Post-infarct treatment with [Pyr\(^{1}\)]apelin-13 improves myocardial function by increasing neovascularization and overexpression of angiogenic growth factors in rats

Yaser Azizi\(^a\), Mahdieh Faghihi\(^a,\)*, Alireza Imani\(^a\), Mehrdad Roghani\(^b\), Ali Zekri\(^c\), Maryam Beigom Mobasher\(^i\), Tayebeh Rastgar\(^d\), Maryam Moghimian\(^e\)

\(^a\) Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
\(^b\) Neurophysiology Research Center, Shahed University, Tehran, Islamic Republic of Iran
\(^c\) Department of Medicine, School of Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
\(^d\) Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
\(^e\) Department of Physiology, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Islamic Republic of Iran

**Keywords:** [Pyr\(^{1}\)]apelin-13, Myocardial angiogenesis, VEGFA, Ang-1, eNOS, Rat

**Abstract**

Ischemic heart disease is the leading cause of mortality in the world. Angiogenesis is important for cardiac repair after myocardial infarction (MI) as restores blood supply to the ischemic myocardium and preserves cardiac function. Apelin is a peptide that has been recently shown to potentiate angiogenesis. The aim of this study was to investigate angiogenic effects of [Pyr\(^{1}\)]apelin-13 in the rat model of post-MI. Male Wistar rats (n = 36) were randomly divided into three groups: (1) sham (2) MI and (3) MI treated with [Pyr\(^{1}\)]apelin-13 (MI + Apel). MI animals were subjected to 30 min left anterior descending coronary artery (LAD) ligation and 14 days of reperfusion. Twenty-four hours after LAD ligation, [Pyr\(^{1}\)]apelin-13 (10 nmol/kg/day) was administered i.p. for 5 days. Hemodynamic functions by catheter introduced into the left ventricle (LV), myocardial fibrosis by Masson’s trichrome staining, gene expression of vascular endothelial growth factor-A (VEGFA), VEGF receptor-2 (Kdr), Ang-1 (angiopoietin-1), Tie2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2), VEGF receptor-1 (Kdr), Ang-1, Tie2 and eNOS were assessed at day 14 post-MI. Post-infarct treatment with [Pyr\(^{1}\)]apelin-13 improved LV function and decreased myocardial fibrosis. [Pyr\(^{1}\)]apelin-13 treatment led to a significant increase in the expression of VEGFA, Kdr, Ang-1, Tie2 and eNOS. Further, treatment with [Pyr\(^{1}\)]apelin-13 promoted capillary density. [Pyr\(^{1}\)]apelin-13 has angiogenic and anti-fibrotic activity via formation of new blood vessels and overexpression of VEGFA, Kdr, Ang-1, Tie2 and eNOS in the infarcted myocardium which could in turn repair myocardium and improve LV function.

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1. Introduction

Myocardial infarction (MI), the main cause of morbidity and mortality in the developed countries, is the most usual representation of cardiovascular disease (Huusko et al., 2010; Mitsos et al., 2012). MI is followed by left ventricular (LV) enlargement and reduced capillary density in the infarcted myocardium. Studies have indicated that the capillary density in the border zone is profoundly decreased as compared to the remote areas of the infarcted myocardium (Fukuda et al., 2004). There have been major advances for preventing and treating cardiovascular diseases such as coronary artery bypass grafting. Nevertheless, many patients experience disabling signs in spite of intense pharmacotherapy which makes them unsuitable for invasive revascularization therapies (Grass et al., 2006; Mitsos et al., 2012). Therefore, there stands a need for novel therapeutic strategies to treat these patients. However, the challenge to improve blood flow to the ischemic heart has led to the development of many innovative strategies. Among them, the use of several compounds to increase

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**Abbreviations:** (Ang-1), angiopoietin-1; (Apel), [Pyr\(^{1}\)]apelin-13; (MI), myocardial infarction; (LAD), left anterior descending coronary artery; (Tie2), tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2; (VEGFA), Vascular Endothelial Growth Factors; (HIF-1α), Hypoxia-Inducible Factor-1α; (NOS), nitric oxide synthases; (NO), Nitric oxide; (GAPDH), Glyceraldehyde 3-phosphate dehydrogenase; (LV), Left ventricle; (dLVP), Left ventricular end-diastolic pressure; (LVSP), Left ventricular systolic pressure; (HR), heart rate; (RPP), Rate pressure product

* Corresponding author. Tel./fax: +98 21 66419484.

