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ORIGINAL RESEARCH

COL3A1 induces ischemic heart failure by activating AGE/RAGE pathway

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Abstract

This study aimed to screening different expression genes (DEGs) related to ischemic heart failure (IHF). For screening DEGs in gene omnibus (GEO) dataset, limma methods were used to screen the significant DEGs between IHF and sham groups. Venn plot was used for the analysis of the intersection DEGs among three GEO datasets (GSE107568, GSE107569, and GSE116250). String and KEGG (kyoto encyclopedia of genes and genomes) pathway were used for the analysis of key DEGs and their associated signal pathway. IHF rats model were established by high ligation of the anterior descending branch of the coronary artery. Functional indices were analyzed using echocardiography. IHF rats were then administrated with AAV-sh*COL3A1* to knockdown its expression. The expression of *COL3A1* was measured by RNA-sequencing and western blotting. Transmission electron microscope (TEM) assay was used to measure the apoptosis of cardiomyocytes. Serum AGE (advanced glycation end-products), SOD (superoxide dismutase), MDA (malondialdehyde), and LDH (lactate dehydrogenase) levels were determined by enzyme-linked immunosorbent assay (ELISA) kit. Limma analysis discovered 14 upregulated DEGs were involved in three GEO datasets (GSE107568, GSE107569, and GSE116250), and *COL3A1* was identified as one of the key genes, which was associated with AGE/RAGE (receptor for advanced glycation end-products) pathway by KEGG enrichment analysis. Western blotting and RNA-Seq indicated that *COL3A1* protein and mRNA were highly expressed in IHF rats model as compared to that in sham rats. Pathology photograph analysis showed that *COL3A1* deficiency inhibited infarct size in IHF rats. In addition, knockdown of *COL3A1* decreased cell apoptosis in IHF rats by transmission electron microscope (TEM) assay. Mechanically, *COL3A1* deficiency inhibited IHF development through activating AGE/RAGE signal pathway. The present study suggests that *COL3A1* induces IHF progression and development through activating AGE/RAGE pathway.

Keywords

Heart failure; *COL3A1*; AGE/RAGE pathway; Cell damage and apoptosis

1. Introduction

Heart failure is a syndrome characterized by a decrease in the pumping function of the heart and is the final stage in the development of many cardiovascular diseases, such as hypertension, myocardial ischemia, cardiomyopathy, and arrhythmias. Cardiac pumping insufficiency and arrhythmias due to heart failure are the main causes of death in patients with heart failure. It has been shown that in the early stages of heart failure, the systolic and diastolic rates of cardiomyocytes are slowed and the action potential time course is prolonged [1]. For normal myocardium, as the heart rate accelerates, myocardial contraction force increases gradually, showing a positive frequency-force relationship, but in failing myocardium, when the heart rate is slow, myocardial contraction force and myocardial shortening amplitude are normal or even increase; while when the heart rate accelerates, myocardial contraction force and its shortening amplitude decrease or remain unchanged, showing a negative frequency-force relationship [2]. With the continuous improvement of medical technology, the level of treatment for heart failure has also increased substantially, but its mortality rate is still greater than the annual survival rate, even higher than some malignant tumors.

Type III collagen was first discovered and described in 1971. It is an important structural protein and is classified as one of the main fibrous collagens. Type III collagen is the main structural component of hollow organs, such as large blood vessels, uterus, and intestines, where these tissues must be stretched. It is also found in many other tissues related to type I collagen. At all stages of embryonic development, the expression of type I and type III collagen seems to be coordinated. A previous study demonstrated that platelets interact

with type III collagen through specific glycoprotein and non-integrin receptors. Furthermore, Type III collagen plays a role in cell adhesion, migration, proliferation, and differentiation through interaction with cell surface receptor-integrins [3]. Kitamura *et al.* [4] analyzed heart tissue specimens from 35 patients with hypertrophic cardiomyopathy. The amount of type III collagen detected by immunostaining is related to several cardiac indicators of diastolic function and indicated that increased amount of fibrosis was related to poor cardiac function [4]. It has been reported in the literature that *COL1A1*, a homolog of *COL3A1*, contributes to the progression of heart failure in plasma and may be a potential plasma biomarker for predicting heart transplantation (HTx) within one year after the onset of heart failure and may play a role in the development of HF [5]. However, the function of *COL3A1* is poorly understood in ischemic heart failure (IHF).

