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A PNIPAAm-based thermosensitive hydrogel containing SWCNTs for stem cell transplantation in myocardial repair

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ABSTRACT

Poly (N-isopropylacrylamide) (PNIPAAm) hydrogel was a widely used carrier in therapeutic agent delivery. However, its bioactivities for encapsulated cells were not satisfactory. In the study, we aimed to determine whether modification with single-wall carbon nanotubes (SWCNTs) could improve the bioactivitis, especially supportive adhesion of PNIPAAm to encapsulated cells and favor their efficacy in myocardial repair. A thermosensitive SWCNTs-modified PNIPAAm hydrogel (PNIPAAm/SWCNTs) were prepared by incorporating the SWCNTs into base PNIPAAm hydrogel. The bioactivities of the resulted hydrogel to brown adipose-derived stem cells (BASCs) were evaluated and compared with the base PNIPAAm hydrpgel *in vitro*. Then, the PNIPAAm-containing hydrogel was used as carrier for imtromyocardial delivery of BASCs in rats with myocardial infarction. The efficacy of PNIPAAm/SWCNTs hydrogel in stem cell-based myocardial repair was systematically evaluated. In vitro study showed that the PNIPAAm/SWCNTs hydrogel demonstrated significantly higher bioactivities to encapsulated BASCs compared with onefold PNIPAAm hydrogel, including promoting cell adhesion and proliferation. When used as carrier for intramyocardial delivery of BASCs after myocardial infarction, the PNIPAAm/SWCNTs hydrogel significantly enhanced the engraftment of seeding cells in infarct myocardium and augmented their therapeutic efficacies in myocardial infarction (MI). The data provided a supportive evidence for the myocardial application of the SWCNTs-modified hydrogel and offered a new perspective in development or improvement of cardiac tissue engineering scaffold.

1. Introduction

Acute myocardial infarction occurs upon sudden blockage of coronary artery, leading to the death of cardiomyocytes. After MI, lost myocardium will be replaced by extracellular matrix, which would result in the formation of scar tissues and may finally lead to heart failure [1,2]. In the past decades, tissue engineering strategy for myocardial repair or replacement has emerged as a novel therapy and attracted extensive interest due to its promising perspective in clinic [1].

Injectable cardiac tissue engineering involved the application of stem cells and injectable biomaterials in myocardial repair [2]. In previous studies, several stem cells have been used and encouraging results have been achieved in the field, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and various adult stem cells [3–6]. It is relatively easy for electrical couple formation between ESCs/iPSCs-derived cardiomyocytes and host myocardium, but the tumorigenicity and immunogenicity are still the main challenges hampering their clinical application. In comparison, adult stem cells, such as bone marrow mesenchymal

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stem cells (MSCs), adipose-derived MSCs and cardiac stem cells, are more suitable for clinical application. However, MSCs, including bone marrow and adipose source, are limited in cardiomyogenic differentiation, while cardiac stem cells are of clinical unavailability. In recent years, BASCs have been found as novel adult stem cells and were demonstrated to possess a high cardiomyogenic potential [7,8]. Furthermore, they have a similar paracrine capacity as MSCs. Independent groups have investigated the feasibility and efficacy of the cells for myocardial repair and found that transplanted BASCs could effectively engraft into the host myocardium and differentiated into functional cardiomyocytes [7,8]. More importantly, infarct size and heart function were significantly improved, which was even superior to MSCs [7]. Accordingly, BASCs were termed as novel promising seeding cells in cardiac tissue engineering and needed further investigation.

