Construction of engineered myocardial tissues *in vitro* with cardiomyocyte-like cells and a polylactic-co-glycolic acid polymer

YUJIE XING[1], SHUANG SHI[1], YONG ZHANG[1], FUQIANG LIU[1], LING ZHU[1], BINYA SHI[2] and JUNKUI WANG[1]

[1]First Department of Cardiology; [2]Medical Affairs Department, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, P.R. China

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Abstract. The aim of the present study was to explore the feasibility of the construction of engineered myocardial tissues *in vitro* with cardiomyocyte-like cells derived from bone marrow mesenchymal stem cells (BMMSCs) and a polylactic-co-glycolic acid (PLGA) polymer. The PLGA polymer was sheared into square pieces (10x10x1 mm), sterilized by Co^{60} irradiation, and hydrated in Dulbecco's modified Eagle's medium for 1 h. BMMSCs were isolated from the bone marrow of Sprague-Dawley rats and the third passage cells were induced by 5-azacytidine (5-aza). Following successful induction, the cells were trypsinized and suspended at a density of $1x10^9$/ml. Then, the cell suspension was added to the PLGA scaffold and cultured for 14 days. The morphological changes of BMMSCs were observed using phase contrast microscopy. Immunofluorescence staining was used to identify the cardiomyocyte-like cells. Hematoxylin and eosin (H&E) and immunohistochemical staining were used to observe the morphology of the engineered myocardial tissues. The cell adhesion rates and scanning electron microscopy were used to observe the compatibility of the cardiomyocyte-like cells and PLGA. Transmission electron microscopy was used to view the ultrastructure of the engineered myocardial tissues. BMMSCs in primary culture presented round or short spindle cell morphologies. Following induction by 5-aza, the cells exhibited a long spindle shape and a parallel arrangement. Analysis of the cell adhesion rates demonstrated that the majority of the cardiomyocyte-like cells had adhered to the PLGA scaffolds at 24 h. H&E staining suggested that the cardiomyocyte-like cells with spindle nuclei were evenly distributed in the PLGA scaffold. Immunofluorescence staining revealed that the cardiomyocyte-like cells were positive for cardiac troponin I. Scanning electron microscopy demonstrated that the inoculated cells were well attached to the PLGA scaffold. Transmission electron microscopy indicated that the engineered myocardial tissues contained well-arranged myofilaments, desmosomes, gap junction and Z line-like structures. The present study successfully constructed engineered myocardial tissues *in vitro* with a PLGA polymer and cardiomyocyte-like cells derived from BMMSCs, which are likely to share various structural similarities with the original heart tissue.

Correspondence to: Dr Binya Shi, Medical Affairs Department, Shaanxi Provincial People's Hospital, 256 Youyi West Road, Xi'an, Shaanxi 710068, P.R. China
E-mail: sbinya@163.com

Professor Junkui Wang, First Department of Cardiology, Shaanxi Provincial People's Hospital, 256 Youyi West Road, Xi'an, Shaanxi 710068, P.R. China
E-mail: cardiowang@163.com

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Introduction

The incidence of ischemic heart disease is increasing yearly, and heart failure, as a resulting condition, is a worldwide problem that seriously threatens the survival and quality of life of the global population (1). Due to the minimal regenerative capacity of the adult heart, it forms scar tissue to replace the contractile cardiomyocytes upon injury, eventually leading to heart failure (2). The only feasible treatment for patients with end-stage heart failure is heart transplantation, but this is not widely available due to the limited number of donor organs. At present, myocardial cell transplantation is a popular topic of study in the treatment of ischemic heart disease. Following myocardial cell transplantation, the development of myocardial tissue engineering has generated new possibilities for the treatment of ischemic heart disease.

Myocardial tissue engineering aims to reconstruct ideal myocardial tissue by combining cells with scaffolding polymers to replace and repair the damaged myocardium (3-8). It involves 3 stages: Isolation of the donor cells; development of the scaffold material; and construction of the engineered myocardial tissue. The source and species of seed cells are the key factors in myocardial tissue engineering. At present, there are a number of data concerning heart autologous stem cells, pluripotent stem cells, embryonic stem cells, skeletal myoblasts and bone marrow mononuclear cells (BMMSCs) (9-11). Of these cell types, BMMSCs are widely used for cardiac repair due to their easy accessibility and availability. For scaffold design, the pore size, distribution

and interconnectivity are critical factors that determine mass transfer of oxygen and nutrients to support effective vascular ingrowth within scaffolds. Polylactic-co-glycolic acid (PLGA) scaffolds possess high porosity, and good biocompatibility and biodegradability (12), all of which assist to induce tissue formation through cell migration and nutritional diffusion.