In this study, low-output heart failure was established through the construction of a rat heart failure model-coronary artery ligation, and the heart failure screening criteria were based on the relevant heart failure diagnosis guidelines [6–9]. In addition, pathway prediction showed that *COL3A1* is significantly related to the AGE/RAGE signaling pathway. Studies have shown that the accumulation of end-stage glycosylation products (AGE) and the activation of AGE receptors (RAGE) can induce continuous oxidative stress in vascular tissues, which is closely related to the progression of heart failure [10]. However, no studies have reported the role of *COL3A1* and its regulation of AGE/RAGE signaling pathway. This study attempts to reveal the mechanism by which *COL3A1* promotes the progression of heart failure by regulating the AGE/RAGE signaling pathway.

In this study, *CLO3A1* was identified as one of the key genes in the progression of IHF due to its high expression in IHF rats model. *COL3A1* depletion decreased infarct size and cell apoptosis in IHF rats model. Mechanically, *COL3A1* deficiency inhibited IHF through activating AGE/RAGE signal pathway.

2. Materials and methods

2.1 Box plot, Venn diagram, PPI and KEGG enrichment analysis of GEO dataset

Different expression genes (DEGs) from GEO (GSE107568, GSE107569, GSE116250) was determined by bioinformatical analysis (limma analysis different expression genes, data filtering and standardization were performed as described previously [11–14]. ComBat method implemented in the R package ggplot2, GEOquery, and sva were used to account for platform and batch effects. KEGG-GO analysis was performed by using of the clusterProfiler package for R language (version 4.2.2, Bell Laboratories, Murray Hill, NJ, USA) [15]. Pathway enrichment analysis was obtained from metascape online (`https://metascape.org/gp/index.html`) [16]. PPI (protein-protein interaction) analysis of *COL3A1* was obtained from String: functional protein association networks (`string-db.org`).

2.2 Establishment of IHF rat model

After anesthesia in adult male Sprague-Dawley (SD) rats (12–14 weeks), a longitudinal incision was made in the left anterior area of the heart. The thoracic cavity was exposed by blunt separation and breaking the fourth to last rib, the pericardium was torn away to expose the heart, and the left anterior descending branch was permanently ligated 2–3 mm from the aortic root. A change in color of the distal end of the ligated myocardium was observed in the successfully ligated hearts. Rats in the sham operation group (sham) passed under the coronary artery without ligation. After 4 weeks, all rats were killed by severing the necks. The heart functional index was measured by ultrasonic cardiogram. Myocardial tissue sections were stained by TCC (total collagen content) as previous depicted. All animal experiments were approved by the Ethics Committee of Jiangsu Rongjun Hospital for animal use and performed in accordance with the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animal.

2.3 RNA-Seq analysis

RNA-Seq was performed as described previously [17]. After collecting samples, total RNA was extracted using TRIzol reagent (15596018, Ambion, Foster City, CA, USA). The sequencing library was then constructed and DEGs were analyzed.

2.4 western blotting analysis

Western blotting was performed as described previously [18]. And immunoblotted with the following antibodies: anti-mouse *COL3A1* (1:1000, sc-6253, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse Bax (1:1000, sc-20067, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse the B-cell lymphoma-2 (BCL-2) (1:1000, sc-73822, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse Caspase-3 (1:1000, sc-7272, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse RAGE (1:1000, sc-74473, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-mouse β-actin (1:1000, sc-8432, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the Polyvinylidene Fluoride (PVDF) (1620184, bio-rad, USA) membranes were washed and secondary antibodies were applied 1:5000 for 1 h at room temperature, the immunoreactions were visualized with chemiluminescent Electrochemiluminescence (ECL) (1705061, bio-rad, USA) reagent.

2.5 Transmission electron microscope (TEM) assay

TEM (HITACHI, H-7650, Tokyo, Japan) assay was performed as described previously [19]. Cells were seeded in copper mesh, then washed, fixed, embedded, and pictures were captured by TEM.

2.6 Enzyme-linked immunosorbent assay (ELISA)

5×10^3 cells were seeded in 96-well plates with complete medium with FBS (fetal bovine serum) (A4736201, gibco, USA) for 24 h. Then, the supernatant was tested using the AGE, MDA, SOD, and LDH ELISA kit (ab183367, abcam, England) according to the instructions. Finally, the absorbance value was detected using a microplate reader (EL406, BioTek, Winooski, Vermont, USA). The concentrations of cytokines were obtained according to the standard curves.

