Chronic Intermittent Low-Level Transcutaneous Electrical Stimulation of Auricular Branch of Vagus Nerve Improves Left Ventricular Remodeling in Conscious Dogs With Healed Myocardial Infarction

Zhuo et al: Auricular Vagus Nerve and Cardiac Remodeling

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Abstract

Background—Vagus nerve stimulation (VNS) attenuates left ventricular (LV) remodeling after myocardial infarction (MI). Our previous study found a noninvasive approach to deliver VNS by transcutaneous electrical stimulation of auricular branch of vagus nerve. So we hypothesize chronic intermittent low-level tragus stimulation (LL-TS) could attenuate LV remodeling in conscious dogs with healed MI.

Methods and Results—Thirty beagle dogs were randomly divided into three groups, MI group (left anterior descending artery and major diagonal branches ligation to introduce MI, n=10), LL-TS group (MI plus chronic intermittent LL-TS, n=10) and Control group (sham surgery without stimulation, n=10). Tragus stimulation was delivered to bilateral tragus with ear-clips connected to a custom-made stimulator. The voltage slowing sinus rate was used as the threshold for setting LL-TS at 80% below that. LL-TS group was given four hours’ stimulation at 7-9AM and 4-6PM on conscious dogs. At the end of 90-day follow-up, LL-TS group significantly reduced LA and LV dilatation, improved LV contractile and diastolic function, reduced infarct size by about 50% compared with MI group. LL-TS treatment alleviated cardiac fibrosis and significantly decreased protein expression level of collagen I, collagen III, TGF-β1, MMP-9 in LV tissues. The plasma level of hs-CRP, NE, NT-proBNP in LL-TS group was significantly lower than MI group from the 7th day to the end of follow-up.

Conclusions—Chronic intermittent low-level transcutaneous electrical stimulation of auricular branch of vagus nerve can attenuate LV remodeling in conscious dogs with healed MI.

Key Words: vagus nerve stimulation; autonomic nervous system; myocardial infarction; Left ventricular remodeling
Myocardial infarction (MI) leads to molecular, structural, geometrical and functional changes of heart in a process known as post-infarction cardiac remodeling.\(^1\) Despite modern reperfusion strategies, patients who survive an acute myocardial infarction are at higher mortality rate due to tonic sympathetic activities that promote ventricular remodeling.\(^2\) It is well known that chronic vagus nerve stimulation (VNS) could suppress the cardiac sympathetic outflow,\(^3\) prevent and reverse cardiac remodeling.\(^4\) However, VNS treatment has to implant neurostimulator system with a bipolar multi-contact cuff electrode around the cervical vagus nerve by surgery and may cause side effects including neck pain, coughing, swallowing difficulty, and voice alteration along with nausea and indigestion.\(^5\)

Transcutaneous electrical stimulation of auricular branch of vagus nerve (ABVN) located at the tragus, the anterior protuberance of the outer ear, is a noninvasive approach to stimulate afferent vagal nerve fibers. Our previous research\(^6\) found low-level tragus stimulation (LL-TS) could substitute for VNS to reverse acute atrial remodeling. Nevertheless, there was no further research about whether LL-TS could affect ventricular remodeling after MI. The present study was designed to test the hypothesis that chronic intermittent LL-TS could attenuate ventricular remodeling in conscious dogs with healed myocardial infarction.

**Methods**

**Animal preparation**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University
(Permit Number: 2014-0363). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Thirty adult male beagle dogs (body weight, 10 to 15 kg) were supplied by the center of experimental animal in medical college of Wuhan University. All dogs were anesthetized with Na-pentobarbital, 40 mg/kg, and ventilated with room air by a positive pressure respirator (MAO01746, Harvard Apparatus Holliston, USA). Additional maintenance doses of 2 mg/kg Na-pentobarbital were administrated at the end of each hour during the procedure. Normal saline at 50 to 100 ml/h was infused to replace spontaneous fluid losses. Body surface electrocardiogram (ECG) was recorded by using subcutaneous needle electrodes during the whole procedure using a computer-based Lab System (Lead 2000B, Jinjiang Inc., China). A heating pad was used to maintain the core body temperature at 36.5±1.5 °C. The thoracotomy was performed in the left fifth intercostal space. A Harris two-stage occlusion was performed on the left anterior descending coronary artery (LAD) with 3-0 silk above the first diagonal branch to produce myocardial infarction and all the major diagonal branches were ligated to decrease collateral flow to the infarct area. The vessel was partially occluded for 20 minutes and then tied off completely. The LAD artery occlusion was observed in ECG with elevation of ST segment. After three hours of observation, the chest was closed in layers and the pneumothorax was reduced. Antibiotics were given for three days after surgery.

