

# Chronic Peripheral Ghrelin Injection Exerts Antifibrotic Effects by Increasing Growth Differentiation Factor 15 in Rat Hearts With Myocardial Fibrosis Induced by Isoproterenol

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## Summary

This study aimed to investigate the anti-fibrotic effects of ghrelin in isoproterenol (ISO)-induced myocardial fibrosis and the underlying mechanism. Sprague-Dawley rats were randomized to control, ISO, and ISO + ghrelin groups. ISO (2 mg/kg per day, subcutaneous) or vehicle was administered once daily for 7 days, then ghrelin (100 µg/kg per day, subcutaneous) was administered once daily for the next 3 weeks. Ghrelin treatment greatly improved the cardiac function of ISO-treated rats. Ghrelin also decreased plasma brain natriuretic peptide level and ratios of heart weight to body weight and left ventricular weight to body weight. Ghrelin significantly reduced myocardial collagen area and hydroxyproline content, accompanied by decreased mRNA levels of collagen type I and III. Furthermore, ghrelin increased plasma level of growth differentiation factor 15 (GDF15) and GDF15 mRNA and protein levels in heart tissues, which were significantly decreased with ISO alone. The phosphorylation of Akt at Ser473 and GSK-3β at Ser9 was decreased with ISO, and ghrelin significantly reversed the downregulation of p-Akt and p-GSK-3β. Mediated by GDF15, ghrelin could attenuate ISO-induced myocardial fibrosis *via* Akt-GSK-3β signaling.

## Key words

Ghrelin • Myocardial fibrosis • Growth differentiation factor 15 • Akt/GSK-3β pathway

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## Introduction

Despite significant advances in therapy, heart failure (HF), known as the ultimate outcome in cardiovascular diseases including hypertension, myocardial infarction and valvular dysfunction, remains a major clinical problem of high morbidity and mortality worldwide (Shan and Mann 2011). Myocardial fibrosis is characterized by uncontrolled proliferation of fibroblasts and excessive deposition of extracellular matrix proteins in myocardium, in response to pathological stimuli, such as myocardial injury. Myocardial fibrosis is considered an important pathological mechanism in the progression of HF and contributes to myocardial stiffness, cardiac dysfunction, arrhythmias and sudden death (Gonzalez *et al.* 2018). Therefore, inhibiting myocardial fibrosis is an effective way to prevent HF (Travers *et al.* 2017). However current anti-fibrotic therapy is limited, and novel mediators of myocardial fibrosis need to be identified for the development of better therapeutics.

Growth factors and chemokines, including insulin-like growth factor, fibroblast growth factor, and transforming growth factor (TGF), are some of the best-studied mediators and have important roles in proliferation, extracellular matrix deposition and organ fibrosis by interacting with corresponding receptors (Lewis *et al.* 2018). Several recent studies have demonstrated that growth differentiation factor 15 (GDF15), belonging to the superfamily TGFβ, plays a crucial role in the progression of HF (Wollert and Kempf 2012). As a stress-responsive cytokine, GDF15 is weakly produced in the heart under normal physiological

conditions, but its level increases rapidly in response to cardiovascular injury, such as pressure overload, HF, and ischemia/reperfusion. Increased circulating GDF15 levels were found associated with severity of chronic HF as reflected by New York Heart Association class, peripheral edema, and increased level of brain natriuretic peptide (BNP) (Anand *et al.* 2010). In addition, elevated GDF15 plasma level is a surrogate marker of risk of mortality in HF (Kempf and Wollert 2009). However, the responses observed in animal studies are contrary to what is found in humans. In animal studies, GDF15 has protective effects by inhibiting apoptosis, hypertrophy, and adverse remodeling in the injured heart (Heger *et al.* 2010, Xu *et al.* 2014). Thus, further research is essential before considering GDF15 as a therapeutic target in cardiovascular diseases, especially for myocardial fibrosis.

Ghrelin is a 28-amino acid orexigenic peptide released primarily from oxyntic glands of the gastric mucosa. It is an endogenous ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a), which stimulates growth hormone secretion and is well known for its regulation of metabolism and appetite. Ghrelin and its receptor are both expressed throughout the heart and vasculature and play an important role in the cardiovascular system of rodents and healthy humans (Lilieness and Frishman 2016). Several lines of evidence indicated that ghrelin has robust cardioprotective effects including enhancing endothelial and vascular function, inhibiting sympathetic drive, preventing atherosclerosis, and decreasing blood pressure (Shirai *et al.* 2015, Virdis *et al.* 2015, Wang *et al.* 2017, Camargo-Silva *et al.* 2018). Also, in a rat model of HF, ghrelin attenuated ventricular remodeling and improved cardiac function (Eid *et al.* 2018, Mao *et al.* 2015). Ghrelin was found to reduce myocardial fibrosis in rats with myocardial infarction and angiotensin II-induced cardiac fibroblasts by inhibiting NADPH oxidase–reactive oxygen species signaling (Wang *et al.* 2018). In addition, ghrelin could have a protective role by regulating the content of growth factors (Cieszkowski *et al.* 2017, Wang *et al.* 2015). However, whether the anti-fibrotic effects of ghrelin are mediated by GDF15 has not been fully elucidated.