E-mail address: faghihim@sina.tums.ac.ir (M. Faghihi).

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the formation of new blood vessels has captured much attention. (Mitsos et al., 2012; Renault and Losordo, 2007).

Angiogenesis, the formation of new capillaries from pre-existing vessels, plays an essential role in a variety of physiological and pathological processes such as tumor growth and revascularization of the myocardium following MI (Bikfalvi, 2006; Wang et al., 2012; Zaitone and Abo-Gresha, 2012). As a compensatory mechanism, it takes place after ischemia to restore blood flow to the ischemic myocardium and eventually preserve cardiac function (Heil and Schaper, 2004; Zaitone and Abo-Gresha, 2012). On the other hand, impaired angiogenesis contributes to the myocardial fibrosis and myocardial dysfunction in idiopathic dilated cardiomyopathy (Ohtsuka et al., 2005).

Evidences have shown that cytokines such as vascular endothelial growth factors (VEGF), angiopoietin-1 (Ang-1) and transcription factors like hypoxia-inducible factor-1α (HIF-1α) act as angiogenic molecules (Fagiani and Christofori, 2013; Jaumudally et al., 2010; Tuo et al., 2008). VEGF and Ang-1 activate angiogenic and prosurvival signaling pathways and attract stem cell homing to the affected area (Su et al., 2009; Tao et al., 2011). Previous studies have demonstrated that VEGF stimulates Akt/FOXO, which in turn phosphorylates and activates eNOS (Yamahara et al., 2003). Nitric oxide (NO) via cyclic GMP has been seen to participate in this angiogenic effect of VEGF (Fukuda et al., 2006). VEGF is an important regulator of angiogenesis, which stimulates proliferation, migration, survival and proteolytic activity of endothelial cells (Huusko et al., 2010). In addition, Angiopoietin/Tie system also plays a significant role in the formation of vessels. Ang-1 binds to Tie2 receptor in the endothelium and phosphorylates it, thereby preventing the leakage of plasma from the vessels. (Brindle et al., 2006; Fagiani and Christofori, 2013; Shyu et al., 2003).

Apelin, an adipocytokine, was first isolated from bovine stomach tissue extracts by Tatamoto et al (1998). This endogenous peptide is a ligand for the apelin angiotension receptor-like 1 (APJ) (Dai et al., 2006). Apelin which is widely expressed in the endothelium, binds to and activates APJ receptors distributed in endothelial cells, myocardial cells, and some smooth muscle cells (Ashley et al., 2005; Sheikh et al., 2008). Studies have indicated that NOS inhibition can decrease [Pyr1]apelin-13 induced blood flow in forearm. This implies the importance of NO as a signaling molecule after activation of APJ in the endothelium (Japp et al., 2008, 2010). Apelin/APJ system has been found to promote embryonic angiogenesis (Kalim et al., 2007). Apelin deficiency decreases vascular sprouting and impairs sprouting of endothelial progenitor cells, and in vivo myocardial angiogenesis (Wang et al., 2013). Furthermore, low serum apelin levels following MI have also been documented. (Kuklinska et al., 2010; Weir et al., 2009).

Based on these reports, we designed this study to evaluate the effects of post-MI treatment with apelin on VEGFA, VEGF receptor-2 (Kdr), Ang-1, Tie2 and eNOS, myocardial fibrosis and cardiac function in the MI rats.

2. Materials and methods

2.1. Animals

The present study was performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No.85–23, revised 1996). The experimental protocol was approved by the institutional care and use committee of Tehran University of Medical Sciences (Tehran, Iran). In this study, 36 male Wistar rats (weighing 250–300 g) were housed in controlled environmental conditions (22 ± 2°C; light–dark cycle 7 AM to 7 PM). Animals had free access water and standard laboratory food ad libitum. Animals were acclimated to environment for 7 days before the experiments.