At present, there are two predominant methods used for myocardial tissue engineering. The first method involves directly injecting cardiomyocytes or cardiac-like cells into the heart muscle. A number of studies have confirmed that BMMSCs implantation may induce cardiac regeneration and improve cardiac function through myocardial angiogenesis (13-15). However, cell implantation studies are usually limited by restricted cardiomyogenic potential and low cell survival (16-19). The second method involves transplantation of three-dimensional heart grafts. Li *et al* (20) reconstructed myocardial cells in three-dimensional structures using a preformed biodegradable gelatin mesh. A previous study has suggested that cell sheet transplantation may treat heart failure in animal models (21). The present study aimed to construct engineered myocardial tissues *in vitro* using a PLGA scaffold and cardiomyocyte-like cells derived from BMMSCs, which may support the endogenous ability of induced cells to form a cardiac tissue-like structure.

Materials and methods

Isolation and culture of BMMSCs. A total of 40 male Sprague-Dawley rats (age, 4-weeks-old;) were purchased from the Laboratory Animal Center of Fourth Military Medical University (Xi'an, China). The housing temperature ranged between 18 and 26˚C, the humidity ranged between 40 and 70%. All rats had *ad libitum* access to food and water, with a 12-h light/dark cycle. All rats were anesthetized and sacrificed by cervical dislocation. The femurs and tibias of rats were removed under sterile conditions. The marrow cavities were washed with Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), and the mixed suspension was added slowly to the Percoll® solution (1.073 g/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for centrifugation at 2,000 x g for 20 min at room temperature. The enriched cells in the middle layer were collected and mixed with incomplete culture medium for centrifugation at 1,500 x g for 15 min at room temperature. Following removal of the supernatant, the cells were re-suspended with complete DMEM-low glucose medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mmol/l HEPES and 300 mg/l L-glutamine, and then inoculated in a 25 cm^2 culture flask at 37˚C in a humidified atmosphere of 5% CO_2. The non-adherent cells were discarded after 3 days. The culture medium was changed every 3 days. When the cells reached 80% confluence, the cells were digested with 2.5 g/l trypsin and passaged at a 1:2 ratio. The study was approved by the Ethical Review Committee of Shaanxi Provincial People's Hospital (Xi'an, China). All procedures involving animals were in accordance with the ethical standards of the Institutional Research Committee of Shaanxi Provincial People's Hospital.

Induction and differentiation of BMMSCs by 5-azacytidine (5-aza). BMMSCs at third passage were induced using complete DMEM-low glucose medium containing 10 μmol/l 5-aza (Sigma-Aldrich; Merck KGaA). After 24 h, the induction medium was removed and the medium was changed to complete DMEM-low glucose medium without 5-aza at room temperature. The medium was changed every 3 days. After 4 weeks of culture, the cells were prepared for subsequent experiments.

Scaffolds preparation. The PLGA scaffolds consisted of 50% polylactic acid and 50% polyglycolic acid. The porous microstructure was prepared by a particulate extraction method with particle diameter of 150-200 μm, as described previously (22), and the porosity was 90%. The PLGA polymer was cut into squares (10x10x1 mm). Co^{60} irradiation was used to sterilize the PLGA scaffolds prior to cell inoculation, and then the scaffolds were soaked in PBS for 1 h and in DMEM for 1 h, and finally for the next step of the experiment after water drainage.

Detection of cell adhesion rate. A total of 12 pieces of spare PLGA scaffolds were placed in a 24-well culture plate. Then, 1 ml cardiomyocyte-like cells suspension ($1x10^9$ cells/ml) was added to each of the PLGA scaffolds. Following incubation at 37˚C in a CO_2 incubator for 4, 12, 24 and 48 h, PBS was added slowly to flush non-adherent cells. This step was repeated twice. The cells were digested by 2.5 g/l trypsin and then counted using a counting board under optical inverted phase contrast microscope with x100 magnification. The cell adhesion rate was calculated as follows: Number of adherent cells/total number of cells x100%.

