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Ghrelin inhibits post-infarct myocardial remodeling and improves cardiac function through anti-inflammation effect

Cong-Xin Huang^{*}, Ming-Jie Yuan, He Huang, Gang Wu, Yu Liu, Sheng-Bo Yu, Hai-Tao Li, Tao Wang

Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China

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ABSTRACT

Ghrelin is a novel growth hormone-releasing peptide, which has been shown to exert beneficial cardiac effects on chronic heart failure (CHF) recently. In this study, we attempted to investigate the mechanisms for the effect of ghrelin on ventricular remodeling following acute myocardial infarction (MI). Ligation of a coronary artery was used to create an MI in rats. One week after MI, ghrelin (100 $\mu\text{g}/\text{kg}$) or saline were injected subcutaneously twice a day for 4 weeks. When compared to sham groups, ghrelin administration significantly decreased left ventricular (LV) remodeling in post-MI rats, as indicated by increased LV maximum rate of pressure, LV fractional shortening and scar thickness; and decreased LV end-diastolic pressure, LV end-systolic diameter, LV end-diastolic diameter and cardiomyocytes apoptosis. Moreover, ghrelin inhibited the inflammatory response, as shown by decreased mRNA and protein levels of interleukin (IL)-1 β and tumor necrosis factor- α (TNF- α). Subsequently, the expression of matrix metalloproteinase (MMP)-2 and MMP-9 were also inhibited by ghrelin injection. Ghrelin alleviates LV dysfunction and ventricular remodeling in post-MI rats. This suggests that the beneficial effects of ghrelin on CHF may result from an inhibition of the inflammatory response.

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1. Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) leads to progressive ventricular dilatation, resulting in increased myocardial wall stress and ultimately causing heart failure. Evidences have shown that several pro-inflammatory cytokines, such as interleukin (IL)-1 β and tumor necrosis factor- α (TNF- α), are involved in cardiac remodeling and play an important role in the pathogenesis and progression of heart failure [7,24]. These inflammatory cytokines can depress myocardium contractility by inducing hypertrophy and promoting apoptosis. Moreover, sustained presence of inflammatory cytokines leads to activation of matrix metalloproteinases (MMPs), causing a slippage of collagen and hence dilatation, thereby augmenting the further myocardial remodeling process [2,28].

Ghrelin is a novel growth hormone-releasing peptide as an endogenous ligand for the growth hormone secretagogue receptor (GHSR) [16]. It has been demonstrated to exert cardiovascular protective effects in both animal models and humans [12,21]. In a rat model of MI, ghrelin administration significantly improved cardiac function, increased diastolic

thickness of the non-infarcted LV, and inhibited LV enlargement [23,35]. In addition, ghrelin treatment in patients with chronic heart failure (CHF) showed an increase in LV ejection fraction [22]. It should be noted that recent evidence suggests that ghrelin could inhibit pro-inflammatory interleukins expression in human T cells [5] and TNF- α induced cytokines release as well as NF- κB activation in human endothelial cell [17]. Thus, we hypothesized that ghrelin may reduce post-MI remodeling through an inhibition of the inflammatory response and MMPs. This study was designed to test the hypothesis.

2. Materials and methods

2.1. Induction of myocardial infarction

Myocardial infarction was induced in adult male Sprague-Dawley rats, aged 10 weeks, by ligation of the left anterior descending (LAD) coronary artery according to the technique by Pfeffer et al. [26]. Briefly, the rats were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg), and a left intercostal thoracotomy was performed to expose the heart and the LAD was ligated at the origin. Sham animals underwent thoracotomy and pericardiotomy, but not LAD ligation. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the protocol was approved by the

^{*} Corresponding author. Tel.: +86 27 88040334; fax: +86 27 88040334.

E-mail addresses: huangcongxin@yahoo.com.cn, yuanmj8341@163.com (C.-X. Huang).

Institutional Animal Care Committee from Wuhan University, People's Republic of China.

2.2. Drug administration

Rats surviving 7 days after MI were randomized to treatment with ghrelin (s.c.; twice daily, at the dose of 100 µg/kg, Alexis Co., USA) or saline for 4 weeks. The sham-ligated rats that did not undergo LAD ligation were also administered ghrelin for 4 weeks.