2.2. Rat model of myocardial ischemia and reperfusion

Acute intramural (focal) myocardial infarction was induced by ligation of the left anterior descending coronary artery (LAD). After induction of anesthesia (thiopental sodium; 60 mg/kg i.p.), the animals were placed in the supine position and body temperature was maintained as close as possible to 37°C by means of thermal pad and heating lamp. Next, they were intubated and ventilated by room air using a rodent ventilator (tidal volume 2–3 ml; respiratory rate 65–70 per minute, Harvard rodent ventilator model 683, Holliston, MA, USA). Left intercostal thoracotomy (between the fourth and fifth costal space) was performed under sterile conditions. The heart was exposed and pericardium was incised. Acute intramural (focal) MI was produced by ligation of the LAD with 6–0 polypropylene suture approximately 1–2 mm distal from its origin. Both ends of the silk suture were then passed through a small vinyl tube, and the LAD was occluded by pulling the snare, which was fixed by clamping the tube with a mosquito hemostat. Successful LAD occlusion was characterized by ST segment elevation immediately after ligation and cyanosis of the affected myocardium. After 30 min of LAD occlusion, the occluder was removed and restoration of blood flow was verified. After completion of all surgical procedures, the chest was closed in layers. The lungs were inflated by increasing positive end expiratory pressure and the animals were removed from the ventilator and allowed to recover. The sham operated rats underwent the same procedure of thoracotomy, without the LAD ligation. This protocol resulted in the formation of three groups: sham-operated group (sham, n=12), MI group (MI, n=12), and apelin-treated MI group (MI+Apel, n=12). Post-operative, rats were hydrated with normal saline (s.c) and received buprenorphine (0.05 mg/kg) as an analgesic. Tetracycline was used as post-operative antibiotic. [Pyr1]apelin-13 (Sigma) was dissolved in normal saline and administered i.p. 24 h after induction MI (10 nmol/kg/day, once a day) for 5 days (Azizi et al., 2013). Sham and MI animals received normal saline.

2.3. Assessment of myocardial function

Fourteen days after surgery, cardiac hemodynamic parameters were measured. For this, rats were anesthetized with thiopental sodium and ventilated. A small incision was made to the right of the midline in the neck. The right common carotid artery was exposed and cannulated with a PE50 catheter connected to the Powerlab system via pressure transducer. Catheter was pushed down until it had reached the left ventricular lumen. Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), developed left ventricular pressure (dLVP), heart rate (HR), rate pressure product (RPP; HR × dLVP), the maximum rate of left ventricle systolic pressure (+dp/dt) and the minimum rate of left ventricle systolic pressure (−dp/dt) were all monitored and recorded by Powerlab data acquisition system (AD Instrument, Australia).

2.4. Assessment of myocardial fibrosis

After measurement of hemodynamic parameters, the chest was opened and the hearts were arrested during diastole by intravenous injection of KCL (10%). Animal were sacrificed under deep anesthesia and the hearts were rapidly harvested. After washing in normal saline, hearts were fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin. Transverse sections (6 μm)
of hearts were prepared using a microtome. Next, sections were deparaffinized and stained with Masson’s trichrome (Sigma-Aldrich Co., MO, USA) for measurement of fibrosis. The collagen volume fraction in the infarcted and peri-infarcted areas of LV was calculated by measuring the optical density of fibrotic area using Photoshop software (Ver. 7.0, Adobe System, San Jose, CA, USA). Myocardial fibrosis was expressed as a percentage of fibrotic area to left ventricular area (% of LV) in an average of 5 sections in each heart.

