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Transplantation of Umbilical Cord-Derived Mesenchymal Stem Cells Overexpressing Lipocalin 2 Ameliorates Ischemia-Induced Injury and Reduces Apoptotic Death in a Rat Acute Myocardial Infarction Model

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Abstract
Myocardial infarction (MI) is a leading cause of death worldwide and requires development of efficient therapeutic strategies . Mesenchymal stem cells (MSCs) -based therapy of MI has been promising but inefficient due to undesirable microenvironment of the infarct tissue. Hence, the current study was conducted to fortify MSCs against the unfavorable microenvironment of infarct tissue via overexpression of Lipocalin 2 (Lcn2) as a cytoprotective factor. The engineered cells (Lcn2-MSCs) were transplanted to infarcted heart of a rat model of MI. According to our findings, Lcn2 overexpression resulted in increased MSCs survival in the MI tissue ($p < 0.05$) compared to non-engineered cells. Furthermore, the infusion of Lcn2-MSCs mitigated Left ventricle (LV) remodeling, decreased fibrosis ($p < 0.0001$), and reduced apoptotic death of the LVs' cells ($p < 0.0001$) compared to the control. Our findings suggest a potential novel therapeutic strategy for MI, however, further investigations such as safety and efficacy assessments in large animals followed by clinical trials are required.

Keywords Myocardial infarction · Mesenchymal stem cells · Lipocalin 2 · Apoptosis · Exogenous expression

Introduction

Development of efficient therapeutic strategies for myocardial infarction (MI) is necessary since it is the leading cause of deaths and disabilities worldwide. Loss of cardiomyocytes and inability of the myocardium to self-regenerate following MI lead to permanent heart failure [1–3]. Therefore, novel therapeutic approaches to restore cardiomyocytes and their function after MI have always been investigated by both basic and clinical scientists. One of the novel approaches for recovery of infarcted tissue function is transplantation of mesenchymal stem cells (MSCs) [4–8], and several preclinical and clinical studies have been conducted in this regard [9–12]. MSCs are capable of homing and engrafting into damaged tissues, releasing trophic factors [13], promoting neovascularization [14], managing oxidative stress [15, 16], and triggering anti-inflammatory responses [17]. However, despite several advantages of MSCs, their therapeutic application is under question mostly due to their poor survival after transplantation. For example, over 99% of MSCs die within 24 h after transplantation [7, 18, 19]. The major cause of this low survival rate is inevitable exposure of the cells to a variety of stresses during their preparation and following transplantation, including nutrient-poor environment, oxidative stress, hypoxia, and high amount of cytotoxic factors [7, 20–22]. Therefore, in order to improve the efficiency of the MSCs-based therapy of MI, it is essential to employ

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strategies which strengthen the cells against the mentioned stresses. These strategies include pretreatment of the cells with hypoxia, serum deprivation (SD), or some pharmacological agents, as well as genetic manipulation of the cells [7, 23]. Furthermore, another strategy to reinforce MSCs against toxic microenvironment is to equip them cytoprotective factors [24]. Lipocalin2 (Lcn2/NGAL) is a small glycoprotein and a well-known cytoprotective factor [25]. A number of studies indicate that Lcn2 acts as an antioxidant, anti-inflammatory, and anti-apoptotic factor following its engineered overexpression in MSCs (Lcn2-MSCs) [7, 22]. Furthermore, up-regulation of other cytoprotective and growth factors has been shown in Lcn2-MSCs [22, 26]. Conspicuously, it has been well-known that Lcn2 is a key early marker of heart injuries with salutary effects. In other words, Lcn2 is induced following heart injuries, including AMI, to ameliorate the stress condition [27].

In the present study, Lcn2 was overexpressed in human umbilical cord-derived MSCs (hUC-MSCs). Then, the engineered MSCs were transplanted into the myocardium of rats with induced MI. Our results revealed that the Lcn2-MSCs restore functions of the recipient heart tissue by inhibition of apoptosis and decreasing infarct area size. In addition, tissue fibrosis was declined and the heart tissue represented less pathological configuration.

Methods and Materials

hUC- MSCs Isolation and Culture

MSCs from human umbilical cord (hUC- MSCs) were isolated as previously described [28]. The isolated cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), and 1% penicillin and streptomycin antibiotics at 37 °C and 5% CO_2. The cells were sub-cultured by Trypsin-EDTA 0.05% solution (Gibco, USA).

Transfection of the hUC-MSCs

Passage four of the hUC-MSCs was used for transfection by either pcDNA3.1/CT-GFP-Lcn2 plasmid encoding the LCN2 protein, or non-recombinant pcDNA3.1/CT-GFP plasmid as control. For transfection, the cells were cultured in 25cm^2 flasks to 70–80% confluency. Then, they were transfected by 3 μg/ml of either the pcDNA3.1/CT-GFP-Lcn2 (Lcn2-MSCs) or the pcDNA3.1/CT-GFP (V-MSCs) vector [22] using XtremeGENE HP DNA transfection kit (Roche, Germany) according to manufacturer's instructions.

Assessment of Lcn2 mRNA Overexpression by the Transfected Cells Using RT-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, USA). Then, cDNA was synthesized using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, USA) using 2 μg of the extracted RNA according to the manufacturer instructions. Next, to assess the Lcn2 or β-actin (as an internal control) mRNAs expression, RT-PCR was performed using specific primer pairs (Table 1). PCR condition included a primary denaturation step at 95 °C for 5 min, followed by 35 cycles of 30s at 95 °C, 30s at 57 °C, and 30s at 72 °C, and a final step of 5 min at 72 °C. Finally, the PCR products were electrophoresed on 2% agarose gel.