**Low-level tragus stimulation**

Tragus stimulation (auricular vagus nerve stimulation) as previous used (frequency 20Hz, pulse width 1ms) with duty cycle of 5s on and 5s off was delivered to the bilateral tragus in
the external auditory canal with ear-clips connected to a custom-made stimulator (Figure 1). At first, incremental voltages was applied to the tragus until slowing of the sinus rate. The voltage necessary to slow the sinus rate was used as the threshold and LL-TS was delivered at 80% below that. The actual electrical voltage was in the range of 16 to 24V, which did not cause any heart rate changes and resulted in serious adverse reactions that could increase plasma epinephrine level.

**Experiment protocol**

Thirty beagle dogs were randomly divided into three groups before surgery. MI group (n=10) was established by the ligation of LAD and major diagonal branches to introduce MI. LL-TS group (n=10) underwent the same surgery with four hours of LL-TS at 7-9am and 4-6pm that were highest sympathetic tone period proved by Irwin JM every day. Control group (n=10) underwent sham surgery without stimulation. All dogs were followed up to 90 days. None received other background therapy.

**Echocardiographic evaluation of LV function**

Doppler echocardiography (Vivid E9, GE healthcare, USA) was carried out under continuous ECG monitoring with a 3.5 MHz electronic probe on conscious dogs lying in lateral recumbency on the day before surgery, 30th day and 90th day after surgery, respectively. Images were obtained according to echocardiographic criteria and recorded in computer for subsequent analysis. The endocardial borders of LV were measured at the bidimensional right long axis view. The LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV)
and LV ejection fraction (LVEF) were calculated using the Simpson’s biplane equation. The maximal left atrial volume (LA-V$_{\text{max}}$) at the standard left apical four-chamber view was calculated using the Simpson’s monoplane method. The velocity waveforms of the peak mitral inflow velocity in early diastole (E) and during left atrial contraction (A) were measured to calculate the ratio of E to A. Systolic blood pressure (SBP), which was measured on the left forelimb by the Ultrasonic Doppler Flow Detector (Model 811-B, Parks Medical, Ore, USA) and heart rate (HR) was collected during the echocardiographic examination on the day before the surgery and 90th day after surgery. All parameters were measured at least three times and evaluated blind to the intervention by the same experienced echocardiographer.

Myocardial infarct size determination

The infarct size was assessed with 0.5% Evans Blue and 1.0% triphenyltetrazolium chloride (TTC) staining in 5 dogs of LL-TS group and 5 dogs of MI group. In brief, Evans Blue was infused into the left atrial appendage to evaluate the area at risk (AAR) by left thoracotomy under general anesthesia. After giving dogs the euthanasia, the heart was quickly removed and frozen at -20°C for 3 hours, and then was cut into 2-5mm thick slices perpendicular to the LAD occlusion site. Five continuous slice from the occlusion site was incubated in TTC at 37°C for 15 min to discriminate the infarct tissues from the viable myocardium. After overnight fixation with 4% paraformaldehyde, each slice was photographed with a digital camera. The area measurement was performed using Image Tool software version 3.0. The ratio of the area at risk (AAR) to the total ventricular mass and the infarct size normalized to the AAR.
was calculated,\textsuperscript{8} respectively.

\textbf{Histomorphometric Measurements}

At the end of protocol, after the dogs being euthanized, the hearts (5 dogs in LL-TS group, 5 dogs in MI group) were removed quickly from the chest for sectioning from apex to base into three transverse rings of 5mm in thickness. Transmural tissue blocks that were obtained from infarct border zones of the middle slice were mounted on cork using Tissue-Tek embedding medium, and rapidly frozen in isopentane, precooled in liquid nitrogen. Cryostat sections about 8\textmu m thick were prepared and stained with fluorescein-labeled peanut agglutinin (Vector Laboratories Inc) and used to delineate the myocyte border and the interstitial space, as previously described.\textsuperscript{9} Sections were double stained with rhodamine-labeled Griffonia simplicifolia lectin I to identify capillaries. Microscopic fields (magnification\times100) were selected at random from each section and used to measure myocyte cross-sectional area. The surface area occupied by interstitial space and capillaries were measured from each field by means of computer-based video densitometry (Jandel Scientific, Corte Madera, CA) as previous described.\textsuperscript{10} For each histomorphometric measure, the sections obtained from each dog were averaged and that single average was used to represent each dog in the analysis. The volume fraction of replacement fibrosis (VFRF), namely, the proportion of scar tissue to viable tissue, was calculated from trichrome-stained sections as the percent total surface area occupied by fibrosis. The volume fraction of interstitial fibrosis (VFIF) was calculated as the percent total surface area occupied by interstitial space minus the percent total area occupied by capillaries.\textsuperscript{11}
**Western blotting**