2.3. Echocardiographic and haemodynamic measurements

Echocardiographic studies were performed before and 4 weeks after administration of ghrelin. Each rat was lightly anesthetized with an intraperitoneal injection of pentobarbital sodium, transthoracic echocardiography was performed using a sequia 512 (Acuson, Mountain View, CA, USA) equipped with a 3–7 MHz linear transducer. The anterior chest was shaved and a layer of acoustic coupling gel was applied to the thorax. Two-dimensional short-axis views of the left ventricle and M-mode tracings were recorded to measure LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD). LV fractional shortening (FS) was calculated as follows: $(LVEDD - LVESD)/LVEDD \times 100\%$. For haemodynamic measurements, a catheter was inserted into the right carotid artery and then advanced into the LV for recording of LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and LV maximum rates of pressure development (dp/dt).

2.4. Myocardial histopathology

After hemodynamic study, the hearts were excised, and the atria, right ventricle were dissected out. Sections of LV were fixed in the 10% buffered formalin solution, and then embedded in molten paraffin. Five-micrometer sections were cut and stained with Masson's trichrome. Four slides, equally distributed through the infarct area, were taken from each heart as a representative sample and measured for infarct size and infarcted wall thickness. Infarct size was assessed morphologically and calculated as the ratio of surface area of the infarct wall and the entire surface area of the LV. Infarcted wall thickness was determined by measuring the width of the thinnest part of the infarct.

2.5. ELISA

The levels of interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) in non-infarcted LV free wall were quantified using ELISA kits (Biosource; Camarillo, USA) according to the manufacturer's instructions as previously described [8,9]. All samples and standards were measured for three times. The minimum detectable concentration of IL-1β was <3 pg/ml and TNF-α was <0.1 pg/ml.

2.6. Real-time PCR

Total RNA was extracted from non-infarcted LV free wall using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was transcribed from 1 µg of total RNA using a cDNA Archive Kit (Applied Biosystems). Quantification of gene expression was performed using the ABI Prism 7000 (Applied Biosystems). cDNA was amplified under the following conditions: 94 °C for 10 min and then ran for 45 cycles at 94 °C for 10 s and 57 °C for 30 s. The value for each sample was an average of three independent PCR measurements. The mRNA levels of each gene were calculated using the $2^{-\Delta\Delta CT}$ method [18]. SYBR Green Real-Time PCR primers were designed by the Primer Express 3.0 software and according to published sequences from NCBI. The specific primer sequence of the selected genes was listed in Table 1.

Table 1

The sequence of primers used in the real-time PCR assays.

Genes name	Primer sequence	Accession number
IL-1β	F: 5'-GGGATGATGACGACCTGC-3' R: 5'-CCACTTGTGGCTTATGTT-3'	NM_031512
TNF-α	F: 5'-GCCACCACGCTCTTCTGTC-3' R: 5'-GCTACGGGCTTGCTACTCG-3'	NM_012675
MMP-2	F: 5'-GGAAGCATCAAATCGGACTG-3' R: 5'-CACCTCTTAAATCTGAAATCACC-3'	NM_031054
MMP-9	F: 5'-AAGGATGGTCTACTGGACA-3' R: 5'-TTGCGTTTCCAAAGTAAGTG-3'	NM_031055
GAPDH	F: 5'-GCAAGTTCAACGGCAGC-3 R: 5'-CATTGATGTTAGCGGGAT-3	NM_017008

2.7. Western blot analysis

Protein extracts (30 µg) from non-infarcted myocardium were separated on 12% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Non-specific sites were blocked by incubating the membrane in blocking buffer (5% nonfat dry milk in T-TBS containing 0.05% Tween 20) for 1 h. The membranes were incubated with the primary antibodies (MMP-2 and MMP-9, dilution 1:1000; Beyotime Inc, China) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH; 1:2000, Abcam) overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:1500, Beyotime Inc, China) was applied as the second antibody for 1 h at room temperature. Three to five hearts from each group were subjected to Western analysis. Finally, the blots were visualized using an ECL kit (Beyotime Inc, China), and the signals were analyzed by using a Bio-Rad image system (Hercules, CA).

2.8. TUNEL assay

Apoptotic cardiac cells were determined using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in 5 µm thick formalin-fixed, paraffin-embedded sections. The procedure was performed using the TUNEL assay (Roche, Germany) according to the manufacturer's protocol. Each experiment set up by TUNEL reaction mixture without terminal transferase served as a negative control. Slides were examined microscopically at 400× magnification. Twenty random high-power fields in the peri-infarct zone of each sample were chosen and blindly quantified. The apoptotic index was calculated as the number of apoptotic cardiomyocyte nucleus on all cardiomyocyte nucleus.