Here we aimed to investigate the anti-fibrotic effects of ghrelin in isoproterenol (ISO)-induced myocardial fibrosis in rats. Furthermore, we explored whether ghrelin exerted its anti-fibrotic effect *via* GDF15.

## Methods

### *Animals and experimental protocol*

Male Sprague-Dawley rats (180-200 g) were purchased from Vital River Laboratories (Beijing). The rats were maintained at 22-25 °C under a 12-h light/dark cycle with relative humidity 60 % and unrestricted access to food and water for a 1-week acclimatization period. All animal experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

After acclimatization, rats were randomly divided into three groups (n=6) for treatment: control, ISO, ISO + ghrelin. ISO (2 mg/kg per day, subcutaneous) or vehicle was administered once daily for 7 days to induce the rat model of myocardial fibrosis as described (Lai *et al.* 2016). Then ghrelin (100 µg/kg per day, subcutaneous) was administered once daily for the next 3 weeks (Eid *et al.* 2018). The control and ISO groups were administered the equivalent volume of saline at the same times.

### *Hemodynamic assessment of cardiac physiology*

At the end of the experiment, the rats were intraperitoneally anesthetized with urethane (1 g/kg), and a catheter filled with heparin saline (500 U/ml) was inserted into the left ventricle from the right carotid artery to record hemodynamic parameters, including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR), maximum contraction velocity (+dp/dt<sub>max</sub>), and maximum relaxation velocity (-dp/dt<sub>max</sub>) by using a PowerLab data-acquisition system (AD Instruments, Sydney, Australia).

After hemodynamic parameters were measured, hearts were excised and heart weight and left ventricle weight were determined. Ratios of heart weight to body weight and left ventricular weight to body weight were calculated by dividing heart weight and left ventricular weight by body weight as an indicator of cardiac remodeling. Then, heart tissues were quickly frozen in liquid nitrogen and stored at -80 °C. The plasma was prepared by centrifuging blood samples at 3000 rpm for 15 min and was frozen at -80 °C.

### *Masson trichrome staining*

After fixation for 24 h in 4 % paraformaldehyde, heart blocks were dehydrated, embedded in paraffin, and cut into 4-µm-thick slices. Slices were heated overnight at

37 °C, dewaxed, and stained with Masson trichrome by standard procedures. The heart sections were visualized under a light microscope (Olympus, Tokyo, Japan). Images were obtained in five random fields per section and were quantified by using ImageJ. Area of fibrosis was divided by total area of microscopic fields.

#### *Determination of myocardial hydroxyproline content*

Myocardial hydroxyproline content was measured by using a hydroxyproline detection kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) according to the manufacturer's instructions and normalized to total protein.

#### *Measurement of plasma BNP and GDF15*

Plasma levels of the BNP and GDF15 were measured by using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

#### *Western blot analysis*

Protein extracted from heart tissue was quantified by using BCA reagent, and protein samples were denatured at 99 °C for 5 min. An amount of 50 µg protein was fractionated on 10 % SDS-PAGE gels, then transferred to polyvinylidene fluoride membranes, which were blocked with 5 % nonfat milk for 1 h, then incubated with the primary antibodies anti-phospho(Ser473)-Akt (*p*-Akt), anti-Akt, anti-phospho(Ser9)-GSK-3β (*p*-GSK-3β), anti-GSK-3β and anti-GDF15 (1:1000, Cell Signaling Technology, country), with GAPDH as an internal control (1:2000, Poteintech, country) at 4 °C overnight. After washing with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Target bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce, USA). The band intensity was quantified by using ImageJ.

#### *Real-time qPCR*

Frozen heart tissues were suspended in Trizol reagent (Invitrogen, Carlsbad, USA), and total RNA was extracted according to the manufacturer's instructions. Reverse transcription involved using a Reverse Transcription Kit (Toyobo, Osaka, Japan). A SYBRGreen RT-PCR Kit from Toyobo was used for quantitative real-time qPCR analysis with the StepOnePLUS Real-time PCR system (Applied Biosystems, Foster, USA), according to the manufac-

turer's instructions. Gene-specific primers were used to detect

Rat GDF15:

Forward: 5'-GACCTAGGTTGGAGCGACTG-3';  
reverse: 5'-TAAGAACCACCGGGGTGTAG-3';

Collagen I:

forward: 5'-TATGCTTGATCTGTATGTGCCA-CAAT-3'; reverse: 5'-TCGCCCTCCCGTTTTTG-3';

Collagen III:

forward: 5'-CAGCTGGCCTTCCTCAGACT-3';  
reverse: 5'-TGCTGTTTTTGCCTGGTATG-TAA-3'.