The hearts of 10 dogs from control group and the hearts that were sectioned in histomorphometric measurements were used for western blotting. Transmural myocardial tissue sample about 1 square centimeter obtained from the LV free wall outside the infarction area was homogenized in RIPA lysis buffer (Rockland, Gilbertsville, PA) containing proteinase inhibitor (Roche, Basel, Switzerland). The homogenate was centrifuged at 4°C at 2000g for 10 minutes and the resultant supernatant was further subjected to centrifugation at 12,000g for 20 minutes. Protein concentration of each supernatant sample was determined using a DC Protein assay kit (BioRad Hercules, USA). Samples containing equal amounts of protein were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Bio-Rad) and transferred onto Immobilon-P membrane (Millipore, Billerica, USA). After blocking the membranes with BlockAce (Dainippon Pharmaceutical, Japan). The primary antibodies used in the study included polyclonal antibody for matrix metallopeptidase 9 (MMP-9) (TheBinding Site, Birmingham, UK) and transforming growth factor β1 (TGF-β1), anti-COL I, anti-COL III (Abcam, Cambridgeshire, England) and β-Actin (Santa Cruz biotechnology, USA) which served as an internal control. The concentration of primary antibody was obtained from the manufacturer's instructions. Detection was done with enhanced chemiluminescence (Invitrogen, CA, USA). Bands were scanned and analyzed on the ECL detection system. Experiments were performed in triplicate and repeated at least three times.
Blood sampling

Blood samples collection was performed in a dedicated conscious testing room, where dogs were lying on a coach in a quiet, dim-lighted environment, without any sedation or anesthesia. After a stabilization period of 15 minutes, venous blood samples were collected in ice-chilled tubes coated with EDTA (BD Vacutainer K2E, BD Diagnostics, NJ) on the day before the surgery and the 1st, 30th, 90th day after surgery. Plasma was separated by centrifuging at 3000rpm for 15min at 4°C and stored at -80°C until assayed. Plasma high-specific C-reactive protein (hs-CRP) level was measured using a canine-specific high sensitivity CRP ELISA (KT-093, Kamiya Biomedical Company, Seattle, USA), Plasma NE and NT-proBNP concentrations were determined by a validated radioimmunoassay method supplied by American Laboratory Products Company (ALPCO, Salem, USA). Plasma was processed in radioimmunoassay method according to the manufacture’s published procedure.

Data analysis

All data were reported using the median with 25th and 75th percentiles except for the data in the table. If significance was attained by Kruskal-Wallis test between three groups, post-hoc pairwise comparisons with Dunn’s multiple comparison test was performed. Mann–Whitney nonparametric tests were used for comparison of fibrosis measurements (VFRF and VFIF). The data in Table were analyzed using Wilcoxon signed-rank test (SV and CO) and Paired t test (Weight, HR and SBP). For box plots in figures, the lower and upper bounds of the boxes indicate the 25th and 75th percentile (Q.25/Q.75) values, and the horizontal lines indicate the
median. GraphPad Prism 6 for Mac (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. A value of $P<0.05$ was required for statistical significance.

**Results**

**Effects of LL-TS on LV function and myocardial infarct size**

All dogs completed the experimental protocol. As was shown in Figure 2, the baseline of echocardiographic measurement was comparable in three groups. The LVEDV and LVESV were significant higher, LVEF and E/A were significant lower in MI group than control group on the 30th and 90th day, and the LA-V$_{max}$ was significant higher in MI group than control group on the 90th day. But LL-TS group significantly reduced LV dilatation, improved left ventricular contractile and diastolic function compared with MI group on the 30th and 90th day of follow-up. Besides, the treatment of LL-TS reduced the LA dilatation on the 90th day (all $P<0.01$). However, as was showed in the table, there were no significant differences in stoke volume (SV) and cardiac output (CO) at the end of 90-day follow-up between the three groups. Compared with baseline, dogs in MI group had significantly lower SBP and higher HR on the 90th day (Both $P<0.01$), but the differences among three groups did not reach significance. Interestingly, a significant increase in body weight of all dogs was observed at the end of 90-day follow up (all $P<0.01$), but there was no significant difference among the three groups.