2.7 Establishment of *COL3A1* deficiency rats model

The plasmids of sh*COL3A1*-AAV and shNC (sh-negative control)-AAV were purchased from Invitrogen (sh*COL3A1*: GCTACTTCTCGCTCTGCTTCA; Carlsbad, CA, USA). The rats in IHF and sham groups were injected with 100 μL adeno-associated virus (10^12 μg/mL) through caudal vein. After 3 weeks, rats were killed, sectioned, and photographed.

2.8 Statistical analysis

Student's *t*-test and one-way ANOVA (analysis of variance) were used for statistical analysis. Data were presented as means $\pm$ SEM (standard error of mean) of three independent experiments. A *p*-value of 0.05 or less was considered to be significant.

3. Results

3.1 Multi-chip combined analysis to screen DEGs involved in the development of IHF

In order to screen the DEGs in IHF compared with non-failing group, R package (ggplot2 [20] and GEOquery [21]) were used for analysis based on the GEO datasets (GSE107568, GSE107569, and GSE116250). As shown in Fig. 1A–C, all GEO datasets showed downregulated (blue dot) and upregulated (red dot) expression genes. Moreover, 14 upregulated DEGs and 0 downregulated DEGs were respectively involved in the three datasets (Fig. 1D). The online platform-String [22] (`http://string-db.org`) was used to predict the protein-protein interaction among DEGs. The results found that *COL3A1* is the key gene of these DEGs (Fig. 1E). In addition, the DEGs were mainly associated with AGE/RAGE signaling pathway by KEGG enrichment analysis (Fig. 1F).

3.2 *COL3A1* is significantly up-regulated in myocardial cells from rats with IHF

High ligation of the anterior descending coronary artery was used to establish the rats model of IHF. As shown in Fig. 2A, LVEF (left ventricular ejection fraction) [23] was decreased in myocardial cells of rats with IHF compared to that in sham group. On the contrary, both of LVIDs (left ventricular internal diameter) and LVIDd (left ventricular internal dimension diastole) were increased in myocardial cells of rats with IHF compared to that in sham group (Fig. 2B–C). Additionally, FS was decreased in myocardial cells of rats with IHF compared to that in sham rats (Fig. 2D). Based on the results of cardiac function index, the infarct size was significantly increased in IHF group rats compared to that in sham rats (Fig. 2E). Interestingly, *COL3A1* mRNA and protein expressions were upregulated in IHF group compared to that in sham rats (Fig. 2F–G). Taken together, these data indicated that the upregulation of *COL3A1* in myocardial cells of rats with IHF.

3.3 Knockdown of *COL3A1* alleviates myocardial cell damage and apoptosis in rats with IHF.

In order to investigate the function of *COL3A* in IHF rats, the IHF rats model were administrated with AAV-sh*COL3A1* to knockdown *COL3A* expression, which was validated by western blotting (Fig. 3A). As shown in Fig. 3B, knockdown of *COL3A1* in IHF rats remarkably inhibited the infarct size compared with IHF rats with AAV-shNC treatment. Apart from this, *COL3A1* deficiency antagonized the inhibition of LVEF and FS in IHF rat as compared to IHF rats with AAV-shNC treatment. On the contrary, knockdown of *COL3A1* decreased LVIDs and LVIDd in IHF rat as compared to IHF rats with AAV-shNC treatment (Fig. 3C–F).

In addition, TEM assay was used to analyze myocardial cell apoptosis in rats. As shown in Fig. 4A, there were more myocardial cell apoptosis in IHF rats as compared to that in sham rats. *COL3A1* deficiency reversed the enhancement of cell apoptosis in IHF rats with AAV-shNC. Moreover, Bcl-2 expression was decreased in IHF rats as compared to that in sham rats, whereas knockdown of *COL3A1* enhanced the expression of Bcl-2 in IHF rats. Reversely, *COL3A1* deficiency reduced Bax and Caspase-3 expressions in IHF rats with AAV-shNC treatment (Fig. 4B). Taken together, these data suggested that *COL3A1* promotes cell damage and apoptosis in rats with IHF.

3.4 *COL3A1* deficiency suppresses AGE/RAGE signaling pathway and oxidative stress in rats with IHF.