Construction of engineered myocardial tissues in vitro. The cardiomyocyte-like cells induced by 5-aza were digested by 2.5 g/l trypsin, and suspended at a concentration of $1x10^9$ cells/ml. The suspension was slowly instilled onto the prepared PLGA scaffold, and moved into a 5% CO_2 incubator at 37˚C. After 4 h, the initial gelation was observed in the PLGA-cardiomyocyte-like cells compound, and 2 ml culture medium containing 10% FBS was carefully added to completely submerge the compound. The PLGA-cardiomyocyte-like cells compounds were transferred to a 5% CO_2 incubator at 37˚C for subsequent culture. The medium was changed every 2 days. The total culture time was 14 days.

Immunofluorescence staining of the cardiomyocyte-like cells. The immunofluorescence staining of cardiac troponin I (cTnI; cat. no. sc-8118; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was performed to identify whether BMMSCs induced by 5-aza had differentiated into cardiomyocytes. The cells were fixed using 4% polyoxymethylene at 4˚C for 20 min, incubated in 3% hydrogen peroxide and methanol for 10 min at room temperature, blocked in normal goat serum (Abcam, Cambridge, UK) at 37˚C for 30 min, and incubated with cTnI antibody [polyclonal goat anti-mouse immunoglobulin G (IgG); 1:25 dilution] overnight at 4˚C. Subsequent to washing with PBS, the cells were stained by fluorescent isothiocyanate-conjugated rabbit anti-goat IgG (cat. no. sc-2777; Santa Cruz Biotechnology, Inc.;

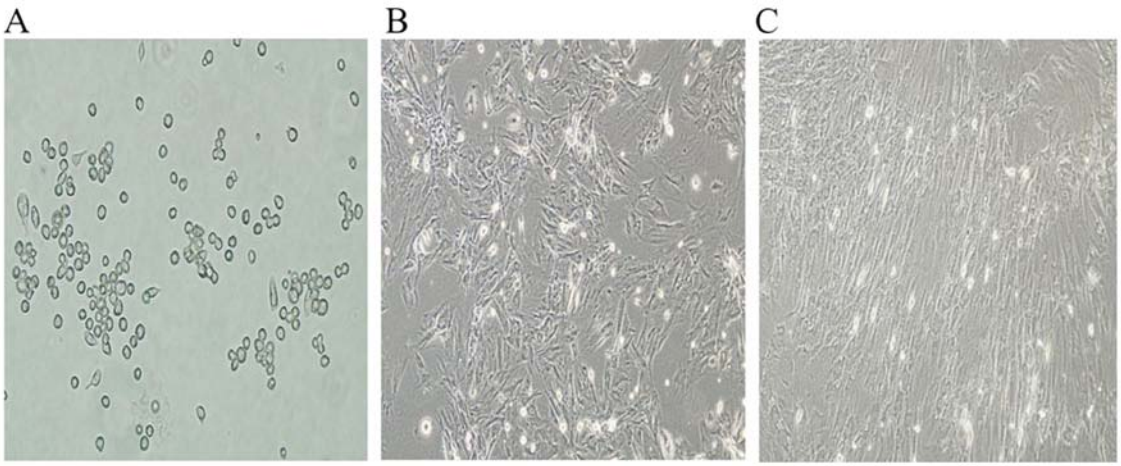

Figure 1. BMMSCs morphological alterations following 5-aza induction. (A) BMMSCs cultured for 3 days (magnification, x100). (B) BMMSCs from passage 3 (magnification, x100). (C) BMMSCs induced by 5-aza exhibited long spindle-shaped cells and parallel arrangement 4 weeks following induction (magnification, x200). BMMSCs, bone marrow mesenchymal stem cells; 5-aza, 5-azacytidine.

1:50 dilution) for 40 min at 37˚C. Finally, the cells were counterstained with Hoechst 33258 at a 1:1,000 dilution, at room temperature, for 10 min, to visualize the nuclei. Fluorescence microscopy (Olympus BX-51; Olympus Corporation, Tokyo, Japan), at an x200 magnification, was used to observe the cells and record the results.

