine, diuretics, mineralocorticoid receptor antagonists (MRAs), as well as antiarrhythmic and anticoagulant drugs, a radical cure remains unattainable. Notably, myocardial remodeling in DCM is irreversible due to the irreparability of cardiomyocyte loss and persistent extracellular matrix dysregulation, and some patients experience rapid disease progression. Therefore, the development of novel therapeutic agents targeting DCM still holds significant clinical value for improving patient outcomes.^{12–15} In this study, it was found that after AGS-IV treatment, the heart weight/body weight ratio, left ventricle/heart weight ratio, and ventricular wall thickness in model rats were significantly reduced, suggesting that AGS-IV can effectively alleviate myocardial tissue lesions in model rats.

The characteristic pathological structural changes in the myocardium caused by DCM are extracellular collagen deposition and matrix remodeling. Excessive collagen formation and progression of myocardial fibrosis exacerbate myocardial sclerosis, thereby affecting cardiac systolic function. This study found that after AGS-IV treatment, the levels of Collagen1 in myocardial tissue of model rats and in model cells were significantly reduced. MMP-2 is an important factor involved in systemic oxidative balance and ventricular remodeling. Related studies have found that a hyperglycemic state leads to the production of a large number of oxygen free radicals, disrupting the oxidative-reduction balance in the body, activating cytokines such as MMP-2, and promoting their degradation of gelatin laminin, fibronectin, etc., as well as the cleavage of fibrous collagen. Therefore, the level of MMP-2 is closely related to the occurrence and development of diabetes. Current studies have confirmed that MMP-2 is an independent risk factor for diabetic foot. In addition, when the myocardium is damaged, MMP activity increases, which can degrade various collagens and elastin in the myocardial extracellular matrix, leading to ventricular remodeling. Studies have shown that reducing the expression of MMP-2 protein in myocardial tissue can inhibit ventricular remodeling and ultimately improve cardiac function. It has been confirmed that MMP inhibitors such as TIMP-1 and TIMP-2 can effectively inhibit ventricular remodeling caused by heart failure without interfering with myocardial structure and function. In this study, the levels of MMP-2 in the serum of model animals and in model cells after AGS-IV treatment were detected. It was found that the levels of MMP-2 in the serum of modeled animals and in cells increased significantly after modeling, while after AGS-IV treatment, the levels of MMP-2 in animal serum and cells decreased significantly, suggesting that AGS-IV has a therapeutic effect on DCM model animals and cells.

ERK is a major member of the MAPK family, and its activation cascade has been extensively studied. ERK is involved in cell growth and apoptosis, so strict regulation of ERK is crucial for cell survival and growth. Reactive oxygen species (ROS), severe injury, and stimuli-induced physiological and pathological disease states can activate ERK. Currently, activation of the ERK signaling cascade has been confirmed to mediate cardiac hypertrophy induced by most stress stimuli. Therefore, compounds that inhibit the ERK pathway, such as MEK-ERK inhibitors (eg, PD98059 and U0126), can be used to suppress excessive cardiac growth in myocardial hypertrophy. Additionally, studies have shown that ERK inhibitors can protect against the development of DCM, myocardial ischemia/reperfusion injury, and ISO-induced cardiac hypertrophy. In this study, detection of ERK mRNA levels in cardiac tissues of model animals and model cells after AGS-IV treatment revealed significantly increased ERK mRNA levels in cardiac tissues and cells following modeling, whereas AGS-IV treatment significantly decreased ERK mRNA levels, suggesting that AGS-IV significantly regulates ERK mRNA levels in DCM model animals and cells.

MRAS is one of the proteins most closely related to classical RAS oncoproteins and possesses most regulatory and effector interaction functions of classical RAS oncoproteins. In the classical RAS/ERK pathway, activated RAS bound to GTP interacts with the RAS-binding domain of RAF proteins through its effector-binding domain, triggering a cascade reaction that ultimately activates ERK to translocate into the nucleus, thereby regulating biological processes such as cell proliferation, division, and the cell cycle. As a protein highly structurally similar to RAS, MRAS also regulates ERK.¹⁶ However, current research on its regulatory mechanisms primarily focuses on oncological diseases such as pancreatic

cancer¹⁷ and colorectal cancer,¹⁸ with fewer studies in the field of cardiovascular diseases. This study found that AGS-IV treatment significantly altered MRAS mRNA levels in cardiac tissues of model animals and model cells.

GNG2 is a subunit of the G $\beta\gamma$ dimer that forms heterotrimeric G proteins with G α subunits, playing important roles in cell proliferation, differentiation, and angiogenesis, and serving as a potential molecular target for treating multiple diseases. Studies have shown that GNG2 can influence ERK activity through interactions with MRAS.¹⁹ In this study, detection of GNG2 mRNA levels in cardiac tissues of DCM model rats and model cells revealed significantly reduced GNG2 mRNA levels in cardiac tissues of model animals and model cells after AGS-IV treatment.

Conclusion

In conclusion, AGS-IV effectively alleviated myocardial tissue lesions in DCM model rats, reduced the levels of Collagen1 and MMP-2 in myocardial tissue, serum, and model cells, and decreased the mRNA levels of GNG2, MRAS, and ERK in cardiac tissues of model rats and model cells. These results indicate that AGS-IV may exert a therapeutic effect on DCM by regulating the GNG2/MRAS-ERK signaling pathway. The present study has initially verified the potential mechanism underlying the therapeutic action of AGS-IV in DCM. In subsequent studies, further validation of this mechanism will be performed through experiments including protein expression detection, transcriptional function investigation, and additional gene knockout assays.

Ethics Statement

This study has been reviewed and approved by the Ethics Committee of Heilongjiang University of Chinese Medicine, with the ethical approval number: 2024102503. The welfare of laboratory animals is in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition.

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Disclosure

Ying Dong and Yidi Ma are co-first authors, and Shi Yan and Qiang Fu are co-corresponding authors. The authors report non conflicts of interest in this work.

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