Enhanced infarct myocardium repair mediated by thermosensitive copolymer hydrogel-based stem cell transplantation

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Abstract

Mesenchymal stem cell (MSC) transplantation by intramyocardial injection has been proposed as a promising therapy strategy for cardiac repair after myocardium infarction. However, low retention and survival of grafted MSCs hinder its further application. In this study, copolymer with N-isopropylacrylamide/acrylic acid/2-hydroxyethyl methacrylate-poly(ϵ -caprolactone) ratio of 88:9.6:2.4 was bioconjugated with type I collagen to construct a novel injectable thermosensitive hydrogel. The injectable and biocompatible hydrogel-mediated MSC transplantation could enhance the grafted cell survival in the myocardium, which contributed to the increased neovascularization, decreased interstitial fibrosis, and ultimately improved heart function to a significantly greater degree than regular MSC transplantation. We suggest that this novel hydrogel has the potential for future stem cell transplantation.

Keywords: Copolymer, hydrogel, mesenchymal stem cells, myocardial infarction, cardiac repair

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Introduction

Bone marrow mesenchymal stem cell (MSC) transplantation has been proposed as one of the potential approaches to treat ischemic heart diseases.¹ Previous studies suggested that intramyocardial injection of stem cells allows targeted cell delivery into the areas of interest, which has been proved to be more advantageous for cardiac repair over intravenous or intracoronary delivery route.^{2,3} However, it is demonstrated that only less than 10% of transplanted cells stayed *in situ* a few hours after intramyocardial injection.⁴ The low cell retention rate and the massive cell redistribution into other organs may lead to the limited efficacy of the stem cell transplantation and hinder its clinical application for infarct myocardium repair.^{5,6} Hence, the approach of intramyocardial injection still needs to be optimized.

Injectable hydrogel has been developed as a novel and effective approach to deliver stem cells for its tissue-like mechanical property, remarkable biocompatibility, and, most important, injectability.⁷ So far, both natural macromolecules and synthetic polymers have been utilized to produce injectable hydrogels.^{8–11} As one of the most widely

used injectable hydrogels, poly-N-isopropylacrylamide (PNIPAAm) is a thermosensitive intelligent material which undergoes a reversible sol-gel transition around 32°C in an aqueous condition, converting from flowable solution to hydrogel when heated above its lower critical solution temperature (LCST).¹² However, PNIPAAm still needs to be optimized to improve the thermosensitive property and biocompatibility for clinical application. Recently, we copolymerized N-isopropylacrylamide (NIPAAm), acrylic acid (AAc), and macromer 2-hydroxyethyl methacrylate-poly(ϵ -caprolactone) (HEMA-PCL) to synthesize poly(NIPAAm-co-HEMA-co-HEMA-PCL) copolymer. And type I collagen was then bioconjugated with the copolymer to enhance the biocompatibility further. We found that the copolymer with NIPAAm/AAc/HEMA-PCL ratio of 88:9.6:2.4 exhibited excellent biological intelligence and low cytotoxicity *in vitro*.¹³

In the present study, we utilized the novel hydrogel to deliver MSCs to infarct myocardium. We hypothesized that the hydrogel can enhance cell retention, decrease cell redistribution to other organs, and, therefore, improve the cardiac repair.

Materials and methods

Preparation of MSCs

All animal experiments were approved by Institutional Review Board and Institutional Animal Care and Use Committee Protocols of Fudan University. The syngenic mouse strain C57/BL6 weighing 20–25 g was used to avoid rejection.

Bone marrow was flushed from tibias and femurs of male C57/BL6. Whole marrow cells were cultured in MesenCult basal medium that was supplemented with MSCs stimulatory supplements (Cyagen Biosciences Inc., CA, USA). The non-adherent cells were removed by a medium change at 36 and 72 h. After the cells grow to fourth passages, the purity of the cells was detected by immunostaining and flow cytometry. The cells were incubated with 1 μ L monoclonal fluorescein isothiocyanate (FITC)-conjugated antibodies against CD34, CD45, CD44, and CD105 (BD, CA, USA), then, analyzed using flow cytometer (FACS, AriaII, BD, NJ, USA) and double immunofluorescent labeling techniques combined with confocal laser scanning microscope (Olympus, Tokyo, Japan).