H&E and immunohistochemical staining. After 14 days of culture *in vitro*, the engineered myocardial tissues were washed with PBS, fixed in 4% paraformaldehyde at 4˚C for 24 h, dehydrated on an ascending series of ethanol, embedded in paraffin, sectioned into 10 μm slices and finally were stained with hematoxylin for 10 min and 0.5% eosin for 1 min at room temperature (H&E). For the immunohistochemical staining of cTnI, the sections were incubated with the primary antibodies directed against cTnI (polyclonal goat anti-mouse IgG; cat. no. sc-8118; Santa Cruz Biotechnology, Inc.; 1:25 dilution) at 4˚C overnight, and then incubated with secondary antibody (rabbit anti-goat IgG; cat. no. sc-2768; Santa Cruz Biotechnology, Inc.; 1:1,000 dilution concentration) at 37˚C for 30 min. A total of 1 mg/ml DAB reagent was added to the cells for 5-10 min at room temperature. The sections were sealed with neutral gum for microscopic examination under optical inverted phase contrast microscope at x200 magnification. Cells with brown granular DAB reaction product in the cytoplasm were considered positive for the protein.

Scanning electron microscopy. After 14 days of culture *in vitro*, the engineered myocardial tissues were fixed in 3% glutaraldehyde at 4˚C for 2 h. Samples were then coated with a thin layer of gold for 5 min in a Sputter-coater JFC-1100. The specimen was viewed using a Hitachi S-3400N scanning electron microscope operating at a typical 5 kV accelerating voltage, 20˚C and 10^{-5} Torr.

Transmission electron microscopy. After 14 days of culture *in vitro*, the engineered myocardial tissues were initially fixed in 3% glutaraldehyde at 4˚C for 2 h, post-fixed in 1% osmium tetroxide at 4˚C for 20 min and embedded in epoxy resin at 45˚C for 12 h. Ultrathin sections of 1 μm were prepared and

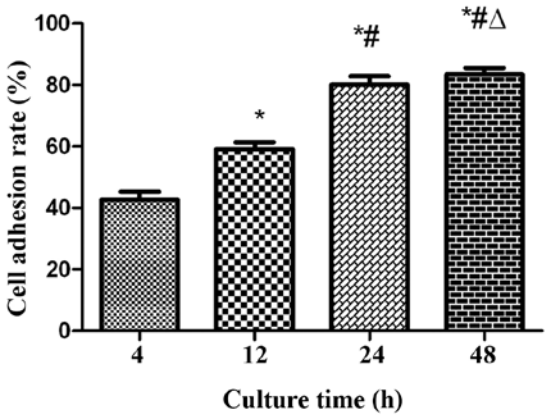

Figure 2. Adhesion rate of cardiomyocyte-like cells in polylactic-co-glycolic acid scaffolds. *P<0.01 vs. 4 h; #P<0.05 vs. 12 h; ΔP>0.05 vs. 24 h.

double stained with 2% uranyl acetate at room temperature for 15 min and 6% lead citrate at room temperature for 15 min. The cellular ultrastructure was observed with a JEM-2000EX transmission electron microscope (JEOL, Ltd., Tokyo, Japan).

Statistical analysis. Data are presented as mean ± standard deviation. Data were compared using standard or repeated measures analysis of variance where appropriate. Pairwise comparisons between groups were performed using the least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cellular morphological changes of BMMSCs induced by 5-aza. After 3 days of primary culture, the adhered BMMSCs were observed to have round or short spindle morphologies (Fig. 1A). Following subculture, the cells became polygonal or long spindle-shaped (Fig. 1B). Subsequent to induction by 5-aza, a proportion of cells died, but the surviving cells began to proliferate and differentiate. Then, the cells aggregated and gradually increased in size 4 weeks later; the adherent