Myocardial infarct size expressed as the percentage of AAR was shown in Figure 3 upside. The AAR, expressed as a percentage of the total ventricular mass, was a little larger in MI group than LL-TS group [M: median = 46.2% (Q.25/Q.75 = 42.3%/49.7%) vs. S: median =
43.8% (Q.25/Q.75 = 40.1%/44.4%), \( P=0.16 \). However, LL-TS treatment significantly reduced mean infarct area by about 50% compared with MI group [M: median = 48.6% (Q.25/Q.75 = 42.3%/49.7%) vs. S: median = 18.9% (Q.25/Q.75 = 17.1%/19.7%)].

Effects of LL-TS on LV remodeling

Histomorphometric findings were shown in Figure 4. Treatments with LL-TS significantly decreased VFRF and VFIF in the tissue of infarct border zones compared with dogs in MI group (All \( P<0.05 \)).

The results of the determination of protein levels in LV free wall tissue were shown in Figure 5, the protein level of TGF-\( \beta \)-1, MMP-9, collagen I and collagen III was remarkably increased in MI and LL-TS group than control group (All \( P<0.01 \)). However, LL-TS group significantly reduced TGF-\( \beta \)-1, MMP-9, collagen I and collagen III protein expression level compared with MI group at the end of follow-up (All \( P<0.05 \)).

Effects of LL-TS on Plasma hs-CRP, NE and NT-proBNP Levels

Plasma levels of hs-CRP increased significantly on the first day after MI, LL-TS therapy markedly attenuated the increase trend on the 30\(^{th} \) and 90\(^{th} \) day. (Figure 6, upper panel, both \( P<0.05 \))

Plasma NE levels also increased from the next day after induction of MI to the 90\(^{th} \) day. However, the plasma levels of NE were significantly lower in LL-TS group than MI group. (Figure 6, middle panel, all \( P<0.05 \))

Plasma NT-proBNP levels were obviously increased from the 1\(^{st} \) day to the 90\(^{th} \) day after
MI. However, LL-TS significantly reduced plasma NT-proBNP levels compared with MI group. (Figure 6, bottom panel, all $P<0.05$)

Discussion

Major findings

Low-level transcutaneous electrical stimulation of auricular branch of vagus nerve has important impaction on cardiac function and ventricular remodeling. We found that chronic intermittent LL-TS treatment significantly improved cardiac function, alleviated cardiac fibrosis, and attenuated left ventricular remodeling in conscious dogs with healed myocardial infarction. Moreover, LL-TS treatment could also reduce plasma level of hs-CRP, NE and NT-proBNP in dogs after MI.

The improvement of LV remodeling by LL-TS treatment

The improvement in global LV remodeling after MI was achieved by LL-TS treatment evidenced by reduced LA and LV dilatation, increased LV systolic and diastolic function. It has been proved that increased vagus nerve activity could reduce the ratio of infarct size to AAR by regulating the nicotinic action.\textsuperscript{13} Recently, Shinlapawittayatorn K et al\textsuperscript{14} also found increased vagus nerve activity could reduce mitochondrial ROS production and mitochondrial swelling, which were responsible for decreasing the infarct size after MI. Our study also indicated the reduction of myocardial infarction size was achieved by chronic intermittent LL-TS treatment. It probably was by means of enhancing vagus nerve activity that LL-TS improved the global LV remodeling.
Reversal of LV cellular remodeling by LL-TS treatment was shown with the reduction of LV fibrosis evidenced by the lower level of VFRF, VFIF. The interstitial fibrosis is the accumulation of collagen in the cardiac interstitium. Collagen I accounts for approximately 70% to 85% of total cardiac collagen in LV and provides tensile strength, while collagen III accounts for about 10% of total cardiac collagen and maintain the elasticity of extracellular matrix (ECM) network.\textsuperscript{15} Synthesis of collagen has been shown to be predominantly enhanced in ischemic cardiomyopathies outside infarct areas.\textsuperscript{16} Our findings indicated that the protein level of collagen I and III were significantly increased at the 90-day follow-up after MI. However, long-term LL-TS treatment markedly reduced the collagen synthesis.

