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Nebulized Delivery of the MAPKAP Kinase 2 Peptide Inhibitor MMI-0100 Protects Against Ischemia-Induced Systolic Dysfunction

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Abstract

Acute myocardial infarction (AMI) results in systolic dysfunction, myocarditis and fibrotic remodeling, which causes irreversible pathological remodeling of the heart. Associated cell death and inflammation cause cytokine release, which activates the p38 MAPK signaling pathway to propagate damaging signals via MAPKAP kinase 2 (MK2). Previously we showed that intraperitoneal injection of a cell permeable peptide inhibitor of MK2, MMI-0100, protects against fibrosis, apoptosis and systolic dysfunction in a mouse model of AMI induced by left-anterior descending coronary artery (LAD) ligation. Here we tested a new route of administration of MMI-0100: inhalation of nebulized peptide. When given within 30 min of AMI and daily for 2 weeks thereafter, both inhaled and injected MMI-0100 improved cardiac function as measured by conscious echocardiography. Limited fibrosis was observed after 2 weeks by Massons trichrome staining, suggesting that MMI-0100 protects the heart prior to the formation of significant fibrosis. These results support a nebulized route of administration of MMI-0100 can protect the myocardium from ischemic damage.

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Keywords

Introduction

Ischemic heart disease contributes to considerable worldwide morbidity and mortality. In the United States alone almost 800,000 myocardial infarctions occur per year. While interventional therapies such as rapid reperfusion have reduced patient death, pathological cardiac remodeling occurs resulting in heart failure in many patients. Current therapies to prevent or reverse this remodeling are lacking.

Post-MI remodeling is characterized by inflammation, hypertrophy, apoptosis and fibrosis in and around the area of ischemic injury. Recent studies have implicated the activation of the p38 MAPK pathway in the initial pathological response to injury and the remodeling process (Arabacilar and Marber 2015). However, it is still controversial whether p38MAPK is an ideal drug target for ischemic injury, as it seems to regulate both beneficial and pathological downstream effectors (Bassi et al. 2008). Because of these dual roles of p38 MAPK, downstream targets of p38 MAPK provide more attractive targets for pharmacological inhibition. One such downstream kinase of p38 MAPK is mitogen activated protein kinase activated protein kinase II (MK2). A MK2 knockout mouse model that was subjected to ischemia–reperfusion injury to the heart exhibited improved cardiac function, reduced infarct size, and reduced cardiomyocyte apoptosis compared to wild type litter-mate controls (Shiroto et al. 2005). However, perhaps due to the similarity between the active (ATP-binding) site of MK2 with other kinases, specific small-molecule inhibitors have not been identified.

A cell-permeable peptide inhibitor of MK2, MMI-0100, is currently in clinical trials for the treatment of pulmonary fibrosis. We have previously demonstrated that intraperitoneal (IP) injection of 50 μg/kg/day MMI-0100 protects against systolic dysfunction, fibrosis and cardiomyocyte apoptosis in an LAD ligation model of AMI (Xu et al. 2014). In this study we hypothesize that inhalation of nebulized MMI-0100 will show similar protection of systolic dysfunction and fibrosis as we previously observed with IP injected administration in an LAD ligation model of AMI.

Materials and Methods

Peptide Synthesis and Delivery

The MK2 inhibitor peptide, MMI-0100 (YAR-AAARQARAKALARQLGVAA), was synthesized with standard Fmoc chemistry, as previously described (Ward et al. 2009). The peptide was prepared in PBS and delivered by IP injections (50 μg/kg/day) or by inhalation of nebulized particles (100 μg/kg/day). Nebulization was performed using an Aeroneb® Lab Nebulizer, which produces a particle size of 2.5–4 μm. Nebulized MMI-0100 peptide was administered in 100 μl PBS at a dose of 100 μg/kg/day for 14 days. The higher dose of

nebulized particles was chosen to compensate for the loss of peptide condensate in the tubing of the nebulizer.