Scaffold is another element in cardiac tissue engineering [9]. It has been demonstrated that suitable scaffolds could improve the myocardial microenvironment, enhance the survival of transplanted stem cells and regulate stem cell fate in *vivo*, all of which contributed to myocardial repair. Many biomaterials, including fibrin [10,11], collagen [12,13], Matrigel [14] and chitosan hydrogel [15], have been successfully applied in injectable cardiac tissue engineering in the past years. Among these reported scaffolds, PNIPAAm hydrogel was an attractive one that has been extensively studied. It was fluid below 32 °C and could gelate rapidly *in situ* when injected *in vivo*, favorable for cell engraftment. However, drawbacks were also observed with PNIPAAm hydrogel that its bioactivity was not so satisfactory for regulating the therapeutic efficacy of delivered stem cells, such as low capacities to support adhesion and proliferation of encapsulated cells [16].

CNTs is one of widely investigated nanomaterials that have good electrical conductivity and suitable mechanical propeities. It has been demonstrated that modification with CNTs could significantly improve the specific biofunction of original materials [17–19], which was appealing in tissue engineering. As is known, hostile factors generated after MI, especially reactive oxygen species (ROS), would severely impair the adhesion of engrafted cells, leading to their anoikis. Therefore, it was considered that adhesion on surrounding matrix is a key step for engrafted stem cells in ischemic myocardium [20]. On this basis, the study was designed to determine whether modification with SWCNTs would improve the bioactivities, especially supportive adhesion of PNIPAAm hydrogel to BASCs, and further favor their efficacy in myocardial repair.

2. Materials and methods

All Sprague–Dawley (SD) rats were purchased from the Experimental Animal Center, Academy of Military Medical Science (Beijing, China). All experiments in the study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Military Medical Science, Beijing, China.

2.1. Preparation and characterization of SWCNTs-modified PNIPAAm hydrogel

First, 0.9 mmol polyethylene glycol diacrylate (M_w = 700), 0.4 mmol 1-(2-aminoethyl) piperazine and 0.08 mmol dodecylamine were added into 4 mL N, N-dimethylformamide. The mixture was incubated at 50 °Cfor 72 h with gentle agitation. During the process, Michael reactions occurred among the substances and after that, the solvents were produced (molecular weight higher than 1000). Then, the products were dialyzed for 72 h with PBS at 4 °C in darkness (molecular weight cutoff: 1kD) [21,22]. After being lyophilized in vacuo, 30 mg/mL solution was prepared in ddH_2O and high purity SWCNTs were added at the concentration of 0.26 mg/mL (less than 2 nm in diameter and 5–30 μm in length). The mixed solution was sonicated for 10 × 1 min in ice bath to uniformly disperse SWCNTs (SWCNTs were dispersed in water as control). Transmission electron microscopy (TEM, HITACHI, H-7650) observation was performed to determine the dispersion of SWCNTs in the solution. Then, dropping the above solution (1 mL) into 10 mL N-isopropylacrylamide aqueous (80 mg/mL in H_2O) and then 200 μL ammonium persulfate (200 mg/mL) and 20 μL N, N, N′, N′-tetramethylethylenediamine were added at room temperature to form hydrogel. The hydrogel was then placed in ice water to liquefy and followed by gelation at 37 °C. The above procedure was repeated for 3 times. After that, the mixture was dialyzed in ddH_2O for 72 h to eliminate the unbound components and lyophilized in vacuo for 48 h to acquire solid product. SWCNTs-modified PNIPAAm hydrogel (PNIPAAm/SWCNTs) were prepared by dissolving the above product into 10 mL H_2O. The surface mophology and the electrical resistance of the hydrogel were detected by scanning electron microscope (SEM, HITACHI, S-3400N) and an electrochemistry workstation (CHI660D), respectively. The control PNIPAAm underwent the same procedure except that SWCNTs were not added. All reagents were purchased from Sigma except for SWCNTs (timesnano, China).

2.2. Isolation, cultivation and characterization of rat BASCs

2.2.1. Cell isolation and cultivation

Interscapular brown adipose tissue was isolated from young SD rats (male, 80–100 g) and washed by PBS. Then, the tissues were cut into 1 mm × 1 mm size and digested with cocktail enzymes consisting of 0.1% collagenase IV (Sigma, USA)/0.1% dispase II (Rotch, Switzerland)/0.05% trypsin (Gibco, USA) for 45 min as previously described [23]. 2 × 10^6 cells were seeded onto 100 mm-diameter dishes and incubated under standard culture conditions (37 °C, 5% CO_2, 95% humidity). The medium was replaced every other day. Cells were observed everyday under inverted phase-contrast microscope (IX70 inverted system microscopy, Olympus Optical, Melville, NY).