The samples were normalized against endogenous rat β-actin (forward: 5'-TACCACATCCAA-GGAAGGCAGCA-3'; reverse: 5'-TGGAATTACCGC-GGCTGCTGGCA-3'), and fold changes were calculated by using the formula  $2^{-\Delta\Delta Ct}$ .

#### *Statistical analyses*

Data are expressed as mean ± SEM. Statistical analysis involved using SPSS v17.0 (SPSS, Inc., USA). Data for three groups were compared by two-way ANOVA. Data for two groups were compared by Students *t*-test. *p*<0.05 was considered statistically significant.

## **Results**

#### *Ghrelin improved cardiac function in rats with ISO-induced myocardial fibrosis*

After 4 weeks of treatment, LVSP and ±dp/dt<sub>max</sub> were decreased and LVEDP was increased with ISO versus control treatment. However, ghrelin treatment increased LVSP and ±dp/dt<sub>max</sub> but decreased LVEDP as compared with ISO alone (Fig. 1A-D). HR did not differ among the three groups (Fig. 1E). Thus, ghrelin treatment ameliorated ISO-induced cardiac dysfunction.

#### *Ghrelin attenuated cardiac remodeling in rats with ISO-induced myocardial fibrosis*

After 4 weeks of treatment, ratios of heart to body weight and left ventricle to body weight were increased with ISO (Fig. 2A-2B). However, ghrelin treatment reduced the ratios. In addition, plasma level of BNP, an important biomarker of HF, was elevated after ISO treatment but was significantly attenuated with ISO + ghrelin (Fig. 2C). Thus, ISO-induced left ventricular remodeling was significantly reduced by ghrelin.

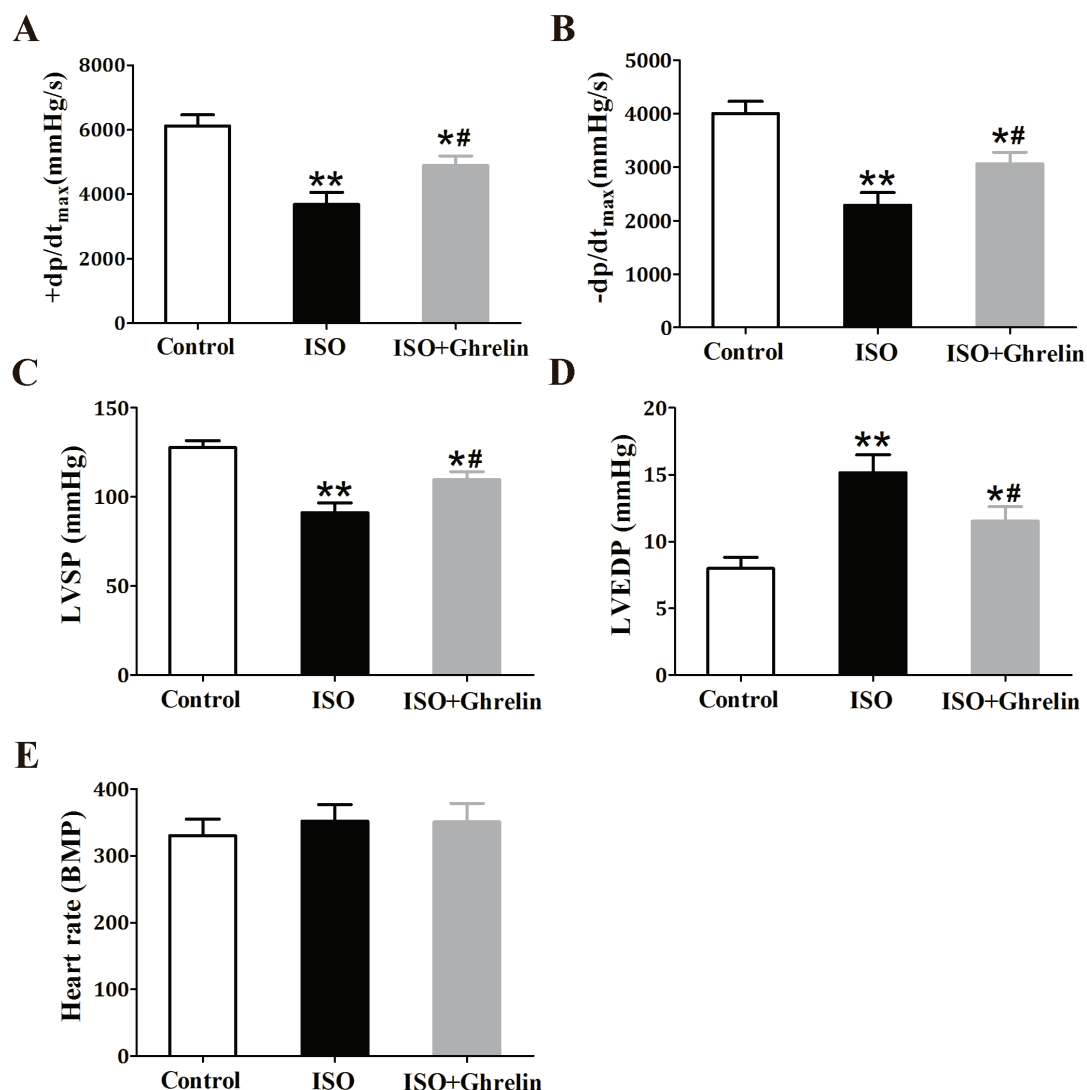
### Ghrelin alleviated cardiac fibrosis in rats with ISO-induced myocardial fibrosis