Based on the results of KEGG pathway enrichment analysis, the DEGs were mainly involved in AGE/RAGE pathway [24]. Therefore, the serum AGE level in rat was analyzed. As shown in Fig. 5A, IHF rat showed high serum AGE level as compared to sham rats, while knockdown of *CLO3A1* suppressed the upregulation of serum AGE in IHF rats with AAV-shNC treatment. Similarly, the expression of RAGE protein was also decreased in IHF rats with AAV-sh*COL3A1* treatment as compared to with AAV-shNC (Fig. 5B). Additionally, increased LDH and MDA protein levels were found in IHF rats as compared to that in sham rats, and *COL3A1* knockdown in IHF rats decreased LDH and MDA when compared with IHF rats with AAV-shNC treatment (Fig. 5C–D). Otherwise, SOD was downregulated in IHF rats compared to sham rats, while *COL3A1* deficiency alleviated the inhibition of SOD in IHF rats with AAV-shNC treatment. Collectively, these results indicated that *COL3A1* positively regulates AGE/RAGE signaling pathway in IHF rats.

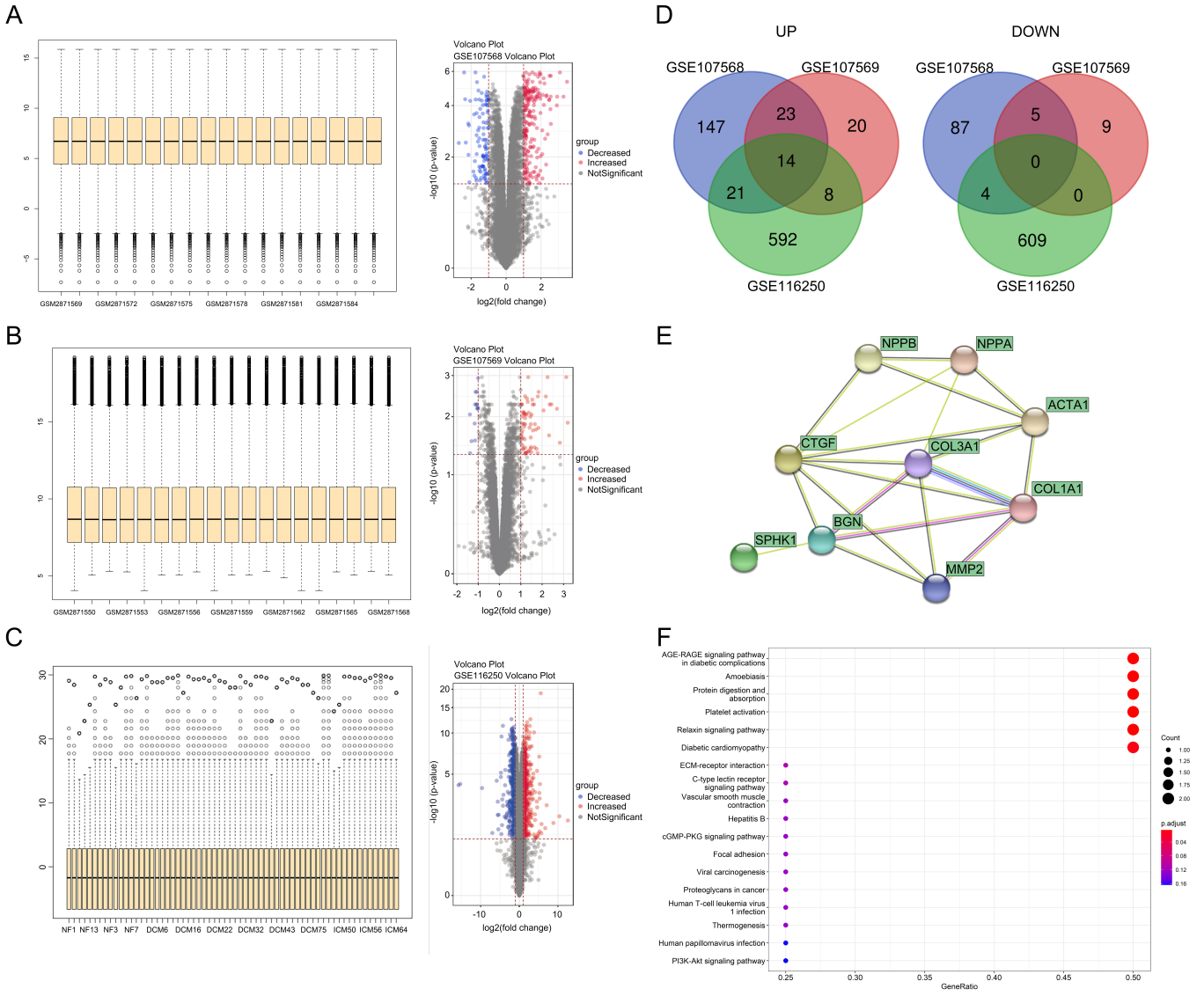

FIGURE 1. Multi-chip combined analysis to screen DEGs involved in the development of IHF. (A–C) Volcano plot was used to screen DEGs (different expression genes) between non-failing group and IHF group in gene omnibus (GEO) dataset (GSE107568, GSE107569, and GSE116250). Blue: decreased expression genes, red: increased expression genes, grey: no significant change expression genes. Based on an adjusted $p < 0.05$ and $|\log \text{fold change}| > 1$. Ns: no significance, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (D) Venn plot analysis of DEGs among three datasets (GSE107568, GSE107569, GSE116250). (E) PPI network showed that *COL3A1* is a key gene in DEGs. (F) KEGG (kyoto encyclopedia of genes and genomes) enrichment analysis of DEGs. IHF: ischemic heart failure.

