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The amelioration of cardiac dysfunction after myocardial infarction by the injection of keratin biomaterials derived from human hair

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ABSTRACT

Cardiac dysfunction following acute myocardial infarction is a major cause of advanced cardiomyopathy. Conventional pharmacological therapies rely on prompt reperfusion and prevention of repetitive maladaptive pathways. Keratin biomaterials can be manufactured in an autologous fashion and are effective in various models of tissue regeneration. However, its potential application in cardiac regeneration has not been tested. Keratin biomaterials were derived from human hair and its structure morphology, carryover of beneficial factors, biocompatibility with cardiomyocytes, and *in vivo* degradation profile were characterized. After delivery into infarcted rat hearts, the keratin scaffolds were efficiently infiltrated by cardiomyocytes and endothelial cells. Injection of keratin biomaterials promotes angiogenesis but does not exacerbate inflammation in the post-MI hearts. Compared to control-injected animals, keratin biomaterials-injected animals exhibited preservation of cardiac function and attenuation of adverse ventricular remodeling over the 8 week following time course. Tissue western blot analysis revealed up-regulation of beneficial factors (BMP4, NGF, TGF-beta) in the keratin-injected hearts. The salient functional benefits, the simplicity of manufacturing and the potentially autologous nature of this biomaterial provide impetus for further translation to the clinic.

1. Introduction

Heart failure is a major cause of death in western countries. It usually results from an acute coronary event such as myocardial infarction, which can cause the death of billions of cardiomyocytes. An effective therapy to ameliorate cardiac dysfunction and regenerate lost myocardium could help millions of patients every year. The emerging field of biomaterials and cardiac tissue engineering has started to provide promising alternatives or adjuncts to conventional pharmacological therapy [1,2]. Injectable biomaterials are appealing choices due to their minimally-invasive nature [3] and the flexibility of being injected alone or mixed with stem cells [4]. Numerous injectable biomaterials have been studied for cardiac regeneration, including fibrin gel [5,6], collagen [7], alginate [8], matrigel [9], self-assembling peptides [10], de-cellularized extracellular matrix [11,12] and synthetic polymer hydrogel [13] or micro-beads [14]. An ideal biomaterial for cardiac regeneration should be biodegradable, non-cytotoxic, cause little or no foreign body reaction, and provide both mechanical and functional support to the injured heart.

Keratin biomaterial is an appealing choice for therapeutic development as it can be easily manufactured, whether as an allogeneic or autologous product. The foundation of keratin biomaterial researches are based on several key properties of keratins that contribute to the overall physical, chemical and biological behavior of these biomaterials [15]. First, extracted keratin proteins have the ability to self-assemble and polymerize into porous, fibrous scaffolds. The injected biomaterial can provide mechanical support to the heart by increasing LV wall thickness and reducing wall tension (Laplace's Law [8]). Secondly, keratin biomaterials derived from wool and human hair have been shown to possess active cell binding motifs, such as LDV and EDS binding residues, which are capable of supporting cellular attachment and motility [15,16]. These properties create a favorable 3D matrix that allows for cellular infiltration, attachment, proliferation and differentiation. In addition, it has been shown that more than 30 growth factors and cytokines are involved in hair morphogenesis [17,18], and the residual of them in the resulting keratin scaffold could be beneficial for cardiac repair. Among many of them are bone morphogenetic protein 4 (BMP4), transforming growth

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factor-β (TGF-β), and nerve grow factor (NGF). Previous studies highlighted these factors play pivotal roles in mediating tissue regeneration including cardiac regeneration. Based on these promising features, the regenerative potential of keratin biomaterials in other tissues (e.g. nerve regeneration, lethal liver injury) has been well-documented [19–21] but potential utility in cardiac regeneration has yet to be tested. We hypothesize that keratin biomaterials may preserve cardiac function and attenuate adverse ventricular remodeling after myocardial infarction (MI).