Masson's trichrome staining showed fibroblast aggregation and increased interstitial collagen volume in the ISO group versus controls, and this fibrotic response was attenuated by ghrelin treatment (Fig. 3A-B). In addition, content of hydroxyproline (an indicator of fibrosis) was higher with ISO than control treatment and was partially decreased by ghrelin treatment (Fig. 3C). The mRNA levels of collagen I and III were higher with ISO than control treatment and were significantly decreased with ghrelin treatment (Fig. 3D-E). Therefore, ghrelin could inhibit the formation of myocardial fibrosis.

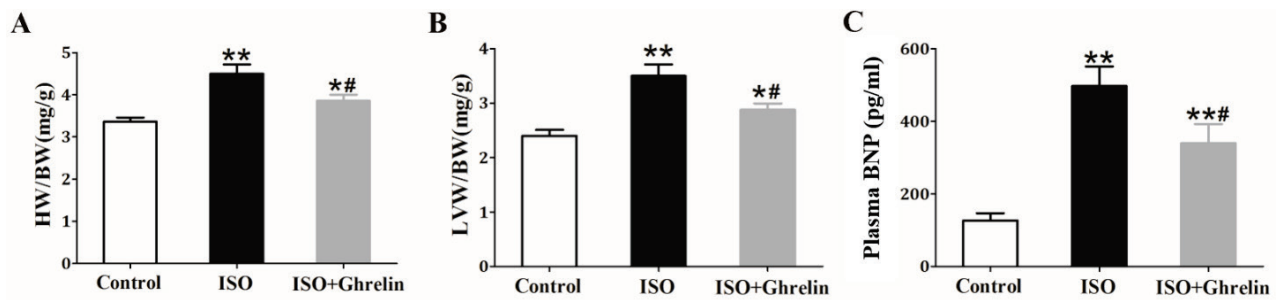
### Ghrelin increased the generation of GDF15 and activated the Akt-GSK-3 $\beta$ pathway

Plasma GDF15 level and GDF15 mRNA and protein levels in heart tissue were significantly lower with ISO than control treatment, and ghrelin treatment could increase the generation of GDF15 (Fig. 4A-D).

