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Injectable biodegradable hydrogels for embryonic stem cell transplantation: improved cardiac remodelling and function of myocardial infarction

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Abstract

In this study, an injectable, biodegradable hydrogel composite of oligo[poly(ethylene glycol) fumarate] (OPF) was investigated as a carrier of mouse embryonic stem cells (mESCs) for the treatment of myocardial infarction (MI). The OPF hydrogels were used to encapsulate mESCs. The cell differentiation *in vitro* over 14 days was determined *via* immunohistochemical examination. Then, mESCs encapsulated in OPF hydrogels were injected into the LV wall of a rat MI model. Detailed histological analysis and echocardiography were used to determine the structural and functional consequences after 4 weeks of transplantation. With ascorbic acid induction, mESCs could differentiate into cardiomyocytes and other cell types in all three lineages in the OPF hydrogel. After transplantation, both the 24-hr cell retention and 4-week graft size were significantly greater in the OPF + ESC group than that of the PBS + ESC group ($P < 0.01$). Four weeks after transplantation, OPF hydrogel alone significantly reduced the infarct size and collagen deposition and improved the cardiac function. The heart function and revascularization improved significantly, while the infarct size and fibrotic area decreased significantly in the OPF + ESC group compared with that of the PBS + ESC, OPF and PBS groups ($P < 0.01$). All treatments had significantly reduced MMP2 and MMP9 protein levels compared to the PBS control group, and the OPF + ESC group decreased most by Western blotting. Transplanted mESCs expressed cardiovascular markers. This study suggests the potential of a method for heart regeneration involving OPF hydrogels for stem cell encapsulation and transplantation.

Keywords: cardiac tissue engineering • injectable hydrogels • cell encapsulation • embryonic stem cell • myocardial infarction

Introduction

Myocardial infarction is one of the main causes of morbidity and mortality and it is responsible for about one third of heart failure cases worldwide [1]. Stem cell therapy is a potential therapeutic option for treating ischaemic heart disease [2–6]. However, unsatisfactory cell retention and transplant survival still plague this technique [7–9]. When delivered to the heart through a needle, approximately 90% of cells were lost to the circulation or leaked out of the injection site [7]. In the cells that successfully entered the heart, almost 90% died within the first week [8,9]. The emerging field of tissue engineering may provide promising alternatives.

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After MI, biodegradable biomaterials can be used, alone or in conjunction with cells or growth factors, to preserve the LV function of the heart [10]. In addition to the basic requirements (biocompatible, biodegradable and biomimetic), the biomaterials used must be injectable and capable of gelation *in situ* after injection to support the LV wall, avoid post-MI negative remodelling and deliver cells directly into the infarcted wall to increase cell survival. In recent years, several types of biomaterials, mainly natural proteins, were used for this purpose, such as fibrin, alginate, Matrigel, collagen and chitosan [11–17]. However, concerns with possible immune rejection, pathogen transfer and availability of large quantities of natural materials have led others to study synthetic injectable materials as cell carriers [18–20].

Recently, an injectable biodegradable macromer, OPF, was developed in our laboratory [21]. Previous research has shown that OPF-based formulations can be crosslinked in the presence of radical initiators under physiological conditions to form a hydrogel [22]. Studies have also demonstrated the degradation of OPF hydrogels both *in vitro* and *in vivo* through the hydrolysis of ester linkages [23]. In addition, the encapsulation of cell populations and particulate drug delivery systems within the hydrogels demonstrated the potential of injectable hydrogel formulations for cartilage, bone and lens tissue engineering applications [22–25]. More importantly, thermal radical initiators can be leveraged to crosslink OPF solutions at 37°C to form hydrogels within 15 min. [26]. The ability to form hydrogels *in situ* with a thermal initiation mechanism is attractive for cardiac repairs because supportive cells and growth factors can be easily incorporated into the polymer solution prior to injection. Once exposed to body temperature, the polymer solution can gel *in situ* within a short time, trapping and aiding in the retention of these factors within the injected area [10]. Considering all these features, we hypothesize that the OPF hydrogel is also preferred for cardiac repairs. Until now, there is no report about its use as a carrier to deliver cells for treatment of MI.

In this study, the OPF hydrogels were used as an injectable scaffold for embryonic stem cells to test (1) whether encapsulated mESCs in OPF hydrogel retain their ability of differentiating with ascorbic acid induction; (2) whether the combination of OPF hydrogels and mESCs could improve cell retention and survival within the ischaemic myocardium after transplantation and (3) whether OPF hydrogels can be used as a carrier to deliver stem cells for the treatment of MI.