4. Discussion

Despite significant improvements in living standards and medical care, heart failure remains a worldwide problem. Treating heart failure is costly and affects the quality of life of patients to varying degrees. Ischemic cardiomyopathy is one of the most common causes of heart failure. In addition, some patients have other diseases, such as type 2 diabetes, which complicates therapeutic interventions for heart failure. Angiotensin-converting enzyme inhibitors, β-blockers, diuretics, contractile drugs, and cardiac resynchronization therapy (CRT) have been widely used in the treatment of IHF. However, a significant number of patients inevitably enter end-stage heart failure for various reasons. Therefore, identification of specific biomarkers of IHF are crucial.

Accumulation of *COL3A1* is a specific marker of several human diseases, including systemic sclerosis, cardiac fibrosis, lung fibrosis, liver cirrhosis and renal fibrosis [3]. Previous studies demonstrated that the accumulation of extracellular matrix proteins in heart tissue can lead to scars caused by myocardial fibrosis, myocardial infarction and other injuries, causing impaired myocardial function, including ischeminc heart failure [25]. Herpel *et al.* [26] studied several extracellular matrix proteins extracted from heart tissue samples from patients diagnosed with dilatation, ischemic, and valvular cardiomyopathy by immunostaining. The results showed that different types of cardiomyopathy have myocardial interstitial fibrosis, but they differ in the distribution of various extracellular matrix proteins [26]. Research specializing in type III collagen have included studies on hypertrophic and dilated