Assessment of Lcn2 Protein Expression by ELISA

Expression of Lcn2 at protein level was evaluated by an ELISA kit (R&D Systems, USA). To determine the Lcn2 protein concentration, cell culture media were harvested 48 h post transfection, centrifuged at 1500 g for 5 min to remove cell debris, and then subjected to the ELISA according to the manufacturer's instruction.

Acute Myocardial Infarction Model and Surgery

All animal studies were conducted in accordance with Animal Care Committee guidelines of National Committee for Ethics in Biomedical Research. Animals were kept at standard animal house at 21–23 °C and 12:12 light/dark cycle conditions with free access to food and water.

Mature male Wistar rats (250–300 g) were used in the current research. The rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Prior to operation, the rats received intramuscular injection of cefazolin (30 mg/kg) to prevent probable infection. The animals' chest was shaved and disinfected by povidone-iodine.

Table 1 The Primer pairs used in RT-PCR analysis of the Lcn2 and B-actin mRNAs

Gene	Forward	Reverse
Lcn2	5′-TCACCTCCGTCCTG TTTAGG-3′	5′-CGAAGTCAGCTCCTTGGT TC-3′
B-actin	5′-TTCTACAATGAGCTGCGTGT GC-3′	5′-GTGTTGAAGGTCTCAAACATGAT −3′

To maintain body temperature, the animals were placed on a warm blanket during the operation. It must be also mentioned that the animals' heart function was monitored by electrocardiography throughout the surgery. Following anesthesia, the rats underwent intubation by a vein detained needle and a mechanical ventilation device using a rodent ventilator (NARCO Bio-Systems, USA). Respiration rate was adjusted to 70 breaths/min and a tidal volume of 3 mL/100 g body weight. Next, thoracotomy was done at the 3rd or 4th intercostal space. The muscles were dissected and the pericardium was eliminated carefully to reach the heart. MI induction was achieved by permanent ligature of left anterior descending (LAD) artery by a 6–0 Prolene suture (SUPA, Iran). Visual confirmation of MI occurrence was performed by observing the heart color change from red to white, and also by alterations in electrocardiogram ten minutes after ischemia induction. The health condition of the animals was monitored carefully, and finally the incision was sealed in layers by stitching (SUPA, Iran**).** The sample size was calculated based on previous similar studies [29–31], and at least four live rats were allocated to each experiment group following the surgery.

Transplantation of the hUC-MSCs

The Lcn2-MSCs and V-MSCs were prepared to transplant into the myocardium. Briefly, the cells were trypsinized and washed twice with PBS by centrifugation at 1500 g for 5 min. Then, the cell pellet (containing 1×10^6 cells) was re-suspended in 100 μl PBS and directly injected into the border zone of ischemic area. In addition, in another experiment, to track and evaluate homing of the cells in the ischemic zone of left ventricle (LV), the hUC-MSCs were labeled by CellTracker™CM-Dil (Invitrogen, USA) according to the manufacturer instruction. Briefly, the cells were treated by 1 μM CellTracker™CM-Dil solution for 5 min at 37 °C, followed by 15 min of incubation at 4 °C. Then, the labeled MSCs were transplanted into the myocardium as described above and the cells were tracked 7 days after transplantation.

The animals were allocated to five groups, of which the sham group was undertaken chest opening without ischemia; the MI group had permanent ligature of LAD after opening the chest; the MI + PBS group received 100 μL PBS after LAD ligation; The MI + V-MSCs group received 100 μL of V-MSCs after LAD ligation; and the MI + Lcn2-MSCs group received 100 μL of Lcn2-MSCs after LAD ligation.

Histological Studies

Three weeks after surgery, all animals were euthanized and their heart was removed and fixed in 4% paraformaldehyde at least for 72 h. After tissue processing, the heart tissue was embedded in paraffin and cut into 3–5 μm sections. The sections were stained by Hematoxylin and Eosin (H&E) to evaluate the cytostructural feature of the heart. In addition, fibrosis formation was detected by Masson's trichrome staining followed by assessment with Image J software (NIH, USA, version 1.8.0_112) to measure fibrotic area in three sections of each heart.

Apoptosis Assay

In situ cell death detection kit (TAKARA, Japan) was used to detect apoptotic cells in different groups. Three μm paraffin embedded sections were treated by Xylene to remove the paraffin. Next, antigen retrieval was performed by treatment with proteinase K solution (Thermo Scientific, USA) at 37 °C for 20 min. Then, the sections were treated by TUNEL enzyme solution at 37 °C for 1 h. Moreover, the cells were further stained with 1 μg/ml of DAPI (Sigma, USA) at 37 °C for 20 min. Finally, the cells were observed under fluorescence microscope (Nikon, Japan) and photographed.

Measurement of Infarct Area Size

Triphenyltetrazolium chloride (TTC) staining was performed to determine the size of infract area. The hearts were washed with sterilized PBS and freezed at −20 to become tightened. Frozen samples were cut into 2–3 mm thickness sections from apex to bottom using heart matrix and incubated in pre-warmed 1% TTC solution at 37 °C for 30 min. Afterward, the sections were fixed using 10% formaldehyde solution for 2 h. The heart slices were photographed and analyzed by the Image J software.