Materials and methods

OPF synthesis

OPF was synthesized from fumaryl chloride and poly(ethylene glycol) (PEG) according to a previously established method [27]. The OPF macromer formulation (OPF 10K) was prepared from PEG of nominal molecular weight 10K. The purified macromer was stored at −20 °C and sterilized prior to use by exposure to ethylene oxide for 14 hrs.

Embryonic stem cells culture

The GFP-labelled mouse ESCs (E14Tg2A ES-hrGFP1 cell line, provided by Prof. Andy Peng Xiang, Center for Stem Cell Biology and Tissue Engineering, Sun Yat-sen University, China) was maintained on mitotic inactive mouse embryonic fibroblast feeder cells. The ESCs culture medium was DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 15% foetal bovine serum (Hyclone, Logan, UT, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Chemicon, Temecula, CA, USA), 1% non-essential amino acids (Chemicon), and 10^3 units/ml of leukaemia inhibitory factor (Chemicon).

Cell encapsulation and culture

Mouse embryonic stem cells were encapsulated into hydrogels at a concentration of 1×10^7 /ml in accordance with previous studies with marrow stromal cells [27]. OPF 10K, poly(ethylene glycol) diacrylate (PEG-DA, nominal MW 2000; Sigma-Aldrich), and 96-well cell culture dishes were sterilized by exposure to ethylene oxide, whereas the initiators, PBS, and medium were filter sterilized by a cellulose acetate membrane filter (0.22 μm pore diameter). OPF 10K (0.1 g) and 0.05 g PEG-DA were dissolved with 300 μl of PBS. After the addition of 46.8 μl of ammonium persulfate (APS, 0.3M) and 46.8 μl of tetramethylethylenediamine (TEMED, 0.3M), 168 μl of the cell suspension containing 7 million cells was added, and the solution was injected into the 96-well cell culture dishes, 100 μl per well. After an 8 min. incubation (37°C), the cross-linked gels were aseptically transferred into a Petri dish containing 10 ml of the differentiation medium. The differentiation medium was DMEM containing 0.1 mg/ml ascorbic acid, 1 mmol/l L-glutamine, 0.1 mmol/l β-mercaptoethanol, 1% nonessential amino acids stock and 20% foetal bovine serum. During the 14-day culture period, the media were changed every 2–3 days.

Immunohistochemical examination for ESC differentiation in OPF hydrogel *in vitro*

On day 14, samples were fixed with 10% formalin at 4°C and washed for paraffin embedding. Afterwards, the samples were dehydrated in a graded concentration of ethanol and then were embedded in paraffin. Sections (10 μm) were deparaffined and rehydrated and then incubated with primary antibodies (antimouse) against cardiac troponin T (cTnT; 1:800; Sigma-Aldrich), GATA-4 (1:800; Sigma-Aldrich), Nkx2.5 (1:800; Sigma-Aldrich) as cardiomyocyte markers, α-fetoprotein (1:500; Santa Cruz, Biotechnology, Inc., Santa Cruz, CA, USA) as endoderm marker, β-III tubulin (1:600; Santa Cruz) as neuroectoderm marker and cytokeratin (1:600; Santa Cruz) as epithelium marker, respectively. After incubating with the primary antibodies overnight at 4°C, the sections were incubated with biotin-labelled secondary antibodies and detected with Diaminbenzidine (DAB) (Sigma-Aldrich). Negative controls were performed by omitting primary antibodies.

Myocardial infarction model

Female Sprague–Dawley rats (6 week of age) were purchased from the Experimental Animal Center, Academy of Military Medical Science (Beijing, China). All experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of

Military Medical Science (Beijing, China). Efforts were made to limit the number of animals used and minimize their suffering. Animals were individually housed in cages (accessible to water and food) with a room temperature of 24 ± 2°C (a normal day/night cycle).

An ischaemia model for this study was described by Miyahara *et al.* [28]. Briefly, S.D. rats were anaesthetized with sodium pentobarbital (30 mg/kg, i.p.). Limb-lead electrocardiography was performed subsequently. The animals were then intubated and ventilated by a volume-regulated respirator during surgery. The surgical approach involved a left lateral thoracotomy as well as pericardectomy and identification of the left anterior descending coronary (LAD) artery for permanent ligation. The left coronary artery was ligated with a 6–0 prolene suture at a location 2–3 mm from its origin between the pulmonary artery conus and the left atrium. Subsequently, the infarcted area of LV paled immediately and beat weakly. The electrocardiography showed MI waves (typical ST-segment changes). Finally, the chest was closed. Twenty-five to thirty-five per cent mortality was observed within the first 24 hrs after occlusion. The surviving rats were maintained on standard rat chow and water *ad libitum*. This laboratory has extensive experience with this model. The histological observation shows that the technique results in an acute infarction size of approximately 40–60% of the LV.