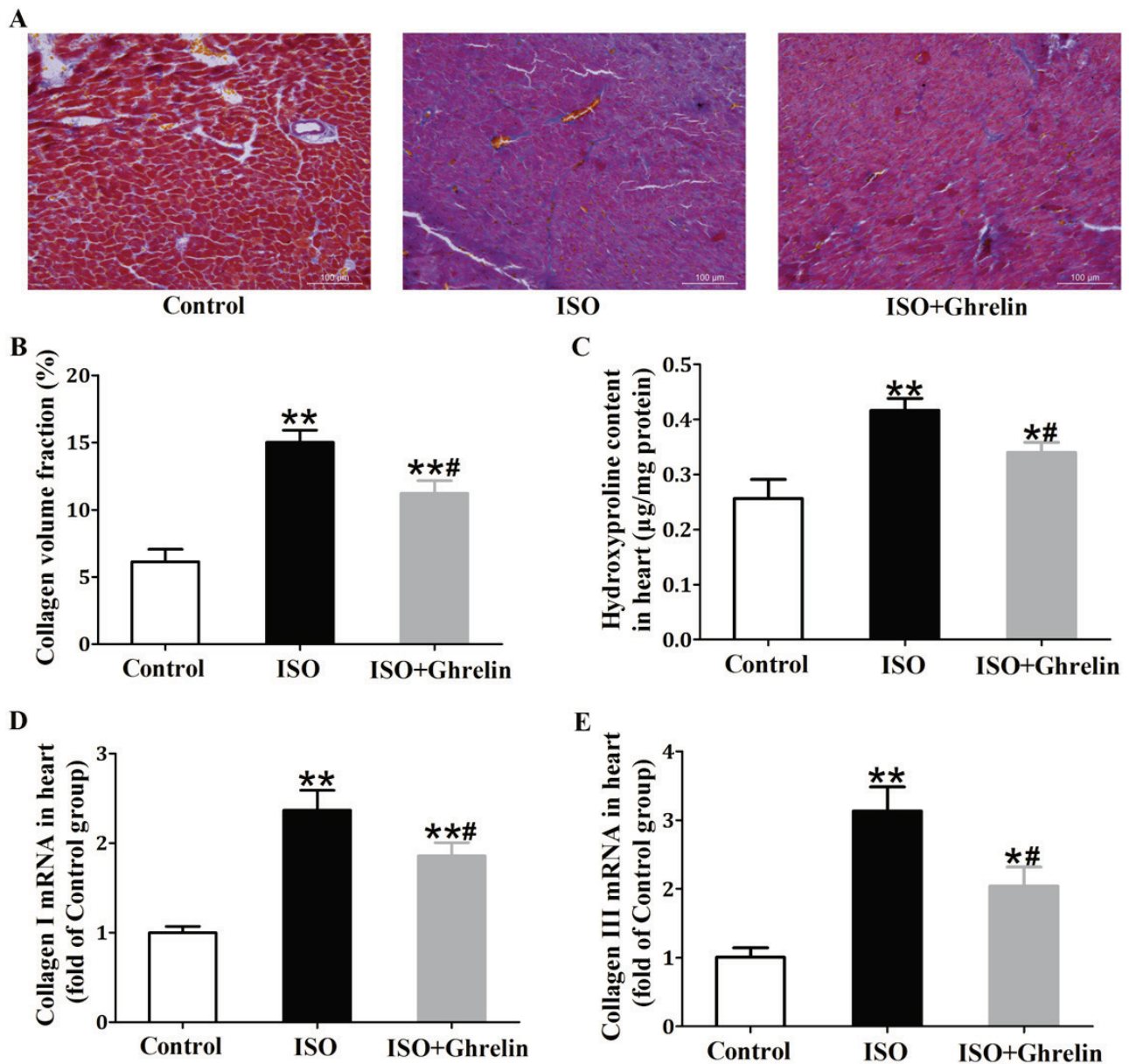
To further explore the potential molecular mechanisms of the cardioprotective effects of ghrelin, we examined *p*-Akt and *p*-GSK-3 $\beta$  levels in heart tissue (Fig. 4E-F). As compared with control treatment, ISO treatment decreased the level of *p*-GSK-3 $\beta$  at Ser9 and decreased that of *p*-Akt at Ser473. However, ghrelin treatment significantly reversed the downregulation of *p*-Akt and *p*-GSK-3 $\beta$  in heart tissue.