Statistical Analysis

Data were analyzed by GraphPad Prism 8 (GraphPad Software, USA), and are represented as mean ± SD. To verify the normal distribution of data, the Shapiro-Wilk normality test was performed ($p > 0.05$ was considered as normal distribution). For data with normal distribution T-Test and one-way ANOVA was performed. For the data deviated from normal distribution Kruskal-Wallis nonparametric test was performed. $P < 0.05$ was considered significant.

Results

Overexpression of Lcn2 in MSCs

Expression of Lcn2 mRNA was confirmed by RT-PCR. A single band of 240 bp on agarose gel was detected in the Lcn2-MSCs, but no expression was noticed in the V-MSCs (Fig. 1a&b). Expression of Lcn2 was also verified at protein level by ELISA. Consistent with the RT-PCR results,

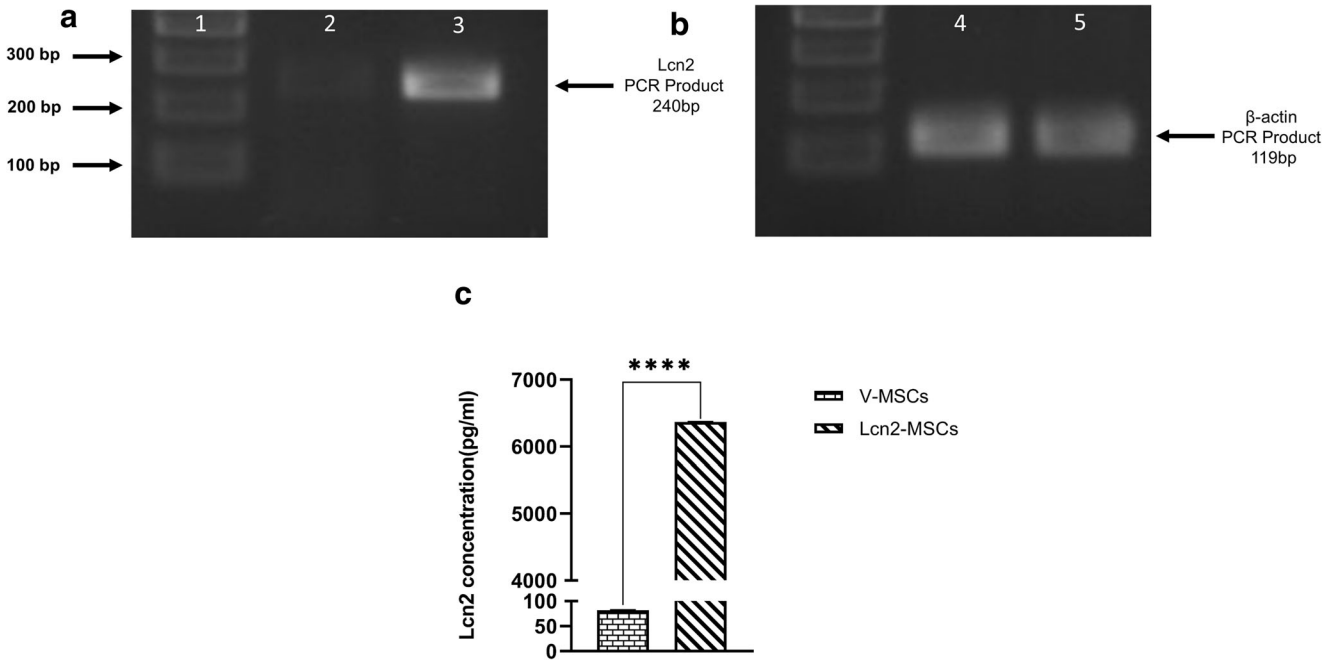

Fig. 1. Evaluation of Lcn2 expression at mRNA and protein Levels. A) RT-PCR was performed to evaluate expression of Lcn2 mRNA. Detection of a single band of 240 bp in the Lcn2-MSCs group and no PCR product in V-MSCs indicated successful exogenous expression of Lcn2. B) Expression of β-actin was used as an internal control (lane1=100 bps ladder, lane2=β-actin of V-MSCs, lane3=β-actin of Lcn2-MSCs, lane4=Lcn2 of V-MSCs, lane5=Lcn2 of Lcn2-MSCs). C) ELISA was performed for evaluation of Lcn2 at protein level. The culture medium of V-MSCs and Lcn2-MSCs were harvested 48 hrs after transfection. The Lcn2 protein expression was significantly ($p<0.0001$) higher in Lcn2-MSCs compared to the V-MSCs. **** $p<0.0001$, (Number of replicates=3).

expression of Lcn2 protein was detected in the Lcn2-MSCs while no expression was observed in the V-MSCs (Fig. 1c).

Lcn2 Renders MSCs Survival in the Harsh Microenvironment after MI

To evaluate survival and homing of the transplanted MSCs, they were labeled by CellTracker™CM-Dil. The number of engrafted cells in the Lcn2-MSCs group was about 20% higher than the V-MSCs group, suggesting that Lcn2 makes MSCs more resistant to the unfavorable ischemic heart microenvironment (Fig. 2).