Injection surgeries

Injection surgeries were performed 1 week after MI. The inclusion criterion was fractional shortening (FS) $<$ 40% by echocardiography 1 week after MI. Rats were placed randomly into groups for the injection surgeries. Rats were anaesthetized with sodium pentobarbital (30 mg/kg). The heart was aseptically exposed by left intercostal thoracotomy to expose the LV wall. The infarcted area was visually identified on the basis of surface scar, wall motion abnormality and lack of vascularization (whitish tissue area). Before injection, mESCs were removed from feeder cultures by differential adhesion and pelleted by centrifugation at 1000 rpm for 5 min. Concomitantly, 1×10^6 mESCs suspended in 100 μL PBS (n = 32 rats, among them 8 were used for 24-hr cell retention observation) or in OPF 10K before crosslinking (the initiator system used for *in vivo* studies was the same as the *in vitro* studies) *in vivo* (n = 32 rats, among them eight were used for 24-hr cell retention observation) were injected along the border zone of the infarcted area at three different locations (below the left atrium, in the middle portion of the LV, and at the apex; the total volume injected into each heart was 100 μl; the volume per injection location was about 30–35 μl) using a 28 gauge needle. The PBS-only (n = 24 rats) or OPF hydrogel-only (n = 24 rats) were injected as controls. The non-infarcted group (n = 10 rats) serving as sham-operation control underwent thoracotomy and cardiac exposure without coronary ligation (suturing without tying the LAD) and injection. Injections were made at an angle to reduce the chance of injection into the lumen of the LV. Injections were verified by a slight lightening in the colour of the myocardium as the solutions entered the infarcted wall. After transplantation, animals were daily immunosuppressed with cyclosporine A (5 mg/kg/day; Sigma-Aldrich) until the end of the study.

Evaluation of 24-hr cell retention and 4-week graft size

The hearts were excised rapidly and frozen in OCT 24 hrs after injection. Five sections (5 μm), equally distributed through the infarcted area (from base to apex in an interval of 1 mm), were taken from each heart as representative samples.

The area covered by the GFP-labelled mESCs was traced using a fluorescent microscope (Olympus BX51; Olympus, Tokyo, Japan). The ratio of area covered by GFP-labelled mESCs on the infarcted area in each sample to the total infarcted area of each sample was measured by using RS Image Pro (version 4.5; Media Cybernetics, Inc., Trenton, NJ, USA). Cell retention was reported as percentage of GFP-labelling area within the infarcted area.

Graft size in the specimens after 4 weeks of injection was determined by immunohistochemical staining. Immunohistochemical staining with rabbit polyclonal anti-GFP (1:400; Abcam, ab6556) was performed to identify the mESCs transplanted in each heart. After incubating with the primary antibodies overnight at 4°C, the sections were incubated with biotin-labelled secondary antibodies and detected with DAB (Sigma-Aldrich). Negative controls were performed by omitting primary antibodies. The ratio of area covered by GFP-positive cells on the infarcted area in each sample to the total infarcted area of each sample was measured by using RS Image Pro. Graft size was reported as percentage of GFP-labelling area within the infarcted area.

Physiological assessment of LV function using echocardiography

Four weeks after transplantation, animals were anaesthetized for function detection with echocardiography (14.0 MHz, Sequoia 512; Acuson, München, Germany). Parasternal short axis views with M-mode were acquired at the ventricular base immediately distal to the mitral valve. LV end-systolic diameter (ESD) and LV end-diastolic diameter (EDD) were measured. LV fractional shortening (FS) was calculated as: FS (%) = [(EDD−ESD)/EDD] ×100. All procedures and analyses were performed by an experienced researcher to whom they were blinded. The animals were euthanized after functional measurements with an overdose of sodium pentobarbital in compliance with above guidelines.

Histopathology

Four weeks after transplantation, the hearts were excised rapidly and frozen in OCT freezing medium freshly. The sections (5 μm) were stained with Masson's trichrome (Sigma-Aldrich) and haematoxylin and eosin.