Animals and Myocardial Infarction Model

C57BL/6 mice (12 week-old, male, 25–30 g) were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in the University of North Carolina at Chapel Hill facilities for at least 7 days with ad libitum access to standard rodent chow and water. Acute myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery as previously described (Maejima et al. 2013; Qian et al. 2012). Mice were immediately treated with lidocaine (6 mg/kg IM) and atropine (0.04–0.10 mg/kg IM) upon surgical closure. Lidocaine and atropine were given every 2–4 h for the first 24 h to prevent arrhythmias. Mice were additionally given 0.1 mg/kg buprenorphine every 12 h for the first 48 h. Within the first hour post-MI, 50 μg/kg/day MMI-0100 peptide (or PBS control) was given IP or 100 μg/kg/day MMI-0100 (or PBS control) was nebulized and inhaled. Treatment was repeated daily for a total of 14 days.

Echocardiography

Cardiac function in mice was assessed by transthoracic, conscious echocardiography performed at baseline and weekly thereafter using a VisualSonics Vevo 2100 ultrasound biomicroscopy system (VisualSonics, Inc., Toronto, Ontario, Canada), as previously described (Willis et al. 2007, 2009, 2014). Briefly, Two-dimensional M-mode echocardiography was performed in the parasternal long-axis view at the level of the papillary muscle on loosely restrained mice by investigators blinded to mouse genotype throughout the process of collection and analysis. Posterior and anterior wall thickness was measured as the distance from the epicardial to the endocardial leading edge. Internal left ventricular diameters were also measured. Left ventricular (LV) systolic function was measured by ejection fraction (LV EF % = [(LV Vol; d-LV Vol; s/LV Vol; d) × 100] and fractional shortening (%FS = [(LVEDD − LVESD)/LVEDD] × 100). Three cardiac cycles were averaged from each mouse to represent a single measurement.

Histological Analysis

Mice were euthanized with isoflurane and cervical dislocation on day 14. The hearts were removed, fixed in fresh 4 % paraformaldehyde for 24 h, stored in 70 % EtOH and paraffin-embedded. *Fibrosis analysis* Hearts were sectioned into 10 levels (3 sections per level) with 75 μm skipped between levels and stained with Masson's Trichrome (MT). Fibrosis was determined in a blinded manner using Aperio Imagescope algorithm analysis (Positive Pixel Count v9, Hue 0.66, Hue Width 0.4). *H&E stain* Three sections were taken from each heart starting 150 μm from the site of the ligation and stained with hematoxylin and eosin (H&E) using standard protocols.

Results and Discussion

In a previous study we demonstrated that injected MMI-0100 peptide exhibits cardioprotection in a mouse model of acute myocardial infarction (Xu et al. 2014). However, IP injection of a peptide therapeutic after infarct in patients is not a feasible treatment

option. In the present, study we tested the efficacy of an alternate mode of administration, inhalation of nebulized peptide, which could be quickly and easily administered to affected patients. Given the close relationship between the heart and lungs via pulmonary circulation, we hypothesized that nebulized MMI-0100 would provide the same cardioprotection that we previously described using injected peptide. Indeed, in this study we observed similar levels of EF and FS protection in both injected and inhaled MMI-0100.

Treatment of AMI with Nebulized MMI-0100 Peptide Preserves Cardiac Function In Vivo

To assess the effect of MMI-0100 on post-MI cardiac function, we investigated four experimental groups: (1) AMI control group (nebulized PBS, IP PBS); (2) AMI experimental group (nebulized MMI-0100, IP PBS); (3) AMI experimental group (nebulized PBS, IP MMI-0100); and (4) sham control group (nebulized MMI-0100, IP PBS). Treatments were given 30 min post-LAD ligation and administered daily for 2 weeks (Fig. 1a). Baseline echocardiography was performed prior to the surgery and at 7 and 14 days post-MI to assess cardiac function and blindly analyzed prior to final statistical analysis (Fig. 1b). Assessment of ejection fraction and fractional shortening (Fig. 1c) demonstrated that myocardial infarction induced by permanent ligation of the LAD (Group 2 LAD + PBS) resulted in declined systolic function compared to sham treated mice (−21.2 EF %, −32.2 FS %). Treatment with MMI-0100 given IP starting 30 min post-LAD ligation resulted in an attenuation of the systolic function loss compared to sham treated mice (Fig. 1c, −9.3 EF %, −16.1 FS %). Interestingly, MMI-0100 delivered by nebulization similarly attenuated the systolic function loss at both 7 and 14 days post-MI (Fig. 1c, −10.8 EF %, −17.9 FS %).