To track the grafted cells, MSCs were transfected with lentiviral vector carrying ubiquitin promoter driving firefly luciferase (Fluc), and then labeled with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) before transplantation.

Synthesis of poly(NIPAAm-co-HEMA-co-HEMAPCL) copolymer

The synthesis of poly(NIPAAm-co-HEMA-co-HEMAPCL) copolymer was carried out by free radical polymerization of NIPAAm, AAc, and HEMAPCL with a feed ratio of 88:9.6:2.4 (NIPAAm/AAc/HEMAPCL). And the copolymer was then used to conjugate type I collagen by 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)/N-Hydroxysuccinimide (NHS) crosslinking.¹³

Morphology of dehydrated poly(NIPAAm-co-AAc-co-HEMAPCL)-type I collagen was observed by utilizing scanning electron microscopy (SEM). The dehydrated hydrogel was immersed into liquid nitrogen and lyophilized at -50°C . Then, the gel was gold-coated and viewed using a JSM-6330F SEM (JEOL, Peabody, MA, USA) operated at 10 kV accelerating. Storage modulus and viscosity were determined from 20°C to 40°C . And water content and remaining of the hydrogel were tested from day 0 to day 14.¹³

Myocardial infarction model and cell transplantation

Myocardial infarction was created in adult female C57/BL6 mice by ligating the left coronary artery.¹⁴ The copolymer was dissolved in phosphate-buffered saline buffer solution with the concentration of 15 wt% at 4°C before injection. Ten minutes after myocardial infarction, saline (group 1, $n=8$), hydrogel solution (group 2, $n=8$), 1×10^6 MSCs (group 3, $n=8$), or 1×10^6 MSCs mixed with hydrogel solution (group 4, $n=8$), were injected into the peri-infarct region.

Optical bioluminescence imaging of survival MSCs in live mice

Bioluminescence imaging (BLI) was performed using the IVIS200 (Xenogen, CA, USA) System to evaluate the survival MSCs in live animals. Recipient mice were anesthetized with isoflurane, shaved, and placed in the imaging chamber. Animals were then scanned for bioluminescence spot at 30 min after intraperitoneal injection of the reporter probe D-luciferin (300 mg/kg body weight). The experiment was performed repetitively at 2 h, 1 day (1 d), and 28 days (28 d). Bioluminescence was quantified in units of maximum photons per second per centimeter squared per steradian ($\text{P}\cdot\text{s}^{-1}\cdot\text{cm}^{-2}\cdot\text{sr}^{-1}$) from a fixed region of interest.

Heart function assessment

Heart function was assessed by transthoracic two-dimensional echocardiographic analysis using an echocardiograph (Vevo770, VisualSonics, Toronto, Canada) immediately after infarction model (Baseline) and 28 d after cell transplantation. Parasternal long-axis views were obtained to view the regional wall-motion abnormalities. Short-axis M-mode views were obtained perpendicular to the midventricular level, with sweep speeds of 200 mm/s confirmed by means of two-dimensional echocardiography. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were computed as: $\text{LVEF} = 1 - (\text{LVIDes}/\text{LVIDed})^2$ and $\text{LVFS} = 1 - (\text{LVIDes}/\text{LVIDed})$.

Histology

After heart function assessment, hearts were sectioned into five segments parallel to the apex-base axis. DAPI-labeled MSCs were observed directly. And interstitial fibrosis was evaluated by Masson trichrome staining. And the peri-infarct capillary densities were measured by staining for PECAM-1 (Santa Cruz Biotechnology) on the DAPI-positive slides.