2.9. Statistical analysis

All values are expressed as mean ± S.D. *t*-test was used for between-group comparisons. One way ANOVA was used to compare more than two groups, and the Newman-Keul's test was used to test for differences between individual groups. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Survival

The survival rate of sham-operated rats was excellent. One of the 15 rats that underwent sham operation died 1 day after operation and 14 survived (93%) until sacrifice. Fifty rats underwent LAD ligation and of these 16 (32%) died during 7 days. The remaining 34 rats were randomly allocated to either saline (*n* = 17) or ghrelin (*n* = 17) treatment. The mortality rate of rats treated with saline (five deaths [29%]) was not statistically different from the ghrelin group (two deaths [12%]).

Table 2
Effect of ghrelin on left ventricular remodeling and function after myocardial infarction.

	Sham-operated (n = 14)	MI-control (n = 12)	MI-ghrelin (n = 15)
Hemodynamic data			
Heart rate (beats/min)	398 ± 25	413 ± 27	407 ± 21
End-systolic pressure (mmHg)	108.7 ± 5.7	85.4 ± 6.6 [*]	87.8 ± 7.2 [*]
End-diastolic pressure (mmHg)	6.1 ± 1.8	18.1 ± 4.4 [*]	12.8 ± 2.9 ^{*,#}
dP/dt _{max} (mmHg/s)	5302 ± 565	3601 ± 673 [*]	4596 ± 645 ^{*,#}
Echocardiographic data			
End-diastolic dimension (mm)	3.34 ± 0.23	5.02 ± 0.43 [*]	4.21 ± 0.49 ^{*,#}
End-systolic dimension (mm)	2.46 ± 0.31	4.33 ± 0.5 [*]	3.14 ± 0.31 ^{*,#}
Fractional shortening (%)	26.3 ± 3.2	13.7 ± 1.1 [*]	25.4 ± 2.7 [#]
Histomorphometric data			
Infarct size (%)	N/A	41.58 ± 3.35	38.20 ± 4.17
Scar thickness (mm)	N/A	0.37 ± 0.02	0.51 ± 0.02 [#]

* $P < 0.05$ vs. sham-operated group.# $P < 0.05$ vs. MI-control group.

3.2. Effect of ghrelin on haemodynamics and LV remodeling

Table 2 summarizes analyses of haemodynamic parameters and cardiac dimensions. FS and dP/dt_{max} were significantly reduced; and LVEDP, LVESD, LVEDD were markedly increased after MI. Ghrelin administration significantly inhibited ventricular remodeling and improved cardiac function, as shown by a significant increase ($P < 0.05$) in FS and dP/dt_{max}, and a significant decrease ($P < 0.05$) in LVEDP, LVESD and LVEDD compared to the MI-saline group.