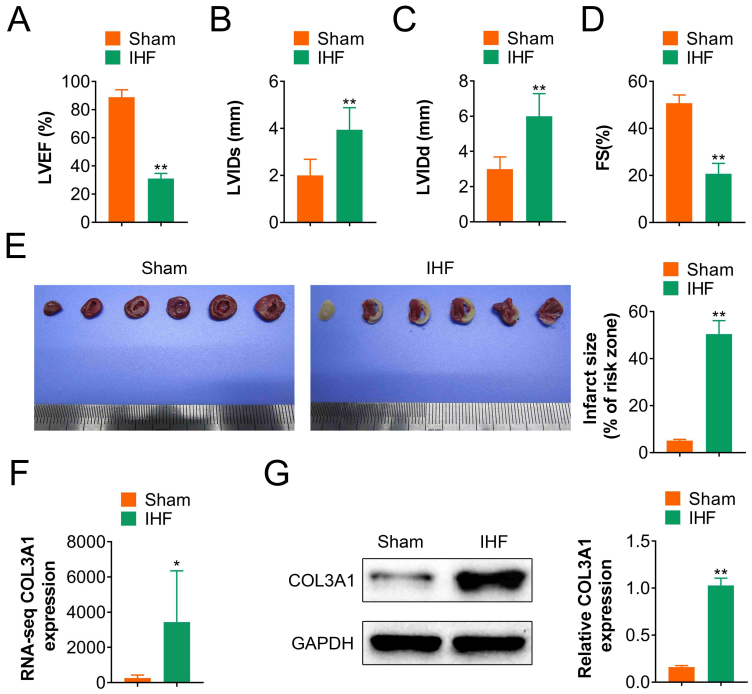

FIGURE 2. ***COL3A1* is significantly up-regulated in myocardial cells from rats with IHF.** (A–D) Rats were divided into sham group and IHF group. Echocardiography analysis of cardiac function index (4 weeks after surgery), including Left ventricular ejection fraction (LVEF), left ventricular end systolic diameter (LVIDs), left ventricular end systolic volume (LVIDd), and left ventricular shortening fraction (LVFS). (E) TCC staining analysis of myocardial infarction size in IHF rats. (F) The mRNA expression of *COL3A1* (type III collagen) was analyzed by RNA-sequencing. (G) Western blotting assay of *COL3A1* protein expression. Data are representative of three independent experiments (mean ± SD). *, $p < 0.05$ and **, $p < 0.01$. *vs.* Sham group. IHF: ischemic heart failure.

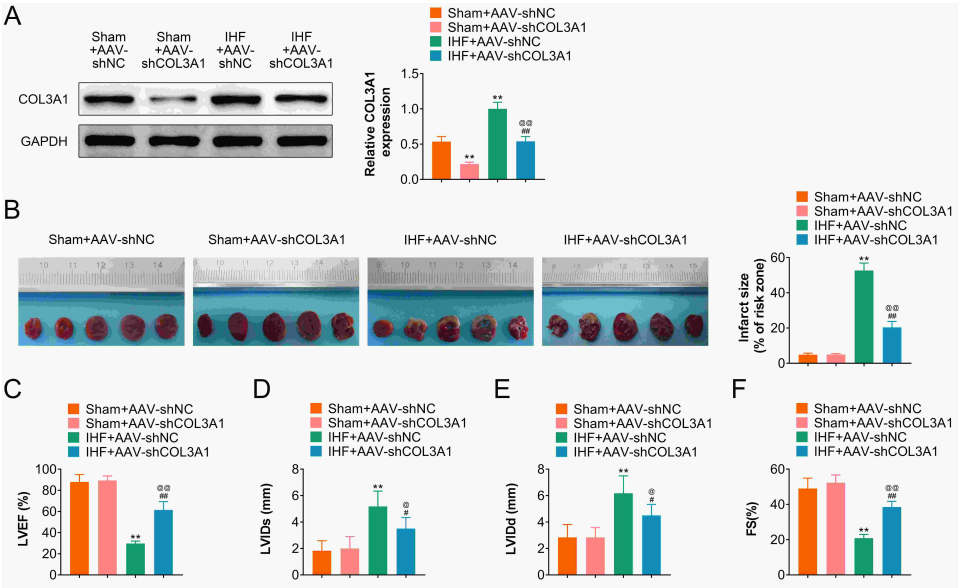

FIGURE 3. Knockdown of *COL3A1* alleviates myocardial cell damage in rats with IHF. (A) Knockdown *COL3A1*-AAV (Adeno-associated virus) plasmid through tail vein injection of sham or IHF model, the expression of *COL3A1* was measured by western blotting. (B) TCC staining analysis of the myocardial infarction size in IHF rats with *COL3A1*-AAV plasmid treatment. (C–F) Echocardiography analysis of cardiac function index in IHF rats with *COL3A1*-AAV plasmid treatment (4 weeks after surgery), including Left ventricular ejection fraction (LVEF), left ventricular end systolic diameter (LVIDs), left ventricular end systolic volume (LVIDd), left ventricular shortening fraction (LVFS). **, $p < 0.01$ *vs.* Sham + AAV-shNC group. $^{\#}$, $p < 0.05$ and $^{\#\#}$, $p < 0.01$. *vs.* IHF + AAV-shNC group. $^{@}$, $p < 0.05$ and $^{@@}$, $p < 0.01$. *vs.* Sham + AAV-sh*COL3A1* group. IHF: ischemic heart failure.