Statistical analysis

The data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). All values were expressed as the mean $\pm$ standard error of the mean. One-way analysis of variance with the *post hoc* Bonferroni test was performed to assess the significant difference among multiple groups. The significant difference between two groups was evaluated using the Student's *t*-test.

Results

Characterizations of isolated MSCs

After four passages of MSCs, flow cytometry and immunostaining results showed absence of CD34, CD45, indicating depletion of hematopoietic cells within the MSC. Moreover, 97.70% and 94.92% of these MSCs expressed the markers CD44 and CD105, respectively, when gated for control at 1% by means of flow cytometric analysis (Figure 1(a) and (b)).

Figure 1 Characterizations of the isolated MSCs. (a) Flowcytometry results of the surface markers of MSCs. (b) Immunostaining of the surface markers of MSCs. (A color version of this figure is available in the online journal.)

The efficiency of DAPI and Fluclabeling was 100% and 89.5%, respectively. Trypan blue dye exclusion showed greater than 95% viability of the cell preparations before transplantation.

Characterizations of poly(NIPAAm-co-HEMA-co-HEMAPCL) copolymer-type I collagen

The interior morphology of the PNIPAAm (Figure 2(a)) and hydrogel (Figure 2(b)) was observed under SEM. The storage modulus and viscosity from 20°C to 40°C were shown in Figure 2(c) and (d). And the water content and

remaining from day 0 to day 14 were shown in Figure 2(e) and (f).

The hydrogel was flowable solution below 27°C and converted to hydrogel with relatively high tensile strength when it was heated to 37°C (Figure 3).

Survival MSCs *in vivo*

In the myocardium, group 4 demonstrated significantly higher optical BLI signals than group 3 [2h: $(7.07 \pm 0.96) \times 10^6$ versus $(1.82 \pm 0.44) \times 10^6$ P s⁻¹ cm⁻² sr⁻¹; 1d: $(2.82 \pm 0.149) \times 10^6$ versus $(8.29 \pm 1.46) \times 10^5$ P s⁻¹ cm⁻² sr⁻¹;

Figure 2 Characterizations of the novel hydrogel. (a) The interior morphology of the PNIPAAm. (b) The interior morphology of the novel hydrogel. (c) Storage modulus from 20°C to 40°C. (d) Viscosity from 20°C to 40°C. (e) Water content from day 0 to day 14. (f) Water remaining from day 0 to day 14

28 d: $(1.13 \pm 0.21) \times 10^6$ versus $0 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$] ($P < 0.01$) (Figure 4(a) and (c)). DAPI-labeled MSCs in group 4 were observed in myocardium at 2 h, 1 d, and 28 d (Figure 4(b)).

In the outside of the myocardium, higher signals [2h: $(3.59 \pm 0.46) \times 10^6 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$; 1 d: $(1.93 \pm 0.50) \times 10^6 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$; 28 d: $(1.30 \pm 0.72) \times 10^5 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$] were found in group 3 as compared to the signals in group 4 [2h: $0 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$; 1 d: $(5.38 \pm 0.11) \times 10^4$; 28 d: $(6.30 \pm 0.25) \times 10^4 \text{ P s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$] ($P < 0.05$ for 2 h and 1 d; $P > 0.05$ for 28 d) (Figure 4(a) and (c)).

Interstitial fibrosis and angiogenesis

At 28 d after infarction, the capillary density based on PECAM-1 immunostaining in group 4 (78.65 ± 5.00 per field) was higher than those in other groups (group 1: 21.88 ± 1.89 per field; group 2: 25.46 ± 4.52 per field) ($P < 0.01$) (group 3: 53.33 ± 5.77 per field) ($P < 0.05$) (Figure 5(a) and (b)). The collagen volume fraction based on Masson trichrome staining in group 4 showed significantly less than other groups (group 1: $82.99 \pm 4.55\%$; group 2: $78.92 \pm 5.87\%$) ($P < 0.01$) (group 3: $53.22 \pm 6.23\%$) ($P < 0.05$) (Figure 5(a) and (c)).