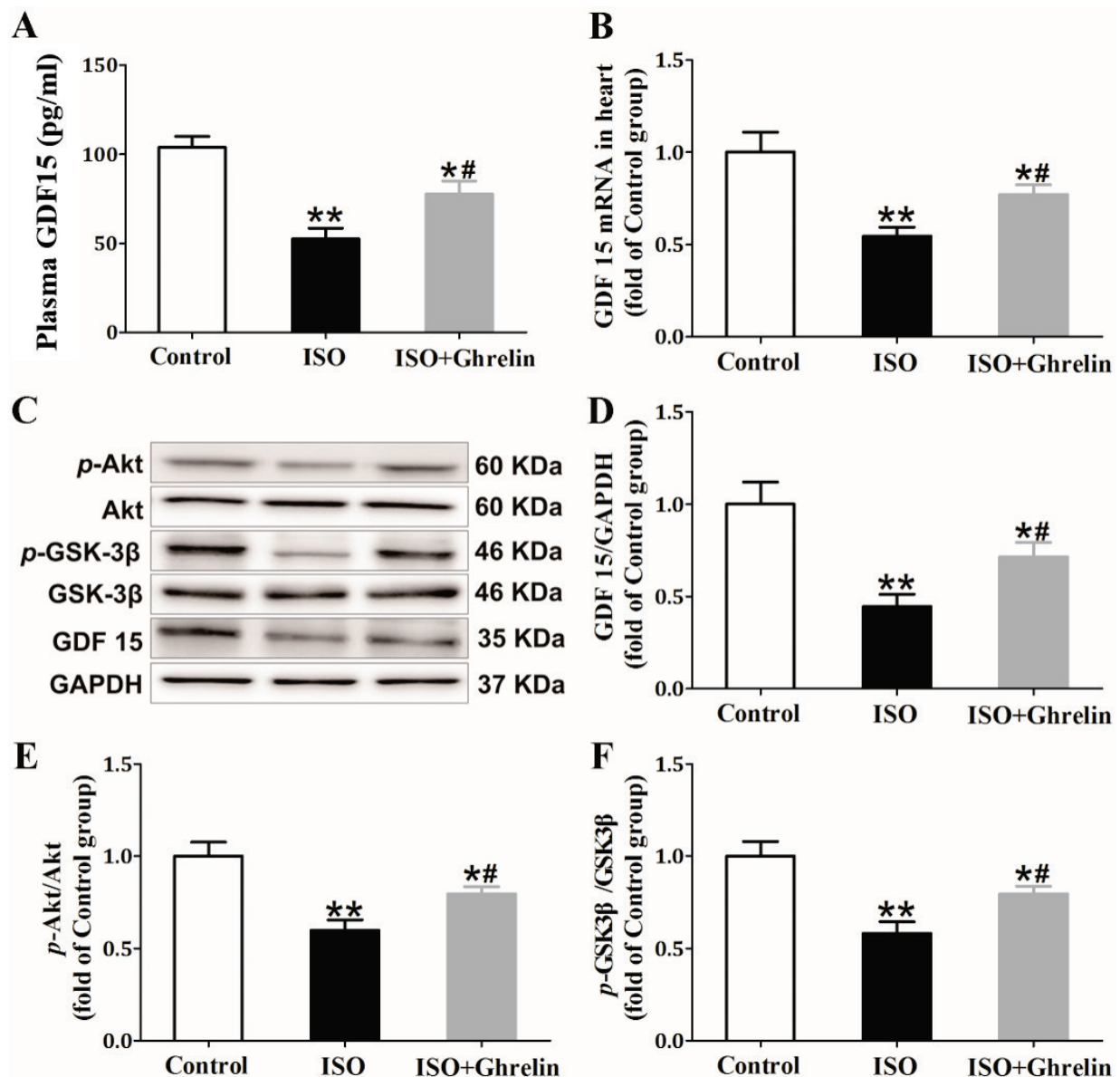
**Fig. 1.** Ghrelin improved cardiac function in isoproterenol-induced myocardial fibrosis rats. Rats were divided into 3 groups: control (saline 2 mg/kg per day, subcutaneous), ISO (2 mg/kg per day, subcutaneous), ISO + ghrelin. ISO or saline was administered for 7 days. Then ghrelin (100  $\mu$ g/kg per day, subcutaneous) was administered once daily for the next 3 weeks. Changes in (A) maximum contraction velocity (+dp/dt<sub>max</sub>), (B) maximum relaxation velocity (-dp/dt<sub>max</sub>), (C) left ventricular systolic pressure (LVSP), (D) left ventricular end-diastolic pressure (LVEDP), and (E) heart rate (HR). Data are mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. Control; #  $p < 0.05$  vs. ISO.



**Fig. 2.** Ghrelin attenuated cardiac remodeling in isoproterenol-induced myocardial fibrosis rats. (A) Heart to body weight ratio (HW/BW). (B) Left ventricular to body weight ratio (LVW/BW). (C) Brain natriuretic peptide (BNP) level in the plasma. Data are mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. Control; #  $p < 0.05$  vs. ISO.



**Fig. 3.** Ghrelin alleviated cardiac fibrosis in isoproterenol-induced myocardial fibrosis rats: (A) Representative Masson's trichrome-stained left ventricular sections (scale bar=100  $\mu$ m). (B) Quantitative analysis for collagen volume fraction (%) in heart tissue. (C) Hydroxyproline content in heart tissue. (D-E) Real-time PCR analysis of collagen I and III mRNA levels in heart tissue. Data are mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. Control; #  $p < 0.05$  vs. ISO.



**Fig. 4.** Ghrelin increased the generation of growth differentiation factor 15 (GDF15) and activated Akt/GSK-3 $\beta$  pathway: **(A)** GDF15 level in plasma. **(B)** Real-time PCR analysis of GDF15 mRNA level in heart tissue. **(C-F)** Western blot analysis and quantification of protein levels of GDF15, p-Akt/Akt, and p-GSK-3 $\beta$ /GSK-3 $\beta$  in heart tissue. GAPDH was an internal control. Data are mean  $\pm$  SEM. \* $p$ <0.05, \*\* $p$ <0.01 vs. Control; # $p$ <0.05 vs. ISO.

## Discussion

This study aimed to investigate the anti-fibrotic effects of ghrelin in ISO-induced myocardial fibrosis and the underlying mechanism. Ghrelin improved cardiac function and reduced myocardial fibrosis in rats with ISO-induced myocardial fibrosis and significantly increased the production of GDF15, which plays an important role in the pathogenesis of myocardial fibrosis. Treatment with ghrelin might prevent myocardial fibrosis and improve outcomes.

Myocardial fibrosis is one of the major pathological changes in failing hearts and results in

increased risk of morbidity and mortality worldwide. Common mediators of myocardial fibrosis include the renin-angiotensin system, TGF- $\beta$  and tumor necrosis factor  $\alpha$  (Prabhu and Frangogiannis 2016, Zhang *et al.* 2011). The excessive activation of the sympathetic nervous system with increased plasma catecholamine level plays a critical role in the process of myocardial fibrosis. Sustained activation of  $\beta$ -adrenergic receptors leads to pathological myocardial fibrosis (Nuamnaichati *et al.* 2018). As well, transgenic mice with cardiac overexpression of  $\beta$ -adrenergic receptors showed myocardial fibrosis (Peter *et al.* 2007).