3.3. Myocardial histopathology

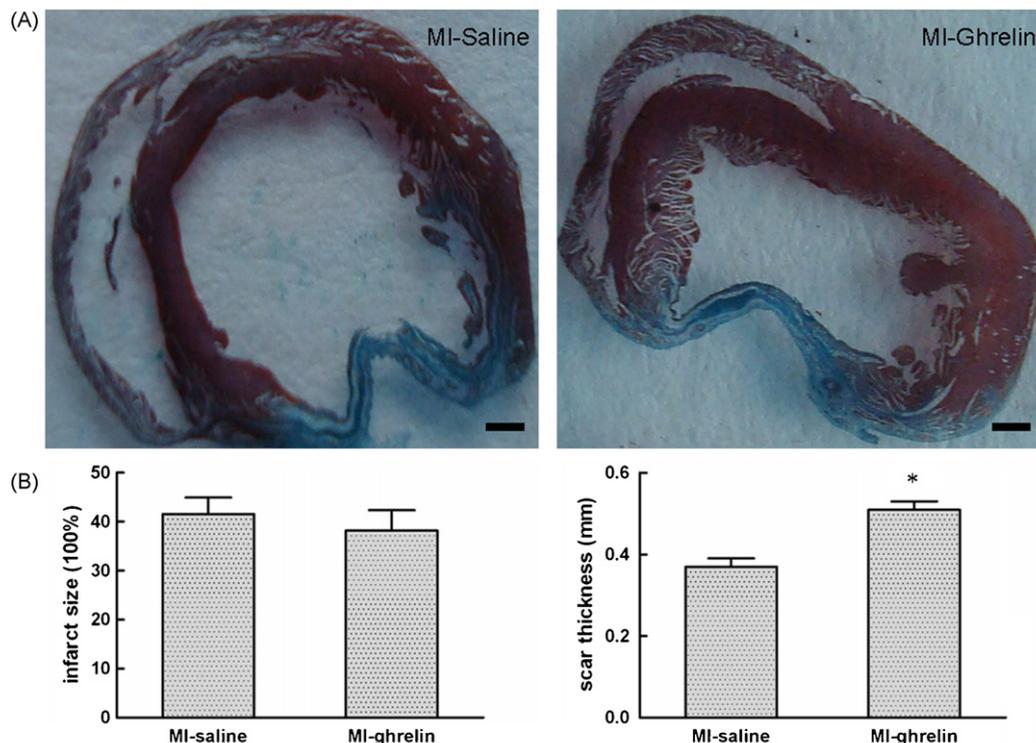
As shown in Fig. 1, myocardial infarct size was similar in the treated and untreated MI groups ($38.20 \pm 4.17\%$ and $41.58 \pm 3.35\%$, respectively; $P > 0.05$). However, ghrelin administration significantly increased infarcted wall thickness compared to saline-treatment MI group (0.51 ± 0.02 and 0.37 ± 0.02 mm, respectively, $P < 0.01$).

3.4. Effect of ghrelin on inflammatory cytokines expression

Since ghrelin possesses anti-inflammatory properties, we examined whether ghrelin administration alters inflammatory cytokines expression in myocardium level. ELISAs revealed that the IL-1 β and TNF- α were significantly higher in saline-treated MI rats than in sham-operated rats, and the levels were partially restored by ghrelin treatment. Real-time RT-PCR revealed that IL-1 β and TNF- α mRNA in LV non-infarcted myocardium were increased in saline-treated MI rats compared to sham-operated rats; ghrelin significantly reduced the elevated IL-1 β and TNF- α mRNA expression in MI rats (Fig. 2).

3.5. Effect of ghrelin on MMP-2 and MMP-9 expression

To further investigate the mechanism of ghrelin on myocardial remodeling, we analyzed the expression of matrix metalloproteinase (MMP)-2 and MMP-9. These factors play important role in

**Fig. 1.** Histological examination of infarcted heart. (A) Masson's trichrome staining of the infarcted myocardium 4 weeks after treatment (scale bar = 1 mm). (B) Infarcted size and scar thickness between the groups. Data are expressed as mean \pm S.D. * $P < 0.01$ vs. the MI-saline group.