Lcn2-MSCs Transplantation Reduced Fibrosis and Alleviated Left Ventricle (LV) Remodeling

LAD was ligated permanently to induce AMI. The color of the ischemic area turned white (from red) immediately after ligation, which confirmed the absence of perfusion beneath the ligated artery. The accuracy of AMI model was further confirmed by elevated ST segment in the electrocardiogram (ECG) (data not shown), and increased serum level of cardiac troponin I (cTnI) (Fig. 3b). The cTnI serum level showed dramatic elevation in the MI group 24 h post-ischemia, but it decreased and approached normal level within 7 days. In addition, infiltration dramatically increased in the MI and MI + PBS groups compared to the MI + Lcn2-MSCs group (Fig. 3a). Remarkably, dispersed Aschoff bodies were observed in the MI, MI + PBS, and MI + V-MSCs but not the MI + Lcn2-MSCs groups, suggesting post-ischemia inflammation in the groups without exogenous expression of Lcn2. Additionally, abnormal cardiomyocytes with puffed cytoplasm and pyknotic nucleus were found in all groups except the MI + Lcn2-MSCs. It is worth mentioning that noticeable remodeling of left ventricle occurred at the infarcted site in the MI, MI + PBS, and V-MSCs groups three weeks post-ischemia, resulting in considerable ECM dissociation, losing cardio myocytes, and progressive narrowing of infarcted cicatrix. However, as is shown by Fig. 3c, thick LV wall was apparently present in the V-MSCs group. Besides, declined left ventricular remodeling was detected in the hearts which received LCN2-MSCs. The Masson's trichrome staining revealed acute fibrosis in the MI (61.44 ± 6.175%), MI + PBS (56.76 ± 11.788%), and even V-MSCs (50.55 ± 15. 515%) groups. However, the fibrosis rate in the Lcn2-MSCs group (19.47 ± 4.888) was significantly lower than the other groups ($P < 0.0001$) (Fig. 3d).

Transplantation of Lcn2-MSCs Reduced the Infarct Size in Left Ventricle

Three weeks after induction of MI, the infarct size of left ventricle was determined by TTC. In Fig. 4a, the white region shows the ischemic area, and the red one represents the normal

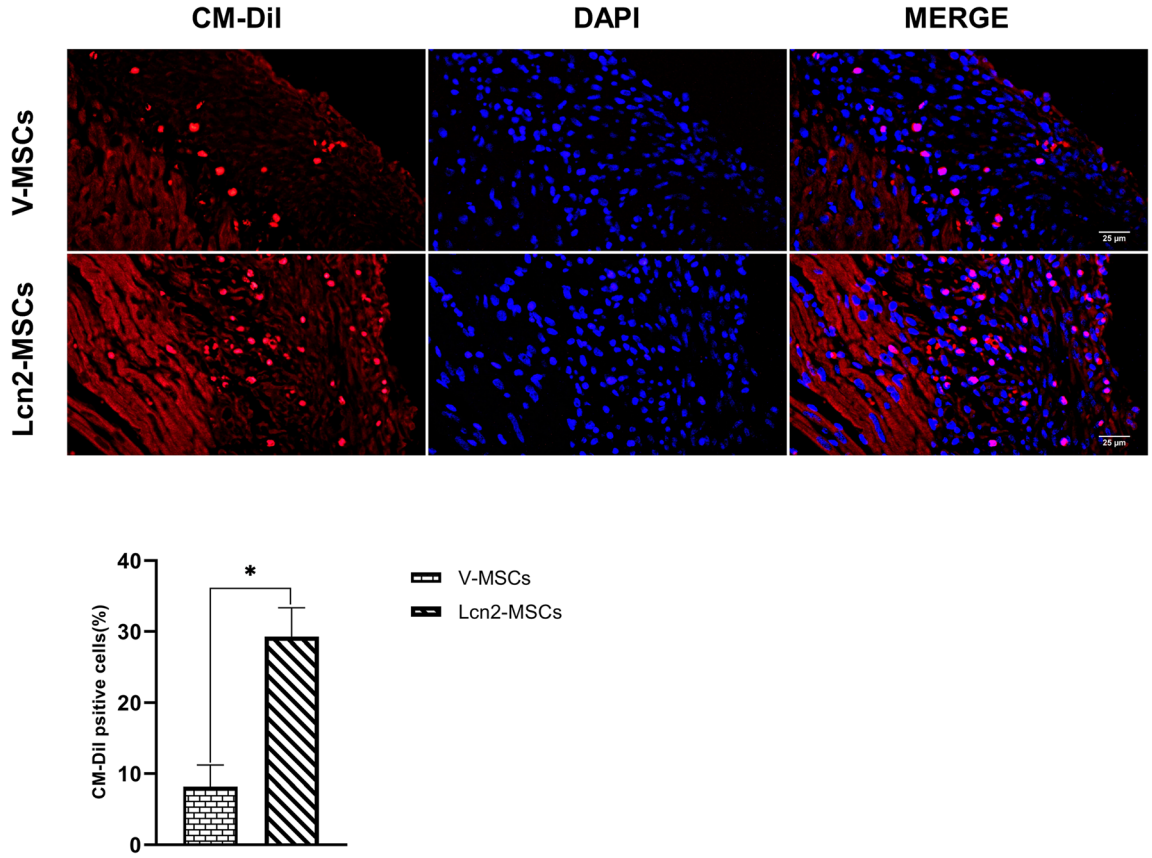

Fig. 2 Survival and homing of transplanted CellTracker™CM-Dil labeled hUC-MSCs in the transplantation site of left ventricle. A) Tracking of CellTracker™CM-Dil labeled V-MSCs and Lcn2-MSCs 7 days after transplantation. The heart tissue was stained with DAPI and the two figures were merged. B) The number of positive cells of labeled V-MSCs and Lcn2-MSCs seven days post transplantation. The number of engrafted cells in the Lcn2-MSCs group was significantly ($p<0.05$) higher compared to the V-MSCs group. Lcn2-MSCs; the hUC-MSCs transfected with pcDNA3.1/CT-GFP-Lcn2 plasmid, V-MSCs; the hUC-MSCs transfected with the empty vectors (Number of replicates = 3, * $P<0.05$)

heart tissue. Our findings disclosed a significant shrinkage ($10.58 \pm 0.975\%$) in the ischemic site of the Lcn2-MSCs group compared to those of MI ($25.31 \pm 2.058\%$), MI + PBS ($24.23 \pm 2.708\%$), and V-MSCs groups ($19.95 \pm 0.101\%$) (Fig. 4b). It must be noted that the shrinkage in the MI + Lcn2-MSCs group was significantly higher ($p < 0.0001$) than the MI-V-MSCs group. In addition, the infarct size was almost similar in both MI and MI + PBS groups ($p > 0.05$).