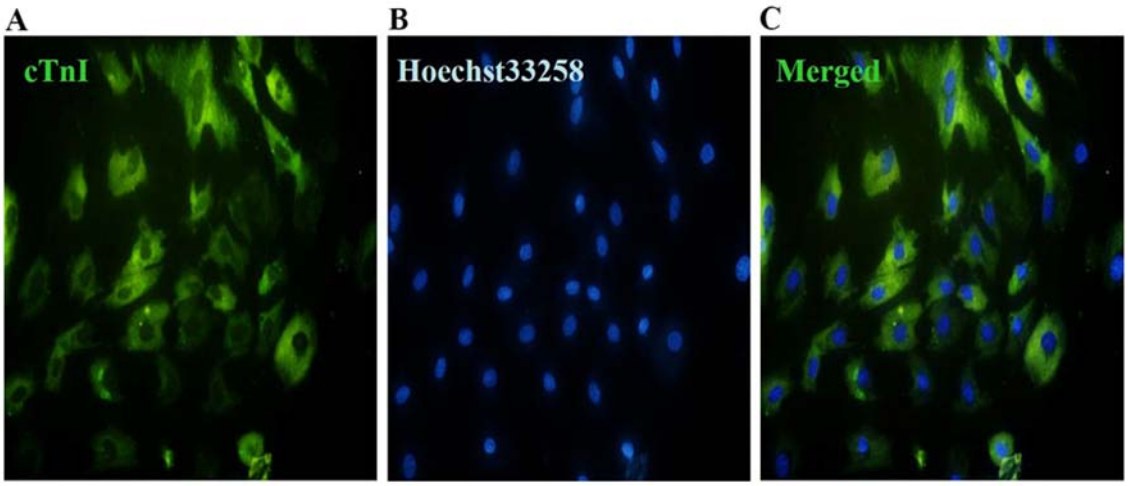

Figure 3. Immunofluorescence staining for cTnI in induced bone marrow mesenchymal stem cells. (A) The induced cells were positive for cTnI, as indicated by the green fluorescence signals (magnification, x200). (B) The nuclei were counterstained with Hoechst 33258, as indicated by the blue fluorescence signals (magnification, x200). (C) The merged data from images in A and B (magnification, x200). cTnI, cardiac troponin I.

cells were completely in contact with neighboring cells and arranged in a uniform direction (Fig. 1C).

Adhesion rate of cardiomyocyte-like cells. The adhesion rates of cardiomyocyte-like cells in PLGA scaffolds were as demonstrated in Fig. 2. It was identified that the adhesion rates at 12, 24 and 48 h were markedly increased compared with that at 4 h ($P<0.01$), and the adhesion rates at 24 and 48 h were increased compared with that at 12 h ($P<0.05$). However, the adhesion rate of 48 h was not statistically significant compared with 24 h ($P>0.05$). It was indicated that the adhesion rate of the cardiomyocyte-like cells increased gradually with increases in culture time. The majority of the cardiomyocyte-like cells had adhered to PLGA scaffolds at 24 h.

Immunofluorescence staining for cTnI. The expression of cTnI was estimated by immunofluorescence staining following 5-aza induction. The results suggested that the cells at 4 weeks after induction by 5-aza exhibited green fluorescence (Fig. 3A). The nuclei of the cells exhibited blue fluorescence (Fig. 3B). Fig. 3C represents the merged data from Fig. 3A and B.

H&E and immunohistochemical staining of the engineered myocardial tissue. H&E staining demonstrated the presence of an elongated pattern, marked organized striations, and a low nucleus: Cytoplasm ratio (Fig. 4A), which indicated the structural maturation of the engineered myocardial tissue. Immunohistochemical staining revealed that the cells in the engineered myocardial tissues contained a number of differentiated cardiomyocytes that were positive for cTnI (Fig. 4B), revealing the presence of cardiomyocyte-like cells within PLGA scaffolds.

Scanning electron micrographs of the engineered myocardial tissue. The results of the scanning electron microscopy suggested that the inoculated the cells were well attached to PLGA, and the cells grew and proliferated in 3 dimensions in the engineered myocardial tissues (Fig. 5A). A large number of cell enations in the surface of PLGA were observed,