In addition to preserving cardiac function, MMI-0100 treatment attenuated the calculated LV Mass post-MI that occurs after LAD ligation (Fig. 1d). Since these measurements depend largely on the dilation and wall thickness, it reflects the preservation of muscle mass normalized to body weight (Fig. 1e), which, along with heart rate, did not significantly change during the 2 weeks of the study (Fig. 1f, g). Anterior wall thickness significantly decreased in both systole and diastole in the MI control group by 7 days and improved by 14, reflecting the remodeling process occurring (Fig. 2a, b). Similar effects were seen in the posterior wall thickness in systole and diastole (Fig. 2c, d). This decrease in anterior and posterior wall thickness due to MI is attenuated by MMI-0100 treatment delivered IP and inhalationally (Fig. 2a–d). MMI-0100 treatment (IP and inhalationally delivered) similarly attenuate the changes in MI-induced dilation in systole (Fig. 2e) but not diastole (Fig. 2f), reflecting changes in LV Volumes in systole and diastole (Fig. 2g, h). Taken together, these studies illustrate that the dilated LV (Fig. 2e–h) characteristic of systolic dysfunction (Fig. 1c), support the interpretation that MMI-0100 treatment attenuates MI-induced heart failure when delivered IP or inhalationally.

Histological Analysis of After 2 weeks of Daily MMI-0100

In our initial prior studies, we tested MMI-0100's efficacy in a severe myocardial infarction model with the idea that daily MMI-0100 would only prevent fibrosis (Xu et al. 2014). To our surprise, however, daily MMI-0100 given IP resulted in significant functional protection, including prevent cardiomyocyte apoptosis (Xu et al. 2014). A drawback to using this severe model of MI model is that it resulted in a mortality nearing 50 %. Therefore, in the current

study, we used a permanent ligation model that was less severe by placing the LAD ligation at a lower level, resulting in depressed function but minimal fibrosis (3–4 % compared to our prior study of 20 %) (Xu et al. 2014).

In mice challenged with this modified LAD ligation and treated daily with vehicle control (LAD + PBS), we observed significantly increased collagen on Masson's Trichrome stained histological sections of mouse hearts 2 weeks post-ligation. Cardiac sections were assayed from the site of the LAD ligation to the apex at ten equally distributed levels (3 sections at each level) as outlined in Fig. 3a. Sections were then stained with Masson's tri-chrome, where minimal significant increases in collagen were seen (3–4 % collagen positive vs. 1 % sham controls) (Fig. 3b). With this minimal increase in focal fibrosis, MMI-0100 given IP or inhalationally did not significantly reduce the amount of total collagen (Fig. 3c, d). Histological analysis of H&E stained slides for inflammation did not detect any differences in inflammation between the LAD + PBS and LAD + MMI-0100 groups (IP and inhalationally delivered) at 14 days post-LAD ligation (Fig. 4a–d). This may reflect the 14 day harvest timing, where acute inflammation would likely have visually dissipated, but may have contributed to the decline of cardiac function in the AMI control group at earlier time points. Further study is necessary to determine the contribution of inflammation in this model.