Transplantation of the Lcn2-MSCs- Decreased Apoptotic Death in Ischemic Area of LV

Apoptosis was detected in heart tissue after ischemia using TUNEL method. As it is shown by Fig. 5a-and b, apoptotic cells were detected in the infarcted tissue of the MI, MI + PBS, and V-MSCs groups 24 h and 1 week after induction of ischemia. It is interesting that transplantation of Lcn2-MSCs reduced apoptosis in the heart tissue (Fig. 5a). The number of apoptotic cells were significantly ($p < 0.0001$) higher in the MI, MI + PBS, and MI + V-MSCs groups compared to the MI + Lcn2-MSCs group 24 h post-ischemia. Interestingly, no reduction in apoptosis was observed in the MSCs group. Apoptosis decreased one week after ischemia in all groups but it was still significantly ($p < 0.0001$) higher in the MI, MI + PBS, and MI + V-MSCs groups compared to MI + Lcn2-MSCs. In addition, at this time point, the number of apoptotic cells in the MI + V-MSCs and MI + PBS groups were also significantly different ($P < 0.05$).

Discussion

Current therapeutic options for MI are limited and, if any, they are based on supportive cures and/or prevention strategies. Therefore, it is essential to develop new and effective therapeutic strategies for MI. Here, in an attempt to enhance outcomes of MSCs-based therapy for MI, we overexpressed Lcn2 in MSCs and evaluated therapeutic potential of the cells in a rat model of the disease. According to our results, Lcn2 overexpression in MSCs enhanced the cells survival following transplantation. Lcn2 has been shown to act as an antioxidant, anti-apoptotic, and anti-inflammatory agent in various