TGF-\(\beta\)-1 is a crucial regulator of LV cellular remodeling through its direct and potent actions in cardiomyocyte and cardiac extracellular matrix metabolism. TGF-\(\beta\)-1 could regulate fibrous tissue deposition by enhancing collagen I and III synthesis.\textsuperscript{17} This study demonstrated LL-TS reduced TGF-\(\beta\)-1 protein expression to improve the LV interstitial fibrosis.

TGF-\(\beta\)-1 also promotes ECM preservation through increased expression of tissue inhibitors of metalloproteinase that inhibits MMP-9 activity. MMP-9 degrades collagen and contributes to ventricular dilation and remodeling in remote infarction areas after MI.\textsuperscript{18} This study found the protein content of MMP-9 was markedly low on the 90\textsuperscript{th} day after MI and LL-TS could further lower the MMP-9 level in left ventricular tissue.

However, the exact mechanism of regulation of collagen expression level by LL-TS is still not clear. Maybe the renin-angiotensin-aldosterone system plays an important role in that, more study should be done to find out about it.
The plasma evidences for regulation of autonomic nervous system by LL-TS

Chronic intermittent LL-TS could modify non-specific inflammation markers like hs-CRP. It is well known that the term of cholinergic anti-inflammatory pathway has been advanced by the identification of a neural mechanism that inhibits macrophage activation through parasympathetic outflow. It is likely that LL-TS can enhance vagus nerve activity to reduce the plasma hs-CRP level by the cholinergic anti-inflammatory pathway.

Increased plasma NE levels and increased cardiac sympathetic nerve activity can induce myocardial remodeling and the extent of elevation in plasma NE concentration correlates directly with the severity of the LV dysfunction and inversely correlates with prognosis.

The reduction of plasma NE level illustrated that LL-TS decreased sympathetic nerve activities by means of enhancing vagus nerve activities.

The main stimulus for synthesis and secretion of proBNP from cardiac myocytes is myocyte stretch. Convincing evidence in the current literatures suggests that patients with high levels of NT-proBNP are at higher risk of LV remodeling. Our findings indicated LL-TS may weaken left ventricular tension by shifting the autonomic balance from primary sympathetic tone to vagal predominance.

Possible Mechanisms of LL-TS on vagus nerves

The auricular branch of vagus nerve (ABVN) which is the only peripheral branch of the vagus nerve, mainly supplies the auricular concha and most of the area around the auditory meatus. The afferent vagal nerve fibers innervating the tragus area enter the main vagal trunk through the jugular ganglion at level of the skull base. Nomura and Mizuno found that
the afferent fibers of ABVN terminated mainly in the nucleus tractus solitaries (NTS). It is well known that a large number of autonomic nerve fibers, including the fibers from the heart, project to the NTS. Gao et al found acupuncture-like stimulation of ABVN evoked cardiovascular inhibition via activating the cardiac-related neurons in the nucleus tractus solitaries (NTS). Our previous researches pointed out tragus stimulation activated a series of neurotransmission between ABVN, NTS and other nuclei in the brain participating cardiovascular control and eventually activating the efferent vagal fibers. Plenty of researches had proven that stimulation of cervical vagus nerve could improve LV systolic function, prevent progressive LV enlargement in dogs with heart failure, prevent sudden death in conscious dogs with a healed myocardial infarction and attenuate ventricular dysfunction and infarct size during acute ischemia-reperfusion injury. The present study reveals that LL-TS has the same effects as invasive cervical vagus nerve stimulation on the vagal efferent fibers, which are part of the final pathway responsible for the regulation of intrinsic cardiac autonomic nervous system (CANS). Electric stimulation of ABVN could augment vagal tone by means of activating the afferent vagal nerve and modulate intrinsic CANS to attain cardio-protective effect.

Clinical implications
The beagle dog model of myocardial infarction used in this study manifested many of the hemodynamic and neurohormonal phenomenons in post-ischemic diseases observed in humans (i.e. marked and progressive depression of LV systolic, diastolic function and ventricular fibrosis and remodeling). Also transcutaneous electrical stimulation of auricular
branch of vagus nerve had already been used to treat epilepsy and depression, reduce the amount of anesthetic used during operative procedures and suppress sepsis in a murine model of endotoxemia. The findings from this study offer an important step forward for translating the results to clinical applications. The therapeutic modality used in the present study also brought delightful results by manipulation of autonomic tone through vagal afferent nerve. We therefore propose a new neural interface approach to optimize cardiac autonomic tone for the treatment of post-ischemic heart diseases.