While in our previous study we proposed that the MMI-0100-mediated reduction in fibrosis could be contributing to the protection on cardiac function in LAD mice, these results suggest that the protection of cardiac function seen in Fig. 1 is independent of fibrosis, consistent with our previous studies demonstrating that MMI-0100 can prevent ischemia-mediated apoptosis (Xu et al. 2014). Alternatively, MK2 inhibition may prevent the activation of cardiac stress responses mediated by MK2, as MK2 activation (phosphorylation) can inactivate protective heat shock proteins such as Hsp25/27, causing inflammation and apoptosis (Vidyasagar et al. 2012). Another possible mechanism for the protection afforded by MMI-0100 treatment in this model is due to a direct role of MK2 in cardiac contractility. Activation of p38MAPK has been implicated in depressed contractility due to a downregulation of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2) (Arabacilar and Marber 2015). However, additional research is necessary to implicate MMI-0100 treatment in the preservation of cardiac contractility in this manner.

The benefits of delivering MMI-0100 inhalationally could clinically be beneficial in the context of myocardial infarction for several reasons. First, it would allow delivery of the drug without having to start IV access, providing more rapid initiation of treatment since IV access takes time in even optimal situations and is made more difficult with patients with cardio-circulatory collapse. Second, it delivers the drug directly to the target organ, with the lungs being a direct line to the left ventricle. There are dosing issues to consider when delivering by nebulizer in general related to the dead space that nebulized drug can condensate prior to reaching the lungs, which is something we identified when designing the current studies. Like previous studies, we delivered the MMI-0100 IP at a dose of 50 μg/kg/day (Vittal et al. 2013; Xu et al. 2014). However, the inhalationally delivered MMI-0100 dose we used was 100 μg/kg/day (in 100 μl PBS) because of the dead space between the nebulizer and the oropharynx. In pre-experimental trials, we found that

approximately 50 % of the starting nebulized material (50 μl MMI-0100 in PBS) was left in this dead space, therefore the delivered MMI-0100 dose in the current studies is estimated to be equal between the IP and nebulized daily doses (50 μg/kg/day).

Conclusion

In this study we find that an inhaled, nebulized administration of MMI-0100 protects against AMI-induced systolic dysfunction at 14 days. Using a less severe LAD ligation model compared to our previous study, we observed lower collagen deposition compared to our previous study and no decrease in collagen with or inhaled MMI-0100 treatment, suggesting that fibrosis does not play a significant role in the decline in systolic function. These findings support MK2 inhibition by MMI-0100 as a potential therapeutic in the treatment of acute myocardial infarction and provide an additional method of administration to reach the heart without the need for IV access. This provides a faster mode of administration to MI patients, when rapid treatment time after ischemic injury is of the utmost importance.

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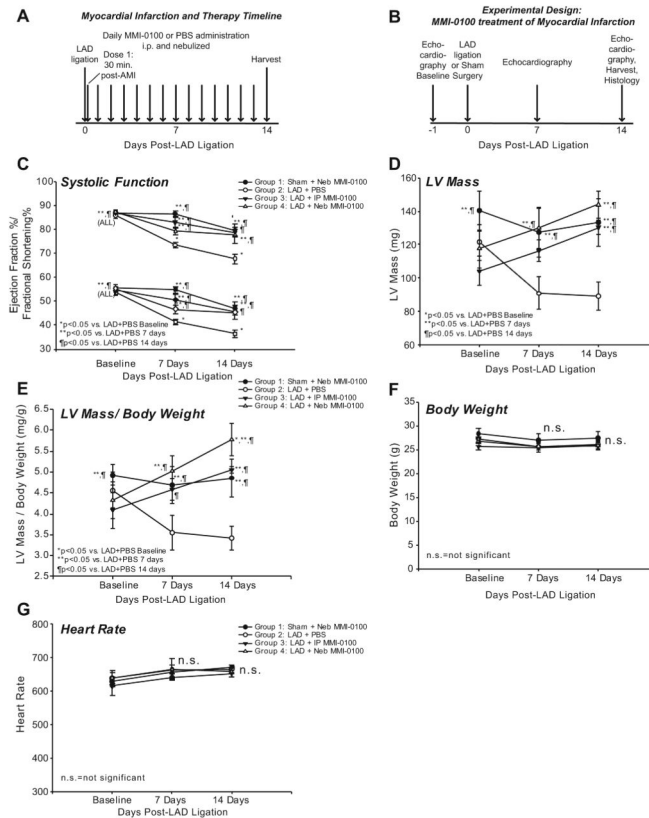
Fig. 1.