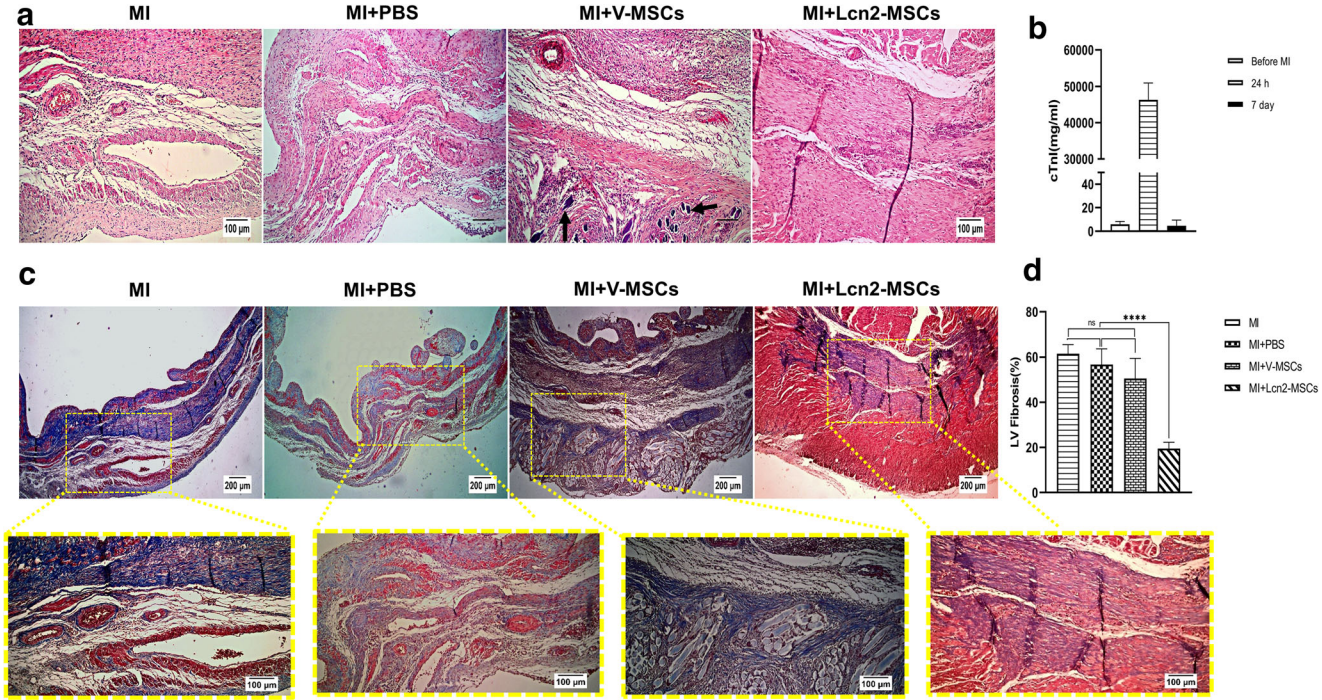

Fig. 3 **a** Photomicrographs of heart tissue stained by H&E. As it is shown by H&E photomicrographs, LV remodeling was less prominent in the Lcn2-MSCs group compared to all other groups. Moreover, although the Aschoff bodies were seen in all groups, but its amount was much lower in the Lcn2-MSCs group (black arrows). **b**) Concentration of cTnI at day zero, 24 h, and 7 days after MI. MI; myocardial infarction, cTnI; cardiac troponin I. (Number of replicate = 4). **c&d**) evaluation of fibrosis in myocardial tissues (blue color) by Masson's trichrome staining. The quantification of fibrosis area shows that the infarct area intended for further expansion in the MI, MI + PBS and MI + V-MSCs groups, while it is evident that the infarct area size was significantly ($p<0.0001$) smaller in the Lcn2-MSCs group. MI: myocardial infarction group in which the LAD was ligated permanently; MI + PBS: the group which received 100 μL PBS after LAD ligation; MI + V-MSCs: the group which received 100 μL of V-MSCs after LAD ligation; MI + Lcn2-MSCs: the group which received 100 μL of Lcn2-MSCs after LAD ligation. (Number of replicates = 4, **** $P<0.0001$)

stress conditions [32]. Therefore, since survival of MSCs is decreased following their exposure to a number of stresses including oxidative stresses and hypoxia, which are well-known stresses during MI, it seems that the overexpressed Lcn2 acts by fortifying the cells to withstand the unpleasant and toxic microenvironments.

It has been known that when MSCs are cultivated outside their own niche, i.e. in vitro cell culture, their capability of homing and engraftment to a damaged tissue decreases mainly due to down regulation of attachment molecules [33, 34]. In fact, one of the main causes of poor cell survival following transplantation of MSCs is probably their decreased adhesion [35–37]. Hence, one of the possible mechanisms of the improved MSCs survival incurred by the overexpression of Lcn2 could be the enhancement of the cells adhesion capability. It is worth mentioning that Lcn2 interacts with some integrins and E-cadherin, and plays an important role in integrin-mediated cell adhesion and signaling [38]. Similar to our findings, Li et al. reported that overexpression of Bcl2 anti-apoptotic gene in bone marrow-derived MSCs (BM-MSCs) increased BrdU positive cells in ischemic heart 4 days, 3 and 6 weeks after transplantation [39]. Furthermore, genetic engineering of BM-MSCs with a Hsp20 coding sequence enhanced post transplantation cell survival to at least two times higher than control cells transfected with an empty vector [40]. Moreover, Zhang et al. overexpressed C1q/tumor necrosis factor-related protein-3 in MSCs and found that the number of viable cells were significantly higher than controls seven days post transplantation [41]. Supporting this notion, we have already found that Lcn2 increases the adhesion capability of MCSs even after their exposure to the stresses [22]. However, the precise mechanism in this regard remains to be investigated in future.

Our results showed that Lcn2-MSCs mitigated Left ventricle (LV) remodeling. Although the precise mechanism underlying this improvement in regenerative potential of the engineered MSCs remains to be investigated, we propose it might be due to paracrine effects of the transplanted cells. Supporting this notion, we previously reported that overexpression of Lcn2 in MSCs results in up regulation of a variety of antioxidants as well as growth factors/cytokines [22]. MSCs secret a number of growth factors/cytokines such as HGF, FGF-2, IGF-1, FGF-4, FGF-9, and TGF-β1. These growth factors/cytokines play anti-inflammatory, anti-apoptotic, angiogenic, and mitogenic roles in damaged tissues [42]. We also reported that overexpression of Lcn2 in MSCs resulted in up regulation of a number of growth factors/cytokines