Heart function

There was no obvious difference in baseline heart performance (LVFS and LVEF) among the different groups ($P > 0.05$). LVFS and LVEF in group 3 ($17.48 \pm 0.91\%$, $36.83 \pm 1.68\%$) improved significantly compared with those in group 1 ($10.59 \pm 0.51\%$, $23.22 \pm 1.10\%$, $P < 0.01$) and group 2 ($11.60 \pm 0.70\%$, $25.34 \pm 1.41\%$, $P < 0.01$). Furthermore, LVEF and LVFS in group 4 ($27.55 \pm 0.88\%$, $53.81 \pm 1.24\%$) showed greater enhancement than those in group 3 ($P < 0.01$) (Figure 5(a) and (b)).

Discussion

As a widely used transplantation strategy, intramyocardial injection of stem cells needs to be optimized. In our present

study, we constructed a novel thermoresponsive intelligent hydrogel by poly(NIPAAm-co-AAc-co-HEMA PCL) copolymer and type I collagen. The novel hydrogel was then used to deliver MSCs to mice ischemic myocardium and promoted cardiac repair successfully.

An ideal hydrogel for stem cell transplantation should be injectable with an intelligent thermosensitive property.¹⁵ Our novel hydrogel was composed of poly(NIPAAm-co-AAc-co-HEMA PCL) copolymer and type I collagen. To be specific, NIPAAm provided the thermal sensitivity of the hydrogel, HEMA PCL served as biodegradable pieces, and AAc was used to enhance the hydrophilicity and adjust the LCST.^{13,16} In our previous work, we constructed a series of thermosensitive copolymers, which were prepared by varying the molar ratio (R ratio) of the hydrophilic monomer AAc and the hydrophobic macromer HEMA PCL. We found that the copolymer with NIPAAm/AAc/HEMA PCL ratio of 88:9.6:2.4 was considered as the best candidate for injectable properties.¹³ In the present study, poly(NIPAAm-co-AAc-co-HEMA PCL) exhibited satisfied biological intelligence property. It was flowable solution at room temperature and converted to hydrogel with relatively high tensile strength when it was heated to 37°C . Hence, we suppose that the properties could be suitable for stem cell transplantation.

Low cytotoxicity is very important for the clinical application of biomaterials. Collagen, as the dominating composition of extracellular matrix, could offer a good adhesive surface that promotes cellular attachment, proliferation, and expansion. However, highly hydrated collagen gels exhibit poor structural integrity, which is insufficient for tissue replacement.^{17,18} In this present work, we conjugated type I collagen with copolymer of poly(NIPAAm-co-AAc-co-HEMA PCL). And it is demonstrated that the bioconjugation showed improved rigidity according to the storage modulus and viscosity, which may be more suitable for myocardium replacement. In our previous study, it is suggested that the bioconjugation did not change the injectable property of copolymer. Furthermore, this copolymer

Figure 3 The *in situ* thermosensitive gelation of the novel hydrogel. (A color version of this figure is available in the online journal.)

Figure 4 The retention and redistribution of grafted MSCs. (a) BLI signals in live animals at 2 h, 1 d, and 28 d. (b) DAPI-labeled grafted MSCs in myocardium of group 4. Scale bar = 50 μm . (c) BLI signals of retention at 2 h, 1 d, and 28 d. (d) BLI signals of redistribution at 2 h, 1 d, and 28 d. (* versus group 4, $P < 0.01$; # versus group 4, $P > 0.05$). (A color version of this figure is available in the online journal.)

collagen-based hydrogel exhibited extremely low cytotoxicity for MSCs.¹³ Therefore, this tissue-like hydrogel had good biocompatibility for stem cell transplantation in myocardium.