Study limitations

We did not measure the neurotransmitters and associated signal transduction pathways, which may elucidate the mechanism underlying the treatment effects, further researches need to be done. The absence of background therapy with angiotensin converting enzyme inhibitors, beta adrenergic receptor blockers and/or aldosterone antagonists focus attention on the direct effects of therapy, it does limit extrapolation of the results to patients who would surely be receiving established post-ischemic heart diseases therapy.

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Disclosures

None.

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**Table.** Evaluation of body weight, systolic blood pressure (SBP), heart rate (HR) and hemodynamic variables of stroke volume (SV) and cardiac output (CO) at baseline and 90-day follow up.

<table>
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All results are presented as mean±SD. † P<0.05 vs. baseline;
Figure Legends

Figure 1 (A). Schematic diagram of stimulation site of ABVN highlighted by the red arrow; (B) high-intensity stimulation reduces spontaneous sinus rate by about 20 beats/minute, suggesting the activation of the vagus nerve. ABVN: auricular branch of vagus nerve.

Figure 2. Transthoracic echocardiographic evaluation at the baseline, 30 days and 90 days follow up. All results are presented as the median with 25th and 75th percentiles. The LVEDV and LVESV were significantly higher, LVEF and E/A were significantly lower in MI group than control group on the 30th and 90th day, and the LA-V\text{max} was significantly higher in MI group than control group on the 90th day of follow-up. LL-TS significantly reduced LA-V\text{max} dilation on the 90th day and significantly reduced LVEDV, LVESV dilation and increased LVEF, E/A compared with MI group on the 30th and 90th day of follow-up.

LA-V\text{max}: maximal left atrial volume; LVEDV: Left ventricular end-diastolic volume; LVESV: Left ventricular end-systolic volume; LVEF: left ventricular ejection fraction. E/A: Peak mitral inflow velocity in early diastole (E) / peak mitral inflow velocity during left atrial contraction (A)

†: P<0.01 vs. baseline; #: P<0.01 vs. MI group.

Figure 3. Representative pictures above show myocardial infarct cross-section with Evans Blue and TTC staining; Blue indicates nonthreatened myocardium; Red indicates the noninfarcted area; white indicates myocardial infarction. Graph below shows comparison of
myocardial infarction size/area at risk between groups. Data are presented as the median with 25th and 75th percentiles. #: P<0.01 vs. MI group.

**Figure 4.** Histomorphometric findings at the end of 90 days in MI group and LL-TS group. (MI group=5, LL-TS group=5) VFIF=volume fraction of interstitial fibrosis; VFRF=volume fraction of replacement fibrosis; §: P<0.05 vs. MI group #: P<0.01 vs. MI group.

**Figure 5.** Representative picture above of western blots from LV free wall tissues in each group (control group=10, MI group=5, LL-TS group=5) showed effects of LL-TS treatment on protein expression level of TGF-β1, MMP-9, collagen I and collagen III. Protein analyses below were showing (A) Relative protein level of TGF-β1. (B) Relative protein level of MMP-9; (C) Relative protein level of collagen I; (D) Relative protein level of collagen III. *: P<0.01 vs. control group; §: P<0.05 vs. MI group; #: P<0.01 vs. MI group.

**Figure 6.** Effects of LL-TS on plasma level of hs-CRP, NE and NT-proBNP during the study; LL-TS therapy markedly attenuated the increase trend in plasma hs-CRP levels at both 30th and 90th day. (Figure 6, upper panel, both P<0.05); LL-TS also significantly reduced both plasma NT-proBNP and NE level from the next day after induction of MI to 90th day. (Figure 6, middle and bottom panel, all P<0.05) Data are presented as the median.

*: P<0.05 vs. control group; #: P<0.05 vs. MI group.
Figure 2

- LA-V$_{max}$ (ml)
- LVEDV (ml)
- LVESV (ml)
- LVEF (%)
- E/A

Legend:
- control
- MI
- MI+LL-TS
Figure 4

VFRF %

MI

LL-TS

VFIF %

MI

LL-TS
Figure 5

Control  MI  LL-TS

13KD  ← TGF-β1
92KD  ← MMP-9
130KD  ← Collagen-I
134KD  ← Collagen-III

B  MMP-9

C  Collagen I

D  Collagen III

*  MI vs Control
#  LL-TS vs MI
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