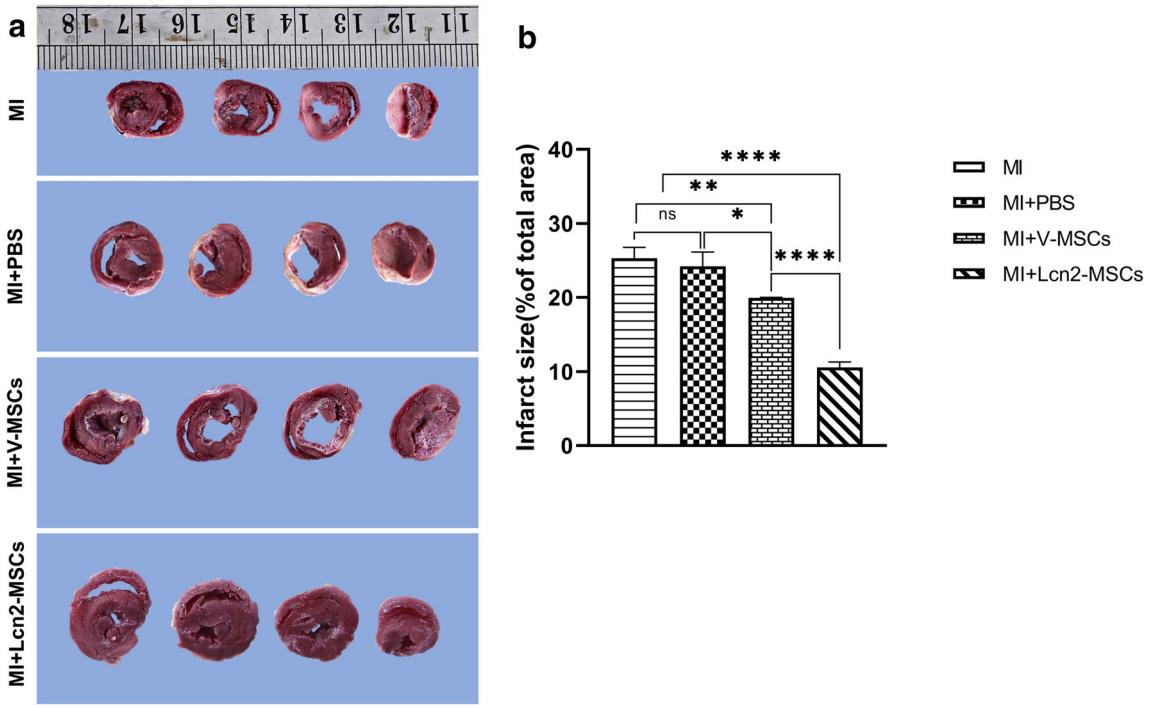

Fig. 4 Evaluation of infract area size in different groups. The measurement of infracted area disclosed that the Lcn2-MSCs group had smaller infracted area than the other groups MI: myocardial infarction group in which the LAD was ligated permanently; MI + PBS: the group which received 100 μL PBS after LAD ligation; MI + V-MSCs: the group which received 100 μL of V-MSCs after LAD ligation; MI + Lcn2-MSCs: the group which received 100 μL of Lcn2-MSCs after LAD ligation. (Number of replicates = 4, *P<0.05, **<0.01, **** P<0.0001).

such as TGFB1, HGF, IGF-1 and FGF-2 after their exposure to hypoxia, H_2O_2, or serum deprivation (SD) stresses [22]. In other words, Lcn2 might act by up regulation of beneficial cytokines/growth factors to alleviate the I/R-induced tissue damages. Recently, we reported that over-expression of Lcn2 in MSCs improves regenerative potential of the MSCs in a rat model of acute kidney injury [26]. In addition, consistent with our previous in vitro study, the Lcn2 overexpression enhanced secretion of a number of critical cytokines/growth factors including IGF-1, HGF, FGF, and VEGF in the AKI rats receiving Lcn2-MSCs [26].

Similar to our work, MSCs were also genetically engineered to express a number of cytoprotective genes in other studies. For example *Park* et al. overexpressed hepatocyte growth factor (HGF) in BM-MSCs and investigated their therapeutic potential following their delivery into infarcted heart. They found beneficial therapeutic effects for the HGF-BM-MSCs as reflected by improvement of left ventricular function, increased angiogenesis, and decreased infarct area scar compared to control groups in a rat model of MI [43]. *Yang* et al. transfected vascular endothelial growth factor (VEGF) expression cassette to mesenchymal stem cells (MSCs) and studied the effect of VEGF-MSCs on heart function restoration and angiogenesis following MI. They also verified these effects by direct transferring of the expression cassette to cardiomyocytes (gene therapy) or by transplantation of non-engineered MSCs (non-engineered-cell therapy). Their findings reveled that infusion of the VEGF-MSCs results in enhanced improvement of heart function compared to either the gene therapy or the non-engineered-cell therapy [44]. *Zhang* et al. over expressed CXCR4 in BM-MSCs using adenovirus expression system and transplanted the engineered cells to a rat model of MI. They found that overexpression of CXCR4 increases in vivo mobilization and engraftment of MSCs into ischemic area. Furthermore, the CXCR4-BM-MSCs mitigated early signs of left ventricular remodeling and enhanced neomyoangiogenesis [45]. It has been shown that panoptic cell death has been implicated [46] and considerably contributes to myocyte death in AMI [47]. Hence, in order to determine whether Lcn2-MSCs could reduce apoptosis in the infarcted tissue, apoptotic cell death was investigated. Our results **showed** decreased apoptotic cell death in cardiomyocytes following transplantation of the Lcn2-MSCs. We already reported that Lcn2 itself can also exert anti-apoptotic and proliferative functions following exposure of cells to oxidative stress [48]. Gnecchi et al. reported that overexpression of Akt1 in BM-MSCs followed by transplantation of the cells into rat myocardium reduced infarct size, prevented cardiac remodeling, restored performance of the infarcted hearts, and reduced apoptotic cell death [49]. However, in contrast to our findings, Xu et al. showed that recombinant Lcn2 induced apoptosis in both H9c2 cells and