The nonselective  $\beta$ -adrenergic receptor agonist,

ISO, has been widely used to induce a non-invasive reliable model of myocardial fibrosis (Shih *et al.* 2018, Vergaro *et al.* 2016). In the present study, rats were subcutaneously injected with 2 mg/kg ISO once daily for 7 days to establish the myocardial fibrosis model. Subcutaneous administration of ISO produced patchy myocardial necrosis with subsequent hypertrophy, fibrosis, and remodeling leading to HF. After 4-week treatment, we measured hemodynamic parameters such as LVSP,  $\pm dp/dt_{max}$  and LVEDP to assess cardiac function. LVSP and  $\pm dp/dt_{max}$  were significantly decreased and LVEDP was increased, which indicates cardiac dysfunction in the ISO-treated rats. In addition, plasma BNP level and ratios of heart to body weight and left ventricle to body weight were increased, indicating cardiac remodeling in ISO-treated rats. After myocardial injury, cardiac fibroblasts proliferate, differentiate into myofibroblasts and produce excessive extracellular matrix to fill in the gaps created by the removal of dead cells, because cardiomyocytes are nonrenewable. Diffuse interstitial fibrosis, a key morphological change that occurs as part of the structural myocardial remodeling that takes place after myocardial injury disturbs the collagen fiber network of the myocardium. Sections stained with Masson's trichrome in the left ventricle of our rats showed wide areas of excessive collagen deposition between cardiac muscle fibers after ISO treatment. This observation was confirmed statistically by a significant increase in the percentage area of fibrosis and content of hydroxyproline, an indicator of fibrosis. Consistently, collagen I and III mRNA levels were greatly increased after ISO treatment, which indicated cardiac fibroblast activation and excessive extracellular matrix deposition. All these findings indicated successful establishment of the rat model of ISO-induced myocardial fibrosis, which parallels the results of previous studies (Mohamed *et al.* 2015, Yin *et al.* 2015).

Next, we investigated the anti-fibrotic effect effects of ghrelin. Ghrelin is a growth hormone (GH)-releasing polypeptide that was first isolated from the rat stomach in 1999. Since then, ghrelin has been found to have a plethora of physiological effects that go far beyond its initial characterization as a GH secretagogue. In the cardiovascular system, ghrelin has largely cardiac beneficial effects, including protection against ischemia/reperfusion injury, attenuation of left ventricular remodeling after myocardial infarction, and improvement of left ventricular function (Virdis *et al.* 2016).

Exogenous administration of ghrelin improved the cardiac function and prognosis of patients with end-stage HF (Nagaya *et al.* 2004). In addition, ghrelin suppressed the myocardial fibrosis of rats with post-myocardial infarction cardiac failure by adjusting the activating A-follistatin imbalance (Yang *et al.* 2018). However, the role of ghrelin in ISO-induced myocardial fibrosis and the relevant regulatory mechanism have not been well studied.

Interestingly, Camargo-Silva *et al.* (2018) reported that ghrelin potentiated cardiac reactivity to stress by modulating sympathetic control and beta-adrenergic response. However, the suppression of ghrelin on sympathetic hyperexcitation in acute heart failure in rats was also reported in another article (Shirai *et al.* 2015). These differences may be attributed to i) the timeframe, and therefore, acute effects would be potentiation of cardiac function whereas the chronic actions would result in attenuation; ii) ghrelin effects on healthy hearts differ from those sampled during hypertension, cardiac hypertrophy and failure. In fact, literature reports ghrelin acts at the nucleus of the solitary tract to suppress sympathetic activity and to decrease arterial pressure (Lin *et al.* 2004). In rodents with myocardial infarction, ghrelin treatment reduces the increased plasma concentration of norepinephrine, and inhibits activated cardiac sympathetic nervous activity showed by the ratio of low-frequency to high-frequency power in a heart rate variability analysis (Soeki *et al.* 2008).

In our study, ghrelin treatment improved cardiac function, as certified by the increase of LVSP and  $\pm dp/dt_{max}$  and decrease of LVEDP. Ghrelin treatment also reduced plasma BNP level and ratios of heart to body weight and left ventricle to body weight. In addition, ghrelin significantly reduced the percentage area of fibrosis and hydroxyproline content, accompanied by a decrease of collagen type I and III mRNA levels. These results were consistent with Pei *et al.* (2014), who reported that ghrelin prevented doxorubicin-induced myocardial fibrosis and apoptosis *via* a GHSR-independent pathway. Also, chronic administration of hexarelin, a synthetic small peptide ghrelin analogue, attenuated myocardial fibrosis in spontaneously hypertensive rats (Xu *et al.* 2012). Therefore, ghrelin may be an effective treatment for myocardial fibrosis if the anti-fibrosis mechanism of ghrelin is well studied.