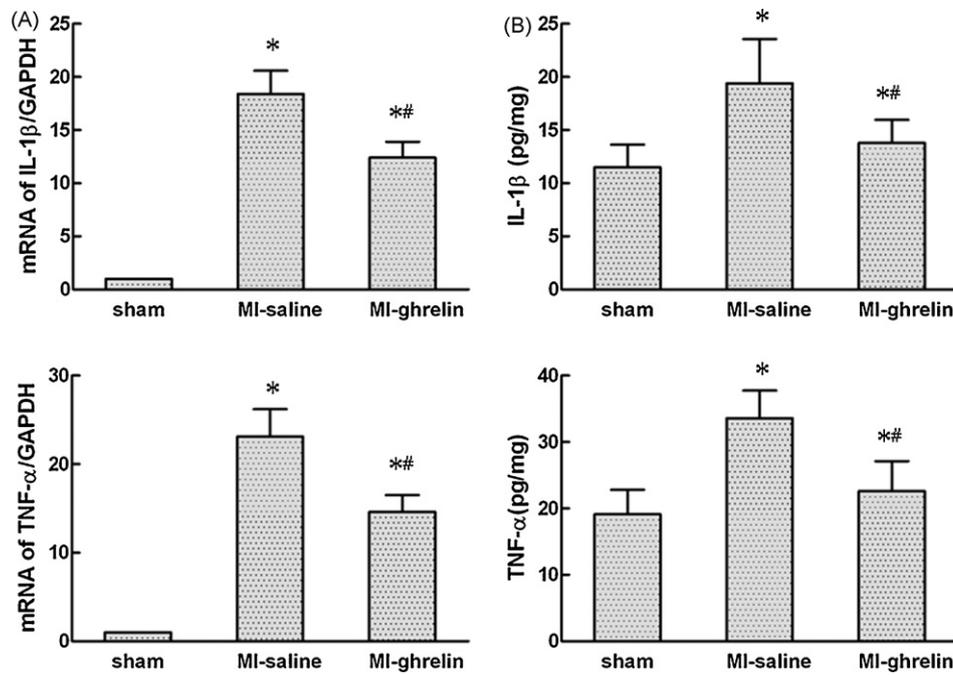


Fig. 2. Real-time PCR and ELISAs analysis of the myocardium from sham-operated rats and MI rats treated with saline or ghrelin. (A) Real-time PCR for IL-1 β and TNF- α . Data are corrected by GAPDH. (B) ELISA analysis of IL-1 β and TNF- α . Data are expressed as mean \pm S.D. * $P < 0.01$ vs. sham group, # $P < 0.01$ vs. the MI-saline group.

extracellular matrix remodeling following myocardial infarction. Compared to sham group, both MMP-2 and MMP-9 expression were significant increased in the MI-saline group, ghrelin treatment significantly decreased the levels (Fig. 3).

3.6. Apoptosis

Apoptotic cardiomyocytes were detected by TUNEL staining in the infarcted border. Apoptosis nuclei were stained brown. The apoptotic index in the MI-ghrelin treated animals was lower than that in MI-saline group (Fig. 4).

4. Discussion

We demonstrated that ghrelin administration, when started 7 days after MI, significantly ameliorated cardiac hemodynamic parameters, which may be the result of improved ventricular remodeling, including less ventricular dilatation, cardiac cell apoptosis and an increase in scar thickness. We also observed that the improved cardiac performance in rats with CHF treated by ghrelin was associated with a decrease in the inflammatory cytokines and a reduction of matrix metalloproteinase expression, such as MMP-2 and MMP-9.

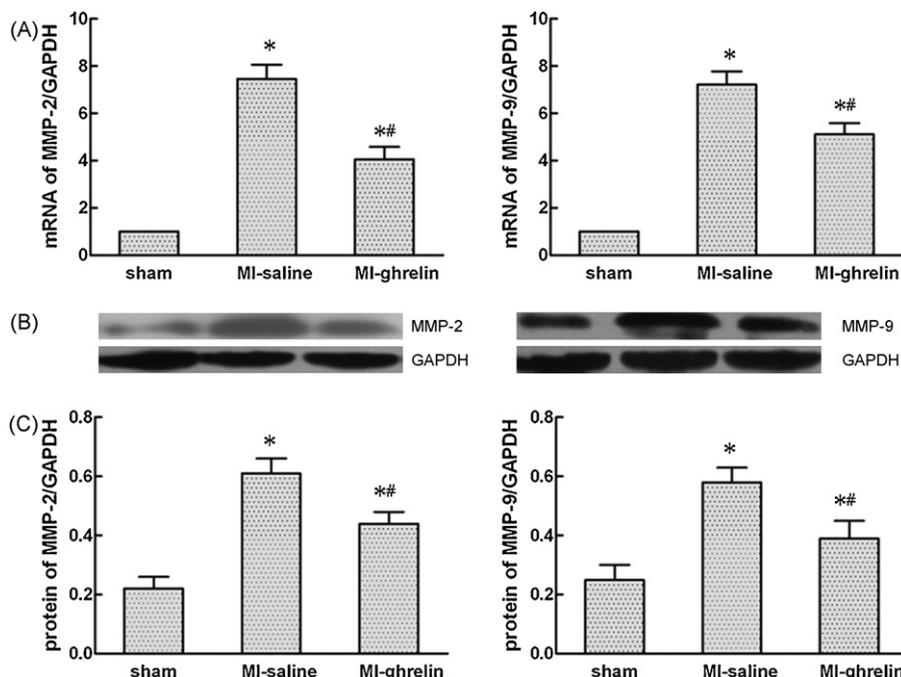


Fig. 3. MMP-2 and MMP-9 mRNA and protein levels, in sham-operated rats and rats with MI, treated with saline or ghrelin for 4 weeks. (A) Relative MMP-2 and MMP-9 mRNA levels. (B) Representative Western blotting bands of MMP-2 and MMP-9. (C) Quantitation of autoradiographic signals of Western blots. Quantitative data were given and the values represent mean \pm S.D. * $P < 0.01$ vs. sham group, # $P < 0.01$ vs. the MI-saline group.