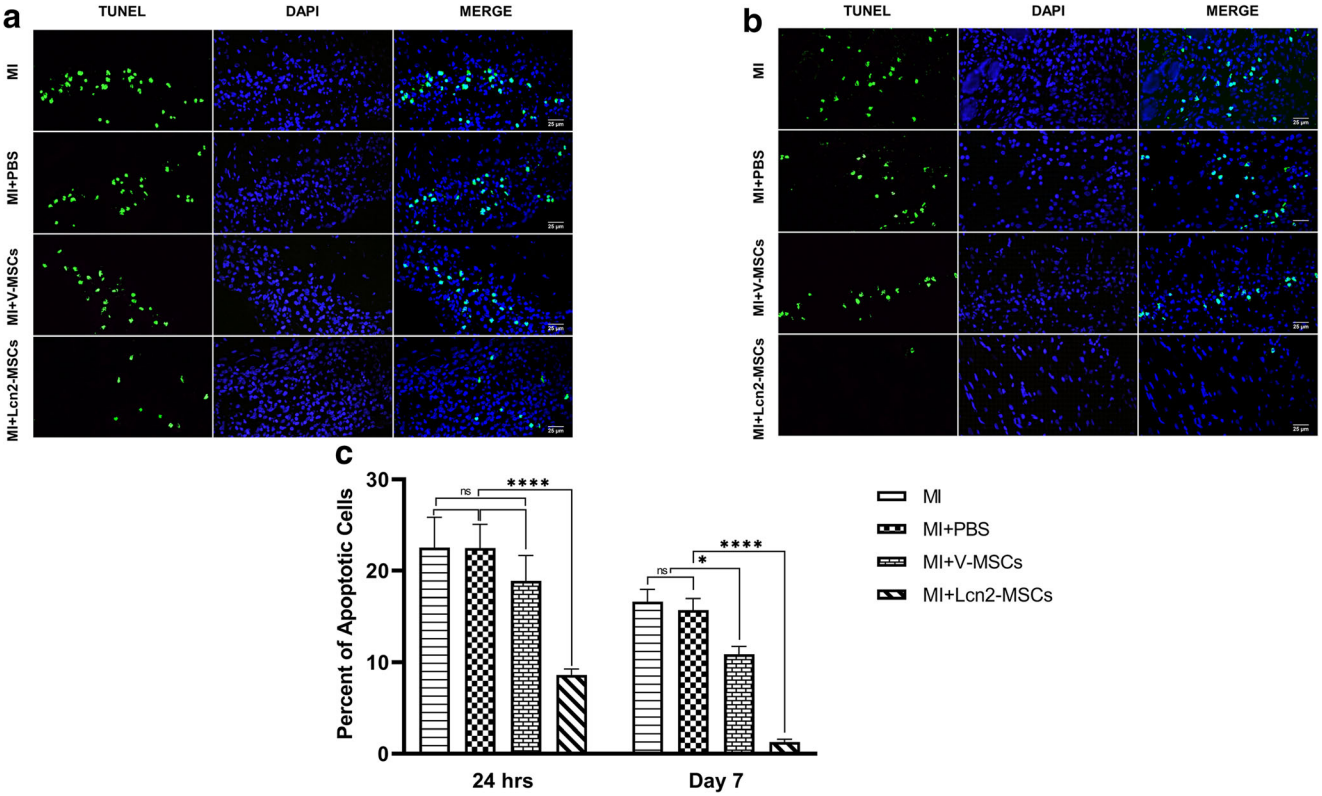

Fig. 5 Assessment of apoptotic cells at 24 h and 7th days post transplantation in different groups. (**a**) Apoptotic cells 24 h after ischemia. The cardiomyocytes were first stained by TUNEL reaction and then by DAPI. The TUNEL and DAPI images were merged. (**b**) Apoptotic cells in the heart tissues 7 days after ischemia. The cardiomyocytes were first stained by TUNEL reaction and then by DAPI. The TUNEL and DAPI images were merged. (**c**) The number of apoptotic cells in different groups. MI: myocardial infarction group in which the LAD was ligated permanently; MI + PBS: the group which received 100 μL PBS after LAD ligation; MI + V-MSCs: the group which received 100 μL of V-MSCs after LAD ligation; MI + Lcn2-MSCs: the group which received 100 μL of Lcn2-MSCs after LAD ligation (Number of replicates = 3, *P<0.05, **** P<0.0001)

primary neonatal rat cardiomyocyte by elevation of intracellular iron levels and contributed to cardiac remodeling [50]. It is worth mentioning that Lcn2 expression is increased following MI, in atherosclerotic plaques, and in coronary artery disease. In fact, Lcn2 is a well-known biomarker of cardiovascular diseases [51, 52]. Although the precise role of Lcn2 overexpression following MI is not well understood, the induction might be a compensatory reaction to re-establish homeostasis. Lcn2 is also one of the well-known biomarkers of AKI [53]. It is considered an early stress response of kidney to acute injuries [54] and dramatic induction of Lcn2 has been reported after kidney damages including acute kidney injury [51, 55]. Interestingly, it has been shown that administration of recombinant Lcn2 mitigates renal damage after AKI in a mouse model, which highlights the compensatory effect of Lcn2 to re-establish homeostasis [56]. In addition, its renoprotective effects showed to be mediated by regulation of several autophagy and apoptosis-related genes [57]. Consistent with the current study, we previously reported that Lcn2 improved MSCs-based cell therapy in a rat model of AKI which confirms the cytoprotective properties of Lcn2 [26]. One of the different methods applied for prevention of AKI secondary to certain procedures and/conditions is remote ischemic preconditioning (RIPC). RIPC includes transient episodes of ischemia induction at a remote site of body before exposure to the procedure/condition. It reduces the ischemia and reperfusion injury of target organs by stimulating endogenous protection [58, 59]. Protective effects of RIPC have been reported on heart as well as kidney, brain, liver, skeletal muscle, small intestine and other organs [60]. Although molecular mechanisms underlying RIPC protective effects remain to be investigated, but as reflected by downregulation of Lcn2, a reduction of subclinical renal damage by RIPC, especially in the early stage of injury, has been reported [59]. This implies the protective effect of up-regulated Lcn2 which subsequently is declined to basal level upon improvement of renal function.

Conclusion

In order to improve the efficacy of MSC-based therapy for MI, we overexpressed Lcn2 in MSCs. Our findings suggested that overexpression of Lcn2 in MSCs could be a novel potential enhancement in this therapeutic strategy of AMI. However,

further investigations are required such as determination of the potential mechanisms underlying the protective effects of Lcn2-MSCs. Moreover, evaluation of heart function by echocardiography must be also performed following transplantation of the cells. Finally, safety and efficacy of the method must be evaluated in larger animals and clinical trials.

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Compliance with Ethical Standards

All experimental procedures were approved by research ethics committee of National Institute for Medical Research Development (IR. NIMAD. REC.1397.474).

Conflict of Interest The authors declare that there is no conflict of interest.

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