2.2.2. Cell characterization

For immunophenotype characterization, freshly isolated cells were resuspended in PBS. Erythrocyte lysis buffer was added to remove red blood cells. Cell aliquots (1 × 10^6 cells in 100uL cell staining Buffer) were put into 1.5 mL Eppendoff tube. Then, anti- CD90, CD45, CD29, CD34 and CD133 antibodies were used according to the manufactuer's guideline and the previous report [23]. Cardiomyogenic differentiation and characterization of BASCs were performed as previously described [23].

2.3. In vitro assay of bioactivities of PNIPAAm/SWCNTs to BASCs under normal and ROS conditions

The dishes were coated with PNIPAAm hydrogel or PNIPAAm/SWCNTs hydrogel.

For the cell adhesion and spreading assay, BASCs were seeded at 3500 cells/cm^2 in α-MEM (10% FBS) with or without 30uM H_2O_2 (exogenous ROS). After 1 h incubation at 37 °C, 5% CO_2, non-adhered cells were removed by slightly washing with fresh medium, the adhered cells were counted under microscope and cell adhesion was expressed as (adhered cells on matrix/adhered cells on PNIPAAm) × 100% [20]. After 4 h' incubation, the cells were fixed with 3% formaldehyde and stained with 0.2% Coomassie Blue as the previous report [20]. Then, the cell spreading was observed under a phase-contrast microscope.

For the cell proliferation assay, 3000 cells/cm^2 BASCs were seeded on the above coated dishes and were cultured for 7 days. MTT (Sigma, USA) assay was performed every two days to determine the cell proliferation.

2.4. Animal models and transplantation surgery

Adult Male SD rats (250 ± 20 g) were anesthetized by intraabdominal injection with 2% sodium pentobarbital (30 mg/kg). Then animals were ventilated by a volume-regulated respirator and a left lateral thoracotomy was performed. The left coronary artery was identified and ligated with a 6-0 prolene suture as previously described [24]. Then, the rats were randomly divided into 4 groups: PBS group (n = 6) receiving 100uL PBS injection at three different sites using a 28-gauge needle; PNIPAAm/SWCNTs group (n = 6) receiving 100uL PNIPAAm/SWCNTs; PBS/BASCs group (n = 6) receiving 2 × 10^6 cells injection in 100uL PBS; and PNIPAAm/SWCNTs/BASCs group (n = 6) receiving 2 × 10^6 cells injection in 100uL PNIPAAm/SWCNTs hydrogel. For *in vivo* tracking, BASCs were labeled using DiI (Gibco, USA) pretransplantation.

2.5. LV function assays

4 weeks after surgery, rats were anesthetized and echocardiography was performed using Sequoia 512 (Siemens, Germany) equipped with a 14.0 MHz linear transducer. M-mode tracings and 2D image were noted. Left ventricular shortening fraction (LVFS) and left ventricular ejection fraction (LVEF) were calculated according to the previous report [15].

2.6. Histology and immunofluorescence

Animals were euthanized, hearts were explanted and cut into 2 transverse slices through infarct. The heart samples were frozen in O.C.T. and 4 μm frozen sections were prepared. The retention of transplanted cells was determined on 1 week's sections under fluorescent microscope by recognizing DiI-positive cells. The 4 weeks' sections were stained with Masson's Trichrome and then, infarct size and left ventricular wall thickness were determined under microscope as previous reports [13,15].

To detect the *in vivo* differentiation of the grafted BASCs, immunofluorescence staining was performed on 4 weeks' sections using anti-α-SA and anti-cTnT