Assessment of systolic dysfunction by conscious echocardiography after inhalational and IP administration of the peptide MMI-0100 inhibitor, MMI-0100 in mice that have undergone AMI. **a** MMI-0100 was administered 30 min after LAD ligation and every 24 h thereafter until harvest at 14 days. **b** Baseline conscious echocardiography was performed prior to LAD ligation, as well as 7 and 14 days post-surgery to calculate. **c** Ejection fraction, calculated as (end Simpson's diastolic volume – end Simpson's systolic volume)/end Simpson's diastolic volume × 100, fractional shortening, calculated as (LVEDD – LVESD)/LVEDD × 100, **d** LV mass, **e** LV mass/BW, **f** BW, and **g** HR. Data represent mean ± SEM. A one way ANOVA was performed between all groups, followed by Holm-Sidak (vs. LAD Ligation + PBS control). *p <0.05 vs. LAD + PBS (baseline). **p <0.05 vs. LAD + PBS (7 days). ¶p <0.05 vs. LAD + PBS (14 days)
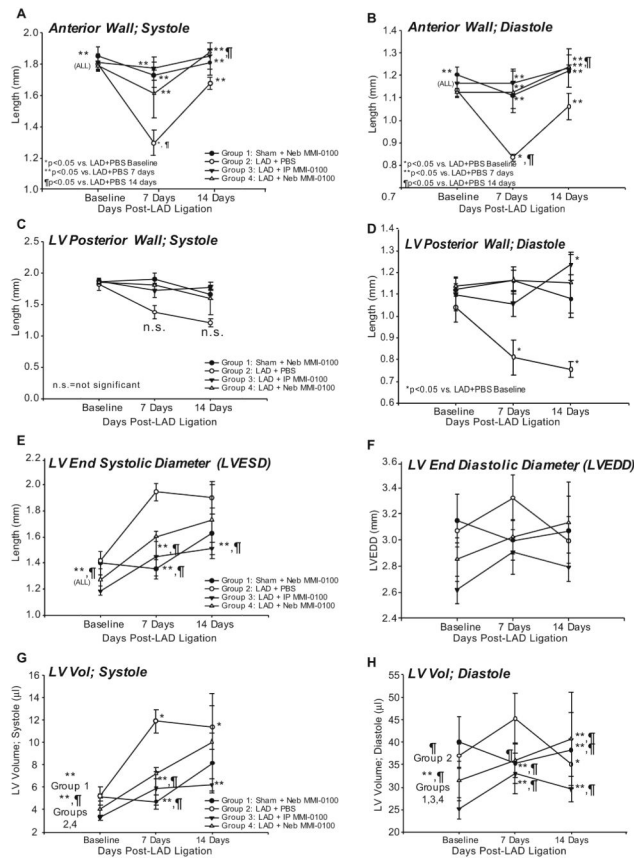
Fig. 2.
Measurement of cardiac dimensions by conscious echocardiography. Measurements were taken of **a** AWTS, anterior wall thickness in systole, **b** AWTD, anterior wall thickness in diastole, **c** PWTS, posterior wall thickness in systole, **d** PWTD, posterior wall thickness in diastole, **e** LVESD, left ventricular end-systolic dimension, **f** LVEDD, left ventricular end-diastolic dimension, **g** LV volume, systole and **h** LV volume, diastole. Data represent mean ± SEM. A one way ANOVA was performed between all groups, followed by Holm-Sidak (vs. LAD Ligation + PBS control). *p <0.05 vs. LAD + PBS (baseline). **p <0.05 vs. LAD + PBS (7 days). ¶p <0.05 vs. LAD + PBS (14 days)
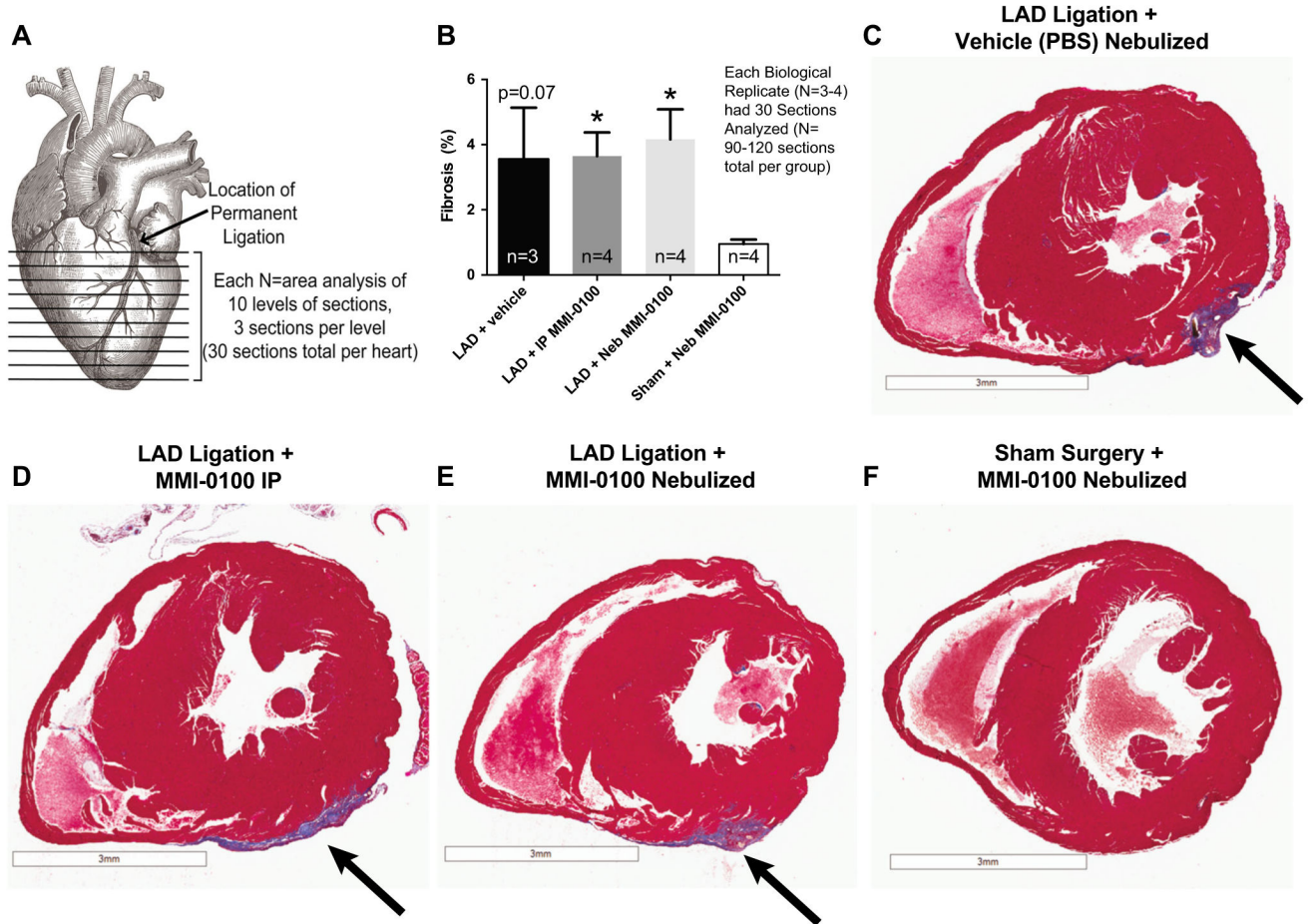
Fig. 3.