2. Materials and methods

2.1. Preparation of keratin biomaterials

Keratin biomaterials were prepared according to previously published protocols with slight modifications [19]. Briefly, human hair obtained from a local barber shop at Zhengzhou was grinded into small pieces and treated with a 2.5% (wt/v) peracetic acid (Sigma–Aldrich, MO, USA) for 10 h. The hair fragments were removed from the solution and washed thoroughly to remove residual oxidant. Proteins were extracted by 150 mM Tris base for 2 h, and then washed in D.I. water for 1.5 h at 37 °C with orbital shaking at 100 rpm. Extracts were then passed through a 400 mm sieve to remove the hair fibers, neutralized, centrifuged, and filtered. Extracts were then combined, purified for 24 h by dialysis (with 1 kD low molecular cut-off), and concentrated and lyophilized with a freeze-dry systems. The keratin hydrogel was formed by re-constitution of the lyophilized material with PBS. To enable *in vivo* histological tracking, the gel was labeled with Texas Red® C2 Maleimide (Invitrogen, CA) before *in vivo* applications.

2.2. Scanning electron microscopy (SEM)

SEM presents a valuable technique for the visualization of the morphological details of the keratin hydrogel. Freshly prepared keratin hydrogel samples were immediately washed in PBS and were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and then rinsed with cacodylate buffer, three times (15 min each). The samples were then dehydrated in 35%, 50%, 70%, 80%, 95%, and 100% ethanol successively for 10 min each and dried in hexamethyldisilazane (Sigma–Aldrich, MO, USA). Scaffolds were sputter-coated with gold and images were captured with a JEOL JSM-6380 LV (JEOL ltd, Japan).

2.3. SDS-PAGE protein analysis

The keratin solution was diluted to 5 mg/mL with D.I. water and mixed 1:1 with SDS loading buffer containing 5% (v/v) 2-mercaptoethanol (Sigma). The samples were denatured by boiling for 5 min. 20 μL of the samples was loaded onto precast 10% gradient Tris–HCl gels (Ready Gel, Bio-Rad). Separation was performed at 120 V for 120 min. After separation, gels were rinsed with D.I. water and then stained with Bio-Safe Comassie stain (G250, Bio-Rad) for 60 min and de-stained in D.I. water for 30 min with gentle shaking. Samples were compared to a standard ladder (Precision Plus Protein™ Standards, Bio-Rad) and the gel was imaged with a Gel Doc XR System (Bio-Rad).

2.4. Mechanical properties

Scaffold mechanical strength was evaluated by determining the capacity to absorb fluid-mechanical energy without damage, according to previously published protocol [22]. Briefly, a syringe pump, connected to a standard 200 μL pipette tip, was used. D.I. water was perpendicularly pumped onto the surface of keratin hydrogel for 5 s through the tip. The flow rate inducing scaffolds rupture was recorded. The force, *F*, experienced by the scaffolds was calculated as follows:

$$F = \rho A v^2$$

where ρ is the density of the water, A is the area of the opening of the pipette tip and v is the fluid flow rate just before impact, which depends on the rate of the syringe piston movement and the diameter of the nozzle tip.

2.5. In vitro growth factor measurement

100 μL keratin hydrogel was formed in each well of a 24-well plate and incubated with 1 mL of serum-free DMEM media (Invitrogen). To study the presence of cytokines and growth factors in the keratin biomaterials, the conditioned media was collected at various time points (day 1, 3, 7, 14, and 28) and fresh media was added back into the well to be conditioned for the next time point. The concentrations of BMP4, NGF, and TGF-β1 in the conditioned media were measured by commercial ELISA kits (R&D Systems, MN, USA), according to the manufacturer's instructions. Serum-free media was used as blank controls.