The infarcted fraction of the left ventricle was calculated according to Pfeffer *et al.* [29]. The infarcted fraction of the left ventricle was calculated from photographs of gross heart slices by measuring the length of the infarcted endocardial circumference and the entire endocardial circumference. Infarction size was reported as percentage of length of the infarcted endocardial circumference in the length of the entire endocardial circumference. The measurements from each slice were averaged together in every heart. All haematoxylin and eosin stained sections were also examined for the possible existence of an immune reaction, as reflected by the observation of lymphocytic infiltrate and giant cells. The degrees of collagen fibre accumulation in the infarcted region were determined by measuring the percentage of fibrotic area in the total LV area, which was calculated by using RS Image Pro.

Western blotting

Western blotting was performed with rabbit polyclonal antibody raised against MMP2 (1:500; Santa Cruz), and goat polyclonal antibody raised against MMP9 (1:500; Santa Cruz) to identify protein expression of MMP2

and MMP9. The left ventricle was obtained from individual rats and used for comparisons among all groups 4 weeks after transplantation. These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor. Sixty micrograms of protein was transferred into sample buffer, loaded on a 7.5% SDS-PAGE, and blotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After being blocked for 120 min., the membrane was incubated with primary antibody (1:250 dilution). After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated appropriate secondary antibodies (anti-rabbit IgG, 1:10,000 dilution, anti-goat IgG; Santa Cruz) followed by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Inc., Piscataway, NY, USA) and measured by densitometry.

Immunofluorescent and immunohistochemical staining

Cryosections were fixed with acetone and endogenous peroxide activity was quenched with 3% H_2O_2. After blocking with 2% normal goat serum, sections were incubated with the primary antibodies [cTnT, Sigma-Aldrich, 1:200; SMA, Sigma-Aldrich, 1:200; vWF, Zymed (South San Francisco, CA, USA), 1:200] at 4°C overnight. Then, sections were incubated with Cy3-conjugated IgG (1:100; Sigma-Aldrich) for 1 hr at room temperature before observation under an Olympus FV1000S confocal laser microscope. For immunohistochemical staining, sections were incubated with peroxidase-conjugated streptavidin and stained with DAB. Negative controls were performed by omitting primary antibodies.

The number of arterioles/venules was determined in the sections immunohistochemically stained with vWF antibody. Five high-magnification fields within the infarcted region of each section were chosen randomly. Arteriole/venule densities were calculated accordingly. Arterioles/venules in each section were quantified using the following criteria: (1) positive for vessel endothelium labelling; (2) within the infarct scar; (3) a visible lumen and (4) a diameter between 10 and 100 μm. The measurements from each slice were averaged together in every heart.

Statistical analysis

Cell retention and graft size were compared using Student's *t*-test. ANOVA and Tukey's multiple comparison tests were used to determine possible significant differences in the cardiac function, infarct size and vessel density between groups. Statistical analyses were performed with SPSS 11.0. Significance was accepted at $P < 0.05$. Results are reported as mean ± S.D.

Results

mESCs differentiation in OPF hydrogel *in vitro*

After a 14-day culture period with ascorbic acid induction, the differentiation of mESCs in the OPF hydrogel was evaluated by immunohistochemical staining (Fig. 1). With ascorbic acid induction, the mESCs encapsulated in OPF hydrogel showed immunoreactivity for a variety of cardiac markers, including cTnT and the cardiac transcription factors GATA-4 and Nkx2.5. The transcription factors GATA-4 and Nkx2.5 are expressed in pre-cardiac mesoderm and persist in the heart during development. GATA-4 and Nkx2.5 immunoreactivity were found in nuclei of cells in OPF hydrogel, indicating the presence of ES-derived cardiomyocytes (Fig. 1A and B). Immunostaining revealed cardiac muscle cell-specific troponinT protein in cells encapsulated in OPF hydrogel (Fig. 1C). In addition, mESCs in OPF 10K hydrogel could differentiate into the endoderm, neuroectoderm and epithelium lineage, revealed by immunostaining of α-fetoprotein, β-III tubulin and cytokeratin, respectively (Fig. 1D–F). There was no positive signal in the negative control in all staining. The results indicated that with ascorbic acid induction, mESCs in OPF hydrogel maintained the ability of differentiating into cardiomyocytes and other cell types of all three lineages *in vitro*.

Cell retention and graft size

Twenty-four hours after injection, GFP-labelled mESCs injected in PBS covered 6.54 ± 2.10% of the infarcted area (Fig. 2A), whereas the co-injection of GFP-labelled mESCs encapsulated in OPF hydrogel covered 16.35 ± 4.13% (Fig. 2B), with the difference between the two groups being statistically significant (Fig. 2C; $P < 0.01$).