Fibrosis analysis. 14 days after permanent LAD ligation and daily drug treatment hearts were harvested and fixed in 4 % PFA for sectioning. **a** 10 levels of sections were taken from the site of the LAD ligation to the apex of the heart. Three replicate sections were taken from each level and stained with Masson's trichrome, for a total of 30 sections per heart analyzed. **b** Percent fibrosis was determined by calculating the area of collagen (*blue stain*) in 3–4 hearts for each group at 10 levels of sections (3 replicates of each level) using Aperio algorithm analysis. Representative images of **c** LAD ligation + vehicle, **d** LAD ligation + IP MMI-0100, **e** LAD ligation + Nebulized MMI-0100 and **f** Sham ligation + Nebulized MMI-0100. A Kruskal–Wallis one-way ANOVA was performed on the fibrosis % from serial sections using 3–4 hearts per group; each percent fibrosis per heart data point was composed of the weighted mean of 30 sections described in **a** above. If significance was reached (p <0.05), a post hoc all pairwise multiple comparison procedures (Tukey test) was performed between each of the groups to determine significance. *p <0.05 vs. all other groups. Heart image in A from: http://www.publicdomainfiles.com/show_file.php?id=13939501819528
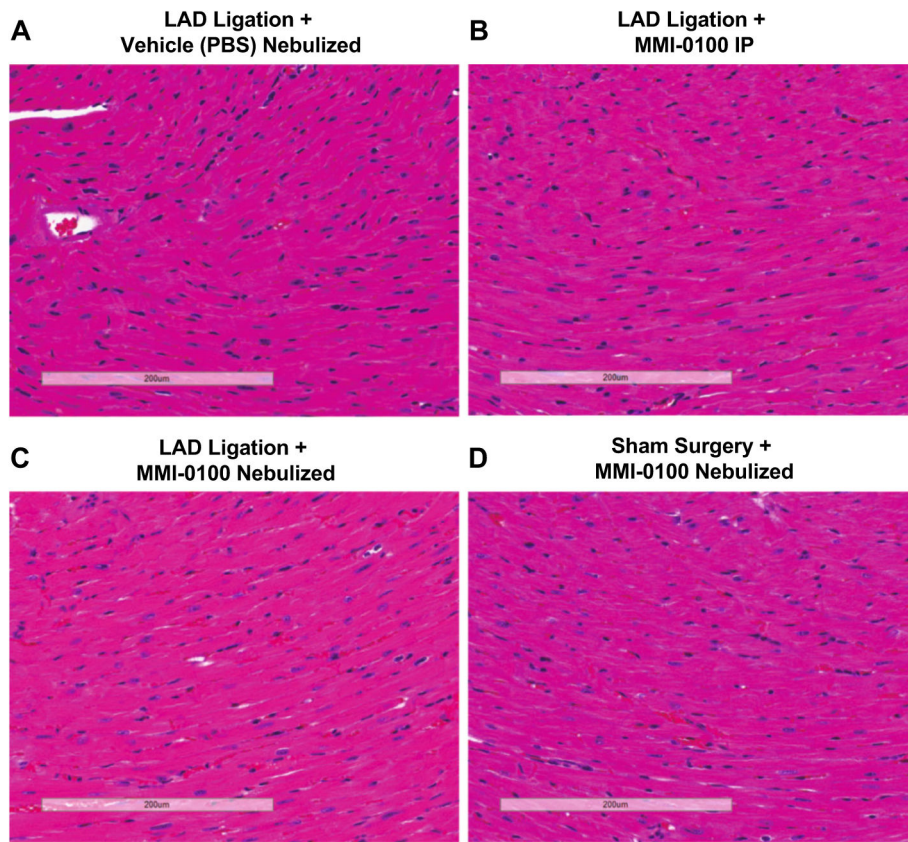
Fig. 4.

Histological analysis of H&E stained heart. Three sections were taken from each heart at the same level, 150 μm from the site of the ligation, and analyzed morphologically for inflammation and other changes. **a** LAD ligation + vehicle, **b** LAD ligation + IP MMI-0100, **c** LAD ligation + Nebulized MMI-0100 and **d** Sham ligation + Nebulized MMI-0100. A one-way ANOVA was performed to assess significant differences using α = 0.05