2.5. Real-time polymerase chain reaction (Real-Time PCR)

After measurement of hemodynamic parameters, the myocardial samples of some animals from each group (n=5) were immediately removed, rinsed in PBS, frozen in liquid nitrogen and stored at −80°C for evaluation of gene expression. Total RNA was extracted from frozen peri-infarct area and border zone of LV free wall using RNX-Plus kit (Cat. No: RN7713C, Cinnagen, Iran). Complementary DNA (cDNA) was synthesized from 1000 ng of total RNA using a Rocket Script RT PreMix (BioNeer). cDNA samples were then used as templates for quantitative reverse transcription polymerase chain reaction (qRT-PCR). Quantification of gene expression was performed using the Rotor-Gene 6000 (Qiagen). Real-Time PCR analysis was provided by the use of AccuPower 2× Green star quantitative PCR Master Mix (BioNeer). The value for each sample was an average of three independent PCR measurements. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The relative expression of VEGFA, Kdr, Ang-1, Tie2 and eNOS were calculated using the 2−ΔΔCt method (Pfaffl et al., 2002). The specific primer sequences are listed in Table 1.

2.6. Immunohistochemistry

On day-14 postMI, capillary density (number/mm²) was measured using a blood vessel staining kit peroxidase system (Cat No. ECM 590, Millipore, USA & Canada) for immunohistochemical staining of cardiac sections. The primary antibody used were monoclonal mouse anti-human CD31, an endothelial cell marker; which cross-reacts with rabbit tissues. Using a light microscope (×400), vascular density was determined from the stained sections by counting the number of vessels within the peri-infarct area, ischemic and border zone, and image of each section was captured using a digital camera. The vessels in 6 random fields within each section of 6 experimental hearts from each group were counted in a blinded fashion. The number of vessels in each field was averaged and expressed as the number of vessels/mm².

2.7. Statistical analysis

All data are presented as means ± S.E.M. All statistical analysis was performed by SPSS software (Version 15.0, SPSS Inc., Chicago, IL). One-way ANOVA was used to compare mean differences among the sham, MI and MI+Apel groups followed by Tukey post-hoc test. A P-value less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of apelin on myocardial function

14 days after surgery, functional recovery of MI hearts was measured by LVEDP, LVSP, dLVP, RPP, +dp/dt and −dp/dt. Hemodynamic data are shown in Fig. 1. Our results showed that there was significant impairment in myocardial function in MI animals in comparison with the sham group. Myocardial function was improved in MI animals by a 5-day treatment with apelin-13. For 5 days could improve myocardial function in MI animals. As shown in Fig. 1, LVSP, dLVP and RPP were markedly declined and LVEDP was significantly increased in MI animals when compared to the sham animals (P<0.001). Treatment with apelin significantly decreased LVEDP (P<0.05), and increased LVSP (P<0.05) and RPP (P<0.001) as compared to MI animals (Fig. 1A–D). Also, our results showed that induction of MI decreased myocardial contractility (+dp/dt) and relaxation (−dp/dt) when compared to the sham group (P<0.001). Treatment with Apelin-13 improved both, contractility and relaxation (P<0.01) when compared to the MI group but could not reverse +dp/dt (P<0.01) to the sham level (Fig. 1E and F).

3.2. Effect of apelin on myocardial fibrosis

Masson’s trichrome staining showed a significant increase in fibrosis and collagen deposition in MI animals than sham group (P<0.001). As shown in Fig. 2A and B, 5-day apelin treatment markedly reduced interstitial fibrosis when compared to MI group (28.090 ± 1.612% vs. 10.712 ± 0.765% in MI+Apel group, P<0.001). Moreover, there was seldom collagen in sham group (Fig. 2B).

3.3. Effect of apelin on gene expression

We investigated the potential mechanisms for apelin’s action on the mRNA expression of growth factors involved in myocardial angiogenesis. A total of 15 hearts were used for Real-Time PCR analysis of mRNA expression of VEGFA, Kdr, Ang-1, Tie2 and eNOS. Fig. 3 shows that 5-day treatment with apelin-13 significantly increased VEGFA, Kdr, Ang-1, Tie2 and eNOS in the peri-infarct area and border zone of LV area in MI+Apel animals when compared to sham and MI animals (P<0.001 for all of them). Except for Ang-1, our data analysis showed no significant differences in mRNA expression between MI and sham animals (P<0.01).