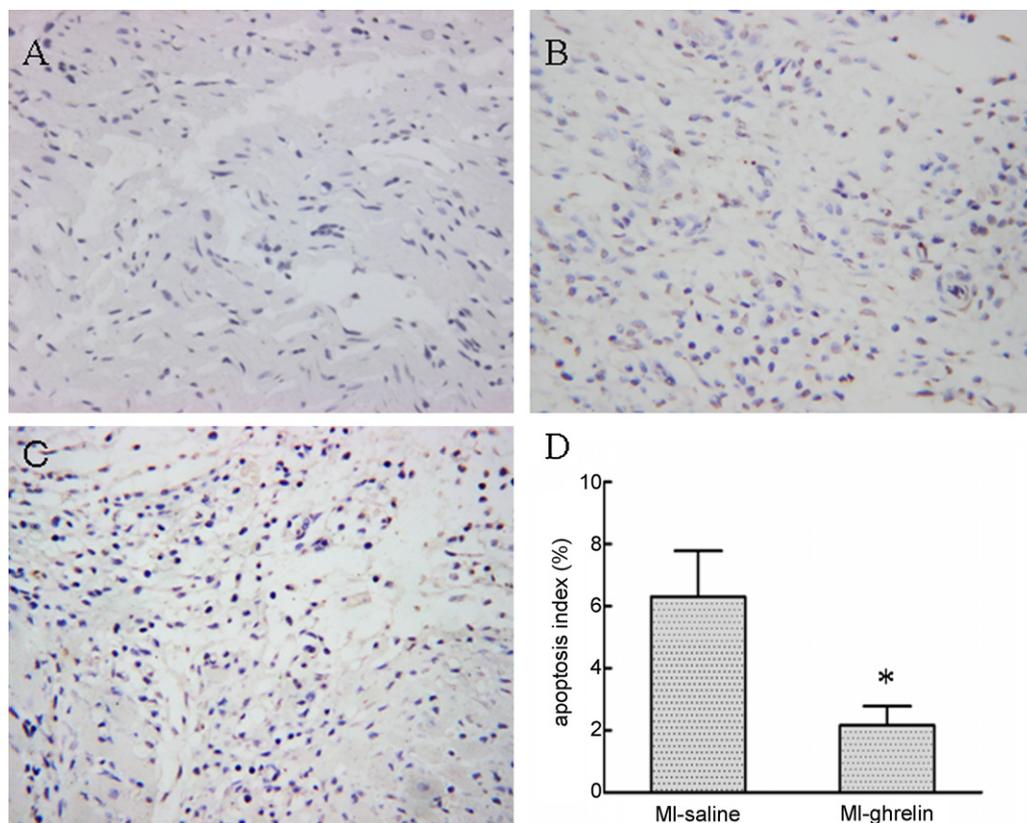


Fig. 4. TUNEL analysis of cardiomyocytes apoptosis. Apoptosis nuclei were stained brown. (A) sham-operated group; no apoptotic cells (B) MI-saline group; significant increase in apoptosis cells. (C) MI-ghrelin group; fewer TUNEL-positive cells. (D) Percentage of TUNEL-positive nuclei. * $P < 0.01$ vs. the MI-saline group.

Previous studies showed that ghrelin has cardiovascular protective effects. However, the exact mechanisms are not fully understood. Nagaya et al. [23] reported that ghrelin increased GH and insulinlike growth factor-1 (IGF-1) levels in rats with CHF and suggested that part of the cardiac effects of ghrelin may be mediated by GH. In this study, ghrelin inhibited many cellular processes such as production of the inflammation and matrix metalloproteinase. Earlier studies have shown that the inflammatory cytokines are involved in myocardial remodeling. IL-1 β and TNF- α , have been demonstrated to be increased during the processing of CHF [11]. Mice with overexpression of TNF develop LV dilatation and anti-TNF therapy can restore the process [30]. Moreover, cytokines not only induce NF- κ B activation but also are themselves induced by NF- κ B. Blockade of NF- κ B improves cardiac function and inhibits ventricular remodeling after MI [14,32]. Furthermore, recent study has indicated that ghrelin inhibited TNF- α induced cytokine release and NF- κ B activation in human endothelial cells [17]. Here, we showed that ghrelin significantly inhibited the inflammatory response following acute myocardial infarction, indicating that ghrelin acts as an anti-inflammatory peptide in the cardiovascular system. However, ghrelin had no effect in sham rats. Taken together, it is possible that ghrelin may play a direct role in ventricular remodeling independent of GH releasing mechanism, but strong evidence is needed to clarify this point.