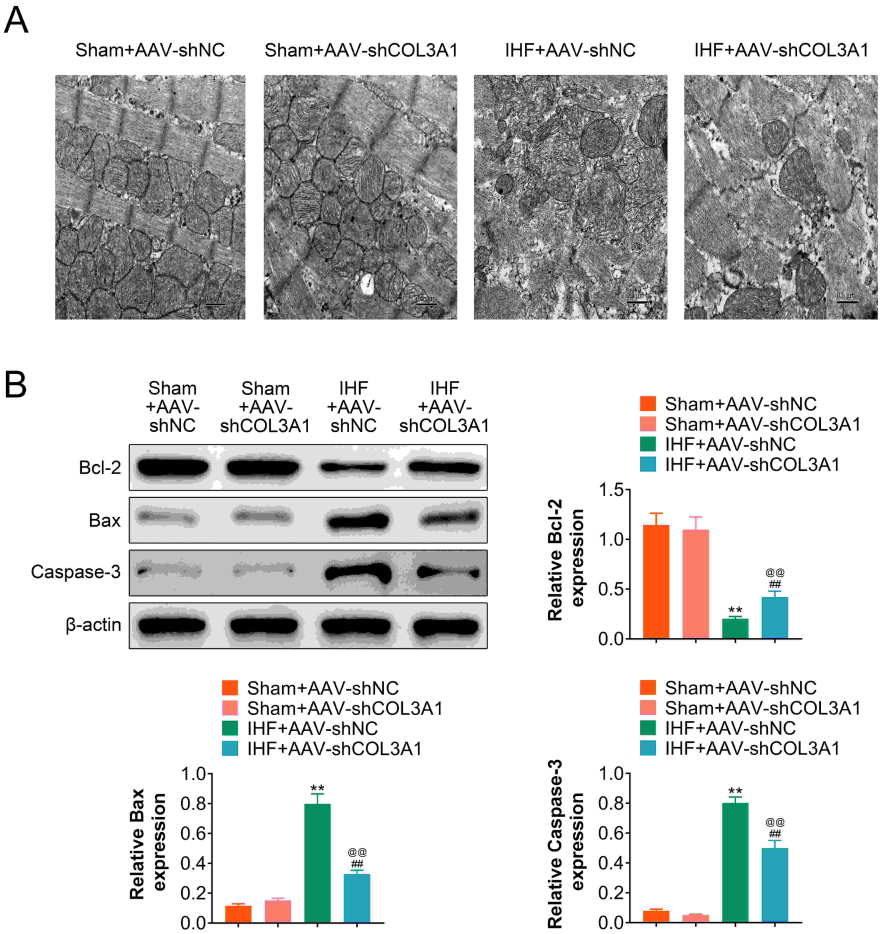

FIGURE 4. Knockdown of *COL3A1* inhibits apoptosis of cardiomyocytes in rats with IHF. (A) TEM assay analysis of cell apoptosis from IHF rats with *COL3A1*-AAV (Adeno-associated virus) plasmid treatment. (B) The western blotting assay determined the expressions of Bcl-2, Bax, and Caspase-3 in IHF rats with *COL3A1*-AAV plasmid treatment. **, $p < 0.01$ *vs.* Sham + AAV-shNC group. ##, $p < 0.01$. *vs.* IHF + AAV-shNC group. @@, $p < 0.01$. *vs.* Sham + AAV-sh*COL3A1* group. IHF: ischemic heart failure.