Improved stem cell retention and survival benefit for the cardiac repair. Some evidences suggest that intramyocardial injection allows targeted cell delivery into the areas of interest, but retention of transplanted stem cells *in situ* can be poor and variable.⁴ After injection, cells could be lost and redistributed through the needle tracks, lymphatic vessels, venous vessels, or endocardium, with the retention ratio of 10% to 15% in the myocardium.^{19,20} In this research, we used BLI, a novel and reliable tool to trace transplanted MSCs in live animals.²¹ In this study, BLI signals at 2 h, 1 d, and 28 d after MSC transplantation in group 4 were significantly higher than those in group 3. This

indicated an improved MSCs retention in myocardium in group 4. Moreover, notable extracardiac signals at liver, spleen, and lung were found in group 3 comparing with group 4. The results also confirmed that the novel hydrogel could diminish redistribution and improve the cell retention *in situ*. Interestingly, we found highest MSCs distribution in liver than in other organs. The mechanism may be attributed to that the liver expresses high levels of chemo-attractant agents, such as stromal derived factor-1.²² Collectively, this novel hydrogel could effectively keep grafted cells in the targeted site. Furthermore, the number of transplanted cell is a serious concern for stem cell therapy. In previous studies on rodent, number of 1×10^6 was a commonly used cell dosage.^{14,23,24} In the clinical trials, however, the number of grafted cells ranged from 2.9×10^6 to 6.59×10^8 .²⁵ Though the optimized

Figure 5 The angiogenesis and interstitial fibrosis. (a) The PECAM-1-based angiogenesis (arrow) and Masson trichrome staining-based interstitial fibrosis. Scale bar = 50 μ m. (b) Capillary density of four groups. (c) Collagen volume fraction of four groups. (* versus group 4, $P < 0.01$; ** versus group 4, $P < 0.05$). (A color version of this figure is available in the online journal.)

Figure 6 Heart function assessment. (a) Left ventricular fractional shortening. (b) Left ventricular ejection fraction. (* versus group 4, $P < 0.01$)

number of grafted cells has not been determined yet, we assumed that the hydrogel could load more than 1×10^6 of cells to meet the clinical requirement. However, the maximum number of grafted cells that could be maintained in good shape in the hydrogel still needs to be investigated in future.

MSC transplantation promotes cardiac repair and improves heart function after myocardial infarction through some mechanisms, such as paracrine effect.²⁶ In the present study, hydrogel-mediated enhancement of cell retention in group 4 may contribute importantly to the

improved paracrine effect from the MSCs. As a result, capillary density increased significantly in the peri-infarct region. Moreover, interstitial fibrosis decreased obviously in left ventricle, which could inhibit left ventricle remodeling process. These beneficial cardiac repair eventually improved heart function in group 4. Therefore, we believed that the novel hydrogel-based MSC transplantation could play an important role in cardiac repair. Although hydrogel provided the excellent retention platform for MSCs, it could not ameliorate the harsh microenvironment in ischemic myocardium, which may hinder the long-term survival of

transplanted MSCs. Hence, the novel hydrogel loading with oxygen- or growth factor-releasing system is being pursued in the subsequent research to improve the MSC microenvironment.

Conclusion

We developed poly(NIPAAm-co-AAc-co-HEMA-PCL)-type I collagen as a thermosensitive and biocompatible hydrogel for MSC transplantation. The hydrogel-mediated MSC transplantation could improve grafted stem cell retention and survival, increase angiogenesis, inhibit left ventricle remodeling, and improve heart function. We believe that this clinically relevant and combined strategy could be of importance for treating patients with myocardial infarction in the future.

Authors' contributions: CG and CW designed the study; YX and KZ conducted *in vitro* experiments; YX, KZ, and HL conducted *in vivo* experiments; ML, YX, and SL conducted the synthesis of hydrogel; and YX and KZ wrote the manuscript. YX and KZ are co-first authors and contributed equally to this work.

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