Growth factors and cytokines produced and secreted from cardiac myocytes and cardiac fibroblasts

play an important role in regulating myocardial fibrosis. Among the factors that contribute to myocardial fibrosis, members of the TGF family are the best characterized and appear to be the key factors in driving myocardial fibrosis. The activation of TGF- $\beta$  promotes myofibroblast differentiation and transformation, enhances the extracellular matrix and inhibits the expression of matrix metalloproteinases by binding to a complex of type II-R and type I-R, then activating Smad pathways (Su *et al.* 2017, Verjans *et al.* 2018). Meanwhile, the counter-regulatory pathways that regulate TGF- $\beta$ 1 activity are activated during the progression of myocardial fibrosis. For instance, bone morphogenetic proteins (BMPs), members of the TGF- $\beta$  family of cytokines, attenuate adverse fibrosis progression by suppressing TGF- $\beta$ 1 via the downstream effector Smad proteins (Wang *et al.* 2012, Morine *et al.* 2018). Ghrelin also attenuates liver or renal fibrosis by inhibiting the TGF- $\beta$ 1/Smad3 pathway (Sun *et al.* 2015, Mao *et al.* 2015). As a TGF- $\beta$ /BMP superfamily member, GDF15 has been described as a promising cardioprotective agent, which is attributed to its anti-apoptotic, anti-inflammatory, or anti-hypertrophic effects demonstrated in animal models (Kempf *et al.* 2011, Kempf *et al.* 2006). GDF-15 may be acted as the compensatory mechanism in the pathological process of myocardial hypertrophy. Expression of GDF-15 is very low in healthy heart, while its expression significantly up-regulated in cardiac remodeling. As negative feedback mechanism, the up-regulation of GDF-15 blocks cardiac hypertrophy by inhibition of epidermal growth factor receptor transactivation (Xu *et al.* 2014). However, clinical studies in humans indicate an association of increased levels of GDF15 with increased all-cause mortality in HF patients (Santhanakrishnan *et al.* 2012). So, whether the anti-fibrotic effects of ghrelin are mediated by GDF15 need to be fully investigated.

In the present study, plasma GDF15 level and GDF15 mRNA or protein levels in rat heart tissue were significantly lower with ISO than control treatment, and ghrelin treatment could increase the level of GDF15. Our results indicate decreased production of GDF15 under the pathological state of myocardial fibrosis in rats, and ghrelin treatment increased the production of GDF15 to exhibit its anti-fibrotic effects. These results are consistent with Lok *et al.* (2012), who reported very low cardiac mRNA and protein levels of GDF-15 in myocardial fibrosis patients with non-ischemic dilated cardiomyopathy.

The Akt-GSK-3 $\beta$  pathway plays a critical role in the pathological fibrotic response. Akt activation inhibits cardiomyocyte apoptosis and improves the survival of cardiomyocytes. Akt exerts its protective effect by directly phosphorylating and inactivating GSK-3 $\beta$ , which regulates the general protein translational machinery. Blood glucose variability can aggravate heart tissue fibrosis, possibly involving oxidative stress by inhibiting Akt signaling (Ying *et al.* 2017). Meanwhile, sonodynamic therapy inhibited fibrogenesis in rat cardiac fibroblasts by activating the AKT-GSK-3 $\beta$  pathway and blocking TGF- $\beta$ 1-SMAD3 signaling (Guo *et al.* 2016). In our investigation, ISO treatment decreased the protein level of phospho-GSK-3 $\beta$  at Ser9, accompanied by a decrease in phosphorylation of Akt at Ser473 as compared with control treatment. However, ghrelin treatment significantly reversed the downregulated *p*-Akt and *p*-GSK-3 $\beta$  levels in rat heart tissue. Several lines of evidence have also shown that ghrelin exerts its protective roles by activating the Akt-GSK-3 $\beta$  pathway in different types of organ damage (Lim *et al.* 2011, Chung *et al.* 2013).

Several articles reported ghrelin could activate GHS-R1a and then stimulate PI3K/Akt signaling pathway in several cells, including cardiomyocytes, endothelial cells and neurocytes (Baldanzi *et al.* 2002, Lacerda-Miranda *et al.* 2012, Popelova *et al.* 2018). Our results also suggested that ghrelin activated PI3K/Akt signaling pathway demonstrated by the increased protein levels of *p*-Akt and *p*-GSK.

Our study suggests that ghrelin could attenuate ISO-induced myocardial fibrosis via the Akt-GSK-3 $\beta$  signaling pathway, which was mediated by GDF15. Therefore, ghrelin might have potential therapeutic value for mitigating excessive myocardial fibrosis, thereby improving cardiac function and outcomes in patients with HF.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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