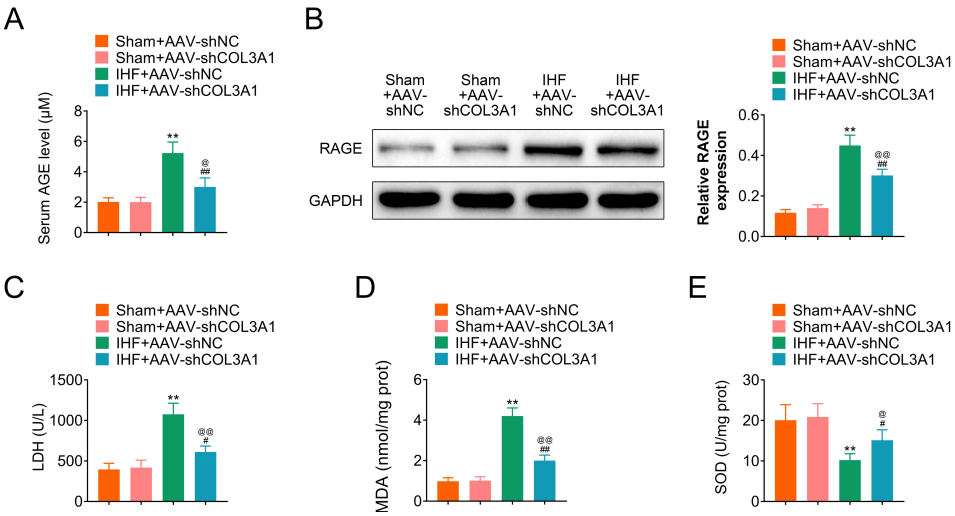

FIGURE 5. *COL3A1* deficiency suppresses AGE/RAGE signaling pathway and oxidative stress in rats with IHF. (A) Serum AGE level in IHF rats with *COL3A1*-AAV plasmid treatment was detected by ELISA. (B) Western blotting assay on the expression level of RAGE (receptor for advanced glycation end-products) in cardiomyocytes from rats with IHF. (C–E) LDH (lactate dehydrogenase), MDA (malondialdehyde) and SOD (superoxide dismutase) levels in IHF rats with *COL3A1*-AAV plasmid treatment were detected by ELISA. IHF: ischemic heart failure.

cardiomyopathy, pulmonary vascular disease, and alternative fibrosis after myocardial infarction. Changes in type III collagen have also been studied in a rare cardiomyopathy, called idiopathic restrictive cardiomyopathy. However, *COL3A1* as a member of type III collagen, remains poorly clarified. This study demonstrated that, there are 14 upregulated DEGs were involved in the three GEO datasets, and *CLO3A1* is one of the key genes in the progression of IHF. *COL3A1* was highly expressed in IHF rats compared to that in sham rats. *COL3A1* deficiency inhibited infarct size and cell apoptosis in IHF rats. Mechanically, *COL3A1* deficiency reduced IHF through activating AGE/RAGE signal pathway.

In the past two decades, many researches have been reported on *COL3A1*, one of the most abundant component of extracellular matrix (ECM) in the TME [27–30]. For example, miR-29 acted as a tumor suppressor in control of methotrexate resistance and cell apoptosis through regulating *COL3A1*, which indicates the role of *CLO3A1* in cell apoptosis [31]. Another study also demonstrated that miR-let-7b is an important regulator in reducing the expression of *COL3A1* to inhibit apoptosis through activating Bcl2 and inactivating Caspase 3 [32]. Here, it is demonstrated that *COL3A1* deficiency negatively regulates cell apoptosis through Bcl-2 upregulation and suppression of Bax and Caspase-3 in IHF rats model. However, the molecular mechanism underlying the role of *COL3A1* in suppressing cell apoptosis in IHF rats model need further investigation.

The AGE/RAGE signaling pathway is a common transduction pathway in regulation of cell migration, cell apoptosis, and cell proliferation [10, 33, 34]. Studies have demonstrated that the AGE/RAGE pathway is associated with fibrosis in the diabetic heart, and cardiovascular disease in patients with chronic kidney disease [35, 36]. Previous studies have reported that RAGE acts a vital role in the apoptosis of cardiomyocytes through stimulation of p38-MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase) pathways [37, 38]. In this study, it is demonstrated that knockdown of *COL3A1* reduced the activity of AGE/RAGE signal pathway. It is thus speculated that *COL3A1* deficiency decreases cell apoptosis through RAGE, which may be regulated by p38-MAPK and JNK pathways. However, the underlying mechanism of *COL3A1* in regulating of AGE/RAGE need further investigation.

5. Conclusions

The present study found that *COL3A1* was identified as a crucial gene according to bioinformatics analysis, and validated to be upregulated in IHF rats model. Moreover, the infarct size was increased in IHF rats model compared to sham rats. Moreover, *COL3A1* deficiency antagonized the induction of infarct, cell apoptosis, AGE/RAGE signaling pathway and oxidative stress levels in IHF rats model. In summary, this study confirmed that *COL3A1*-AGE/RAGE axis played an important role in promoting the progression of IHF. This might provide a promising hallmark for IHF diagnosis and therapy.

AUTHOR CONTRIBUTIONS

XC and JWZ—designed the study, supervised the data collection, TL—analyzed the data, interpreted the data, ZSD and CZ—prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental and animal manipulations are authorized and approved by Jiangsu Rongjun Hospital ethics committee (Approval No. YKT2021001).

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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