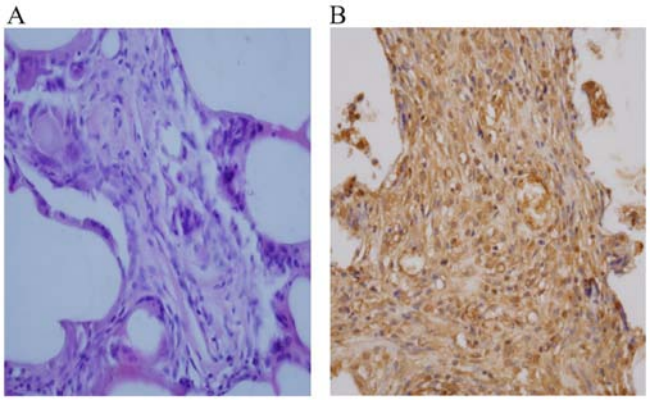

Figure 4. H&E and immunohistochemical staining of the engineered myocardial tissues. (A) H&E staining (magnification, x400). (B) Immunohistochemical staining for cardiac troponin I (magnification, 400). H&E, hematoxylin and eosin.

indicating good adhesion of these cells (Fig. 5B). In particular, the cells were able to secrete a large number of extracellular matrix proteins, which are necessary for cell differentiation (Fig. 5C).

Transmission electron micrographs of the engineered myocardial tissue. The results of the transmission electron microscopy demonstrated the presence uniformly distributed myofilaments (Fig. 6A) and clearly-defined Z line-like structures (Fig. 6B), which are the signs of mature cardiomyocytes. In addition, the presence of specialized junctional structures, including desmosomes and gap junctions, was observed (Fig. 6C and D), which were responsible for electromechanical coupling between neighboring cardiomyocytes.

Discussion

Tissue engineering has become a promising adjunct therapy to the treatment of cardiovascular disease (23-25). It employs certain principles from bioengineering and life sciences fields

Figure 5. Scanning electron microscopy images of the engineered myocardial tissues. (A) The cells were well attached to the polylactic-co-glycolic acid scaffolds, as indicated by the black arrow. (B) Cell enations were observed, as indicated by the black arrow. (C) Extracellular matrix proteins secreted by the cells, as indicated by the black arrow.

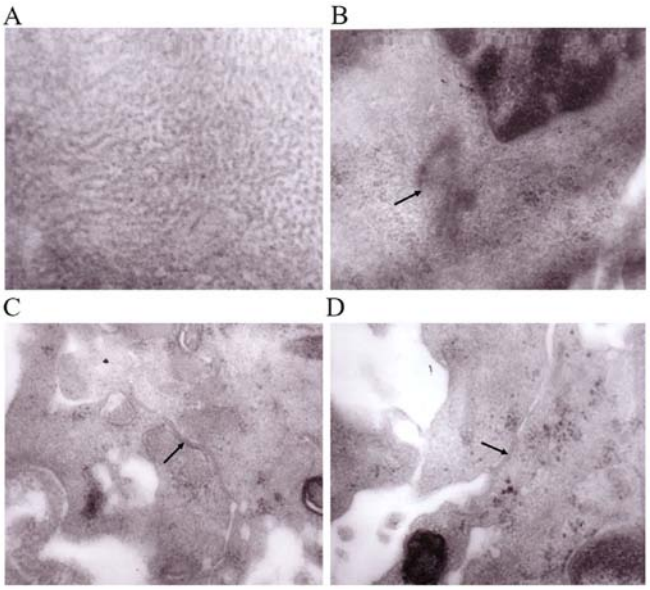

Figure 6. Transmission electron microscopy images of the engineered myocardial tissue. (A) Uniformly distributed myofilaments (magnification, x200,000). (B) Z line-like structures, as indicated by the black arrow (magnification, x60,000). (C) Desmosomes, as indicated by the black arrow (magnification, x80,000). (D) Gap junctions, as indicated by the black arrow (magnification, x80,000).

to generate tissue-like replacements to restore physiological function. The aim of generating engineered myocardial tissue is to repair or regenerate myocardial tissues associated with cardiovascular disease. At present, neonatal cardiac cells are used in myocardial tissue engineering. However, the cardiac cells do not proliferate in high enough numbers to repopulate bio-engineered cardiac tissue. In previous years, stem cells have become an important area of study, as they are multipotent cells with self-renewing ability. Under certain conditions, they are able to differentiate into multiple types of functional cells, including cardiomyocytes. Among these, BMMSCs have become the most promising seed cells, and have been termed the 'repair stem cell' (26). BMMSCs are multipotential cells and are easily obtained. In addition, BMMSCs produce cytokines and certain growth factors, which serve important roles in their differentiation and proliferation abilities (27). In addition, BMMSCs have exhibited immunomodulatory abilities, as demonstrated in bone marrow Graft-verses-host disease (28-30). BMMSCs are able to differentiate into cardiomyocytes under certain specific conditions (31-34). Makino *et al* (35) demonstrated that BMMSCs may differentiate into cells with a cardiac phenotype following treatment with 5-aza. Tomita *et al* (36) revealed that the optimal concentration for cardiomyogenic differentiation was 10 μM for 24 h. Therefore, the present study selected BMMSCs as the seed cells, and induced cardiomyogenic differentiation in these cells using 5-aza.