3.4. Effect of apelin on capillary density

The role of apelin-13 in angiogenesis after induction of MI was assessed by CD31/PECAM-1 immunohistochemical staining. Representative photographs of stained sections are shown in Fig. 4A–C. Myocardial sections of apelin-13 treated animals showed increased CD31-positive microvessels (capillary density) than sham and MI groups (Fig. 4D). Quantitative analysis of data showed more vessels in MI group than in sham group (P<0.05). These results demonstrated that apelin-13 treatment promoted revascularization by increasing capillary density in the LV after MI.

Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>PCR product size</th>
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<td>VEGFA</td>
<td>F: 5′-CATGAACCTTTGCTCTTCTT-3′</td>
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</tr>
<tr>
<td></td>
<td>R: 5′-GGTCAGGCTCTAGTCCGGA-3′</td>
<td></td>
</tr>
<tr>
<td>Kdr</td>
<td>F: 5′-TGGTGTTGCTTGTGGTGTG-3′</td>
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</tr>
<tr>
<td></td>
<td>R: 5′-CGTCACTTGCCGTTGGTGGT-3′</td>
<td></td>
</tr>
<tr>
<td>Ang-1</td>
<td>F: 5′-CTGACAGAGCAACAGAAGCA-3′</td>
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</tr>
<tr>
<td></td>
<td>R: 5′-CTTTTGGGCTTCTGCTGATA-3′</td>
<td></td>
</tr>
<tr>
<td>Tie2</td>
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<td>309</td>
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<tr>
<td></td>
<td>R: 5′-GCCCTCCTGGTTAGG-3′</td>
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<tr>
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<td></td>
<td>R: 5′-GGCTCTCTCTTGCTTGCTGAT-3′</td>
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4. Discussion

In the present study, we investigated the potential mechanisms for apelin’s action on myocardium in the rat model of post-MI myocardial dysfunction. For the first time, this study has shown that 5-day treatment with apelin-13 resulted in improved myocardial function, increased capillary density and mRNA expression of VEGFA, VEGF receptor-2 (Kdr), Ang-1, Tie2 and eNOS and decreased collagen deposition and myocardial fibrosis, after acute intramural MI induction.

Our previous study has demonstrated that 5-day treatment with apelin-13 after 24 h induction of acute intramural (focal) MI, has long term cardioprotective effects related to its antioxidant properties and production of NO (Azizi et al., 2013). So here, we have further studied the mechanisms for the long-term effects of apelin-13 in improving of myocardial function. Our results showed that post-infarct treatment with apelin-13 significantly improves myocardial performance. It markedly increased LVSP, dLVP and decreased LVEDP in comparison with the MI group. Likewise, Li et al. showed that treatment of post-MI mice with myocardial injection of apelin overexpressing bone marrow cells significantly improved end systolic pressure, + dp/dt and −dp/dt and (Li et al., 2013). Furthermore, they also reported that treatment with apelin-13 (1 mg/kg/day) for 3 days before MI and for 14 days post-MI, significantly improved cardiac contractility in post-MI mice and increased end systolic pressure and end systolic pressure–volume relationships (Li et al., 2012).

Fig. 1. Myocardial function (n = 8). (A) LVSP; left ventricular systolic pressure (mmHg). (B) LVEDP; left ventricular end diastolic pressure (mmHg). (C) dLVP; developed left ventricular pressure (mmHg). (D) RPP; rate pressure product. (E) + dp/dt, maximal rate of increase of ventricular pressure and (F) − dp/dt, maximal rate of decrease of ventricular pressure. MI; myocardial infarction, Apel; apelin. Data are presented as means ± S.E.M. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. sham group. $ P < 0.05, $$ P < 0.01 and $$$ P < 0.001 vs. MI group.
Moreover, we found out that apelin-13 exerts beneficial effects on the histology of myocardium. As shown in Fig. 2, acute intramural MI induction has deleterious effects on the myocardial tissue and causes deposition of collagen and increases myocardial fibrosis. This is in line with other studies which have reported that after MI, fibrosis is considerably increased in the affected myocardium (Wang et al., 2012; Xu et al., 2013; Zeng et al., 2012, 2008). This study reveals that apelin-13 decreases fibrosis 14 days post-MI.