We found that ghrelin administration significantly decreased LVEDP, LVEDD and LVESD while dP/dt and FS were significantly increased when compared to MI-saline group. Unexpectedly, we did not see any changes in LVESP and infarct size between the MI-ghrelin and MI-saline groups. It has been previously reported that angiotensin-converting enzyme (ACE) inhibitor and angiotensin II type 1 receptor blocker (ARB) inhibited cardiac remodeling after MI without reduction of infarct size [36]. It is possible that the

improvement of ghrelin on cardiac performance after MI is not attributed to a difference in the infarct size, although there was a trend towards a smaller infarct size in the MI-ghrelin group. In H9c2 cells that do not express the ghrelin receptor, ghrelin can inhibit apoptosis of cardiomyocytes through activation of extracellular signal-regulated kinase (ERK) 1/2 and Akt kinase [1]. Moreover, ghrelin-releasing peptides suppress cardiomyocyte apoptosis in rats with heart failure [35]. In this study, we also found that ghrelin inhibited cardiomyocyte apoptosis. The fact that inhibition of apoptosis in the infarct border zone was accompanied by improvement of LV function in ghrelin treated MI rats, indicating that cardiac myocyte apoptosis might play an important role in mediating cardiac dysfunction during ventricular remodeling. Considering the suppression of LV dilatation and the increased scar thickness, we speculate that infarct size alone may not necessarily be detrimental during cardiac remodeling, whereas the extent of apoptosis may be more critical.

The actions of cytokines on cardiac remodeling seem to be dependent on their influence on the extracellular matrix. The inflammatory cytokines, IL-1 β and TNF- α , can activate MMP [3,19]. Furthermore, the cardiac-specific overexpression of TNF- α develops LV dilatation and prevented by an MMP inhibitor [4], suggesting a potential importance of cytokines on matrix remodeling. It has been demonstrated that IL-1 β and TNF- α induced an upregulation of MMP expression and this increase was reduced with NF- κ B inhibition [13,34], indicating that NF- κ B may mediate in MMP expression. In this study, we found that ghrelin decreased infarcted myocardium IL-1 β and TNF- α level, however, we did not measure the NF- κ B activity. The effect of ghrelin on MMP may be due to inhibiting of pro-inflammatory cytokines and NF- κ B activation.

MMP-2 and MMP-9 are specifically implicated in adverse LV remodeling [27,31]. In animal models of MI, selective MMP

inhibition or targeted deletion of the genes is associated with reduced LV wall thinning and dilatation [6,10,29]. Moreover, plasma MMP-9 levels in chronic heart failure patients are associated with increased LV dimensions [15,25]. In this study, we showed that both MMP-2 and MMP-9 levels were significantly increased 5 weeks after MI compared to sham group. The results are consistent with those earlier findings that MMP-2 and MMP-9 activity remained elevated during the chronic remodeling phase [20,33].

Inhibition of the inflammatory response and matrix metalloproteinase activity early in the post-infarct period may potentially impair healing process, leading to a weaker scar. In this study, ghrelin, initiated 1 week following MI, was not associated with an increase in ventricular dilation. Interestingly, it increased scar thickness and reduced the ongoing remodeling in the infarct border zone. Because the deleterious effects of inappropriate MMP activation are critical in post-MI remodeling progressing, and the control of MMP activation has been suggested as a potential significant target for treating cardiac remodeling, ghrelin may be used as a novel therapeutic factor for the heart failure.

In conclusion, we show that ghrelin administration inhibited ventricular remodeling and improved cardiac performance in rats with CHF following acute myocardial infarction without influencing infarct size. The cardiovascular protective role of ghrelin may be mediated at least in part by its anti-inflammatory effect.

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