2.6. Neonatal rat cardiomyocytes culture and characterization

To test if the keratin biomaterials are compatible with cardiomyocytes, we cultured neonatal rat cardiomyocytes (NRCMs) from Sprague–Dawley (SD) rats in the keratin hydrogel. The NRCM harvesting and culturing methods were described previously in detail [23]. Cells were embedded in the keratin hydrogel during the re-constitution process (75 mg/mL). The culture was incubated at 37 °C and 5% CO_2. For viability assay, rat NRCMs were cultured in keratin hydrogel or on 1 mg/mL laminin-coated surface for 7 days and then stained with a LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, CA, USA), which quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM (live) and red-fluorescent ethidium homodimer-1 (EthD; dead). We next tested the *in vitro* migratory potency of NRCMs incorporated within the keratin hydrogels (75 mg/mL). Cells were cultured in a trans-well plate setup that allowed for cell migration from the upper chamber, through pores in the bottom of the chamber insert, and into the lower chamber where they could be detected by fluorescence plate reader (FlexStation, Molecular Devices, CA). Calcein AM-labeled NRCMs were incorporated at a cell concentration of 10,000 cells/μL in 100 μL of keratin hydrogel or plain media, and FBS was used as a chemoattractant in the lower chamber. For attachment assay, NRCMs were seeded onto surfaces coated with either keratin biomaterials or laminin (both at 1 mg/mL). Non-attached cells were removed by thorough PBS wash and the percentages of adhered cells were reported over the time course. Confocal microscopy (Leica TCS-SP2, Germany) was performed to reveal the spatial distribution of cardiomyocytes in the keratin scaffold. NRCMS were labeled with DiI (Invitrogen) and then incorporated in the keratin hydrogel. Color-coded depth projection and 3D-rended projection images were made from series of z-stack confocal images by Leica's LAS AF software.

2.7. Gene expression analysis

The ability of keratin biomaterials to induce expression of specific genes in cardiomyocytes was examined using quantitative RT-PCR. Cells were cultured for 3 days in basal media without FBS (control group), or keratin hydrogel. Total RNA was extracted from cell cultures using RNeasy® extraction kit (Qiagen, CA, USA) according to the original manufacturer's instructions. Extracted RNA was reverse-transcribed into cDNA using SuperScript II (Invitrogen). Reactions were run on the Applied Biosystems ABI 5700 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The expression of α-myosin heavy chain (MHC), β-MHC, connexin 43 (Cx43), integrin alpha 1 (inta1) and laminin beta 1 (lamb1) genes were examined. The primer sequences for each gene have been described previously [24]. Relative expression of the genes of interest was determined following normalization to the level of a housekeeping gene, GAPDH, and untreated controls.

2.8. Animal model

Animal care was in accordance to Zhengzhou University Animal Care and Usage Committee guidelines. Female SD rats (10–12 weeks; $n = 90$ total) underwent left thoracotomy under general anesthesia, and myocardial infarction (MI) was produced by permanent ligation of the left anterior descending coronary artery. The animals were subjected to intramyocardial injections with a 29-gauge needle in the infarct zone, with one of the two randomly assigned conditions: 1) "MI + Saline" group: injection of 100 μL of normal saline (PBS); 2) "MI + Keratin" group: injection of 100 μL of keratin hydrogel. After injections were completed, the chest was closed, anesthesia was discontinued and the animals were allowed to recover. A Sham surgery group was also included by simply opening and closing the chest.

2.9. Heart morphometric analysis

For heart morphometric analysis, animals were euthanized at 4 weeks and the hearts were harvested and frozen in OCT compound. Quantitative morphometric analysis with Masson's trichrome staining of cryosections (6 sections per heart, collected at 400 μm intervals) was performed as described previously [25]. Images were acquired with a PathScan Enabler IV slide scanner (Advanced Imaging Concepts, Princeton, NJ, USA). From the Masson's trichrome-stained images, morphometric parameters including scar size, infarct wall thickness and viable tissue were measured in each section with NIH ImageJ software. Under Masson's trichrome staining, viable (normal) tissue and scar (infarct) tissue could be distinguished with red and blue, respectively. To evaluate infarct thickness, measures of wall thickness were taken at the thinnest of the infarction and then averaged (6 sections per heart, collected at 400 μm intervals). Viable tissue, expressed as a percentage of risk region, was calculated by the sum of red area in the risk region (defined with previously published methods [25]). Scar size was calculated by the sum of blue area and expressed as a percentage of total LV area.

2.10. Histology

Cryosections were obtained from harvested hearts at various time points. Heart cryosections were fixed with 4% PFA, permeabilized/blocked with Protein Block Solution (DAKO, Carpinteria, CA) contains 1% saponin (Sigma, St. Louis, MO), and