The ideal biomaterial should have the following characteristics: i) Good biocompatibility without causing inflammatory and toxic reactions; ii) good absorption and the ability to be completely replaced by host tissue; iii) good plasticity; and iv) appropriate degradability according to the rate of tissue regeneration (37). PLGA scaffold is a synthetic polymer material, which has the advantages of being non-toxic, with high porosity, and good biocompatibility and biodegradability. In addition, PLGA promotes good cellular interaction, which is useful in cell migration and nutritional diffusion (38). In the present study, cardiomyocyte-like cells differentiated from BMMSCs were seeded on PLGA scaffolds and cultured together to form the engineered myocardial tissue *in vitro*.

It is well-known that cTnI exists only in the atrial and ventricular muscles, and it is a specific protein for the identification of cardiac myocytes. Therefore, the present study detected the expression of cTnI following 5-aza induction in order to verify the differentiation of BMMSCs to cardiomyocytes. Using immunofluorescence staining, it was identified that the cardiomyocyte-like cells differentiated from BMMSCs subsequent to 5-aza induction were positive for cTnI, indicating that the differentiated cardiomyocytes contained myocardium-specific proteins, which may then be used for the construction of engineered myocardial tissue.

The results of cell adhesion rates and scanning electron microscopy demonstrated good compatibility of the cardiomyocyte-like cells and PLGA. Transmission electron microscopy revealed that the ultrastructure of the engineered myocardial tissues constructed *in vitro* was essentially uniform. The existence of Z line-like structures, myofilaments, gap junctions and desmosomes may assist in explaining the ability of the cells for growth and migration within engineered myocardial tissues (39). The contraction of engineered myocardial tissues constructed *in vitro* would require electrophysiological integration with host tissues, so it is important to

note the development of gap junctions between the engineered myocardial tissues.

At present, the mechanism of engineered myocardial tissue remains unclear (40-42). Previous studies have hypothesized that the paracrine effects of the implanted cells are more likely to affect the survival and growth of engineered myocardial tissues (43-45). However, there are a number of doubts that require additional confirmation.

Although significant progress has been made in the study of myocardial tissue engineering, certain problems and technical difficulties remain. For example, the thickness of the three-dimensional myocardium does not match the *in vivo* measurements, and the differentiation rate of BMMSCs is not ideal. In addition, certain limitations exist regarding the angiogenesis of engineered myocardial tissue. The generation of a stable culture system is also required, in order to derive more reliable and more mature engineered myocardial tissue. Furthermore, the function of a myocardial infarction model requires validation using the engineered myocardial tissues constructed *in vitro* to repair the injured myocardium.

In summary, the present study successfully constructed engineered myocardial tissues *in vitro* with cardiomyocyte-like cells derived from BMMSCs following 5-aza induction and PLGA scaffolds, which are likely to share some structural similarities with the original cardiac tissue. The present study also provides encouraging evidence for the general feasibility of engineered myocardial tissue *in vitro* and suggests the potential application for tissue repair in the future. Despite the challenges that remain, the generation of functional engineered myocardial tissue represents a promising supportive therapy in postsurgical heart recovery therapy.

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Authors' contributions

YJX, BYS and JKW conceived and designed the experiments. SS performed the cell culture and induction. YZ performed PLGA scaffold preparation and detection of cell adhesion rate. FQL performed the hematoxylin-eosin staining and immunohistochemical staining. YJX and LZ performed the immunofluorescence staining, scanning electron microscopy detection and transmission electron microscope detection. YJX, SS, YZ, BYS analyzed the data. YJX wrote the paper. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The present study was approved by the Ethical Review Committee of Shaanxi Provincial People's Hospital (Xi'an, China). All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional research committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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