Furthermore, apelin has angiogenic properties (Wang et al., 2013). Activation of APJ has shown to stimulate proliferation and migration of endothelial cells (Ashley et al., 2005; Sheikh et al., 2008). In this study, we investigated post-infarct treatment effects of apelin-13 on the gene expression of important angiogenic proteins. VEGF is one of the most important growth factors involved in angiogenesis. As it binds to VEGF receptors especially Kdr, it causes endothelial cell migration and proliferation and inhibits endothelial cell apoptosis. It stimulates NO production by activating eNOS. In turn, NO contributes to some endothelial functions such as migration, proliferation, anti-apoptosis and vasorelaxation. These events are important processes in the induction of angiogenesis. Furthermore, NO also induces endothelial progenitor cells mobilization (Lakkisto et al., 2010; Mitsos et al., 2012; Zeng et al., 2008). In this study we showed that post-infarct treatment with apelin-13 increases mRNA expression of VFGFA, Kdr and eNOS in the left ventricular myocardium at day 14 post-MI. Li et al. reported that treatment with apelin-13 upregulates VEGF expression and Akt/eNOS phosphorylation 24 h after ischemia in the mice model of MI. Therefore, it is reasonable to infer that apelin-13 binds to APJ receptor, and increases VEGFA, Kdr and eNOS expression in the endothelial cells which in turn may increase endothelial progenitor cell mobilization and endothelial cell migration, proliferation and survival. Through these mechanisms, apelin can promote myocardial angiogenesis, restore blood flow to the ischemic area, decrease fibrosis in the interstitium and improve cardiac function.

It is also well-documented that proteins of angiopoietin (Angs) family such as Ang-1 and Ang-2 and their receptors Tie1 and Tie2 are involved angiogenesis (Bikfalvi, 2006; Fagiani and Christofori, 2013). Ang-1 binds to Tie2 receptor and phosphorylates it. Ang-1 is an endothelial cell survival factor as it prevents cellular apoptosis via PI3K/Akt pathway. Furthermore, it has beneficial effects in the setting of heart diseases and has protective role against ischemic heart disease. Ang-1/Tie2 signaling regulates vascular smooth muscle cells, endothelial cells and pericyte maturation and is critical for the survival, maintenance, stabilization and formation of non-leaky vessels (Fagiani and Christofori, 2013; Zeng et al., 2012).

For the first time, this study showed that post-infarct treatment with apelin increases the expression of Ang-1 and Tie2 in the peri-infarct area and border zone of myocardium at day 14 after induction of MI. We suggest that apelin, via activation of APJ, activates signaling...
pathways that cause elevation of Ang-1 and Tie2 expression and their phosphorylation. In turn, it may increase survival of the endothelial cells that activated by VEGF and eNOS. In addition, these endothelial cells may form non leaky tube like structures and increase blood supply to the affected myocardium. Thus, by this mechanism it may decrease fibrosis after MI and improve myocardial function.

Increased angiogenesis and neovascularization are critical processes in the resupply of myocardium and repair of ischemic damages to the myocardium after MI. These events ultimately result in improved myocardial function. As depicted in Fig. 4, treatment with apelin-13 increases myocardial capillary density and promotes formation of microvessels in the affected myocardium. This in turn is accompanied by decreased collagen deposition in the affected myocardium and an improved cardiac function.

The post-MI heart has marked alteration in both, preload and afterload. Therefore, pressure–volume loop analysis is ideal. One of the limitations of this study was pressure–volume loop measurement. Western blot is another limitation of our study.

5. Conclusion

Taken together, our findings suggest that post-infarct treatment with apelin-13 can improve myocardial function, its contractility
and relaxation via decreasing myocardial fibrosis. Further, it can promote angiogenesis by increasing the expression of VEGF-A, Kdr, Ang-1, Tie2 and eNOS. Thus, apelin-13 via its angiogenic actions, improves myocardial function, decreases fibrosis and preserves its structural integrity. Our results suggest that apelin-13 can be used as a novel therapy for the treatment of patients with ischemic heart diseases.

Disclosures

None.

Conflict of interest

None.

Acknowledgments

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References