After 4 weeks of implantation, the mESCs population was significantly higher in the ESCs + OPF hydrogel group compared with that of ESCs + PBS co-injection ($P < 0.01$; Fig. 2D–F). Cells encapsulated in OPF hydrogel covered 11.83 ± 3.26% (Fig. 2E) of the infarcted areas. In contrast, only 4.56 ± 1.82% (Fig. 2D) of the infarcted areas were covered by the cells co-injected with PBS. In addition, mESCs injected in PBS were frequently found scattered in the infarcted areas, whereas those in OPF hydrogel generally aggregated.

Cardiac function

Cardiac function for each group was compared with the PBS control group by echocardiography. LVEDD (Fig. 3A), LVESD (Fig. 3B) and LVFS (Fig. 3C) were analysed, respectively. Improved heart function could be observed in OPF + ESC, PBS + ESC and OPF groups at 4 weeks after injection. All treatments have significantly greater LVFS compared to the PBS-control group ($P < 0.01$, respectively). The rats transplanted with OPF + ESC exhibited the best improvement in LV function (measured by LVFS) compared with other groups ($P < 0.01$). The group treated with OPF-only exhibited similar LV systolic function to the one receiving PBS + ESC cells ($P > 0.05$).

Histopathology

Infarct size

The infarcted size in the PBS-only group was 52.4±4.6% (Fig. 4A). Injection with OPF hydrogel reduced the infarcted size to 39.1 ±

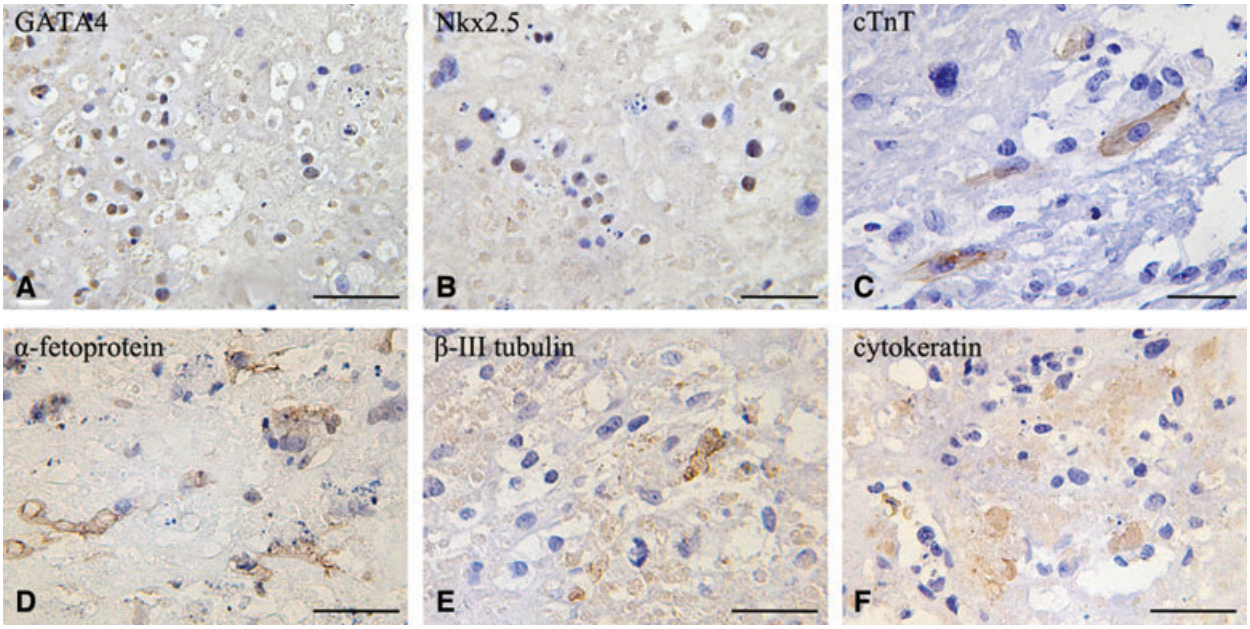

Fig. 1 mESCs differentiation in OPF hydrogel *in vitro*. (**A**–**C**) With ascorbic acid induction, the mESCs encapsulated in OPF hydrogel showed immunoreactivity for a variety of cardiac markers, including cardiac troponin T (cTnT) and the cardiac transcription factors GATA-4 and Nkx2.5. (**D**–**F**) mESCs in OPF hydrogel could differentiate into the endoderm, neuroectoderm and epithelium lineage, revealed by immunostaining of α-fetoprotein, β-III tubulin and cytokeratin, respectively. Scale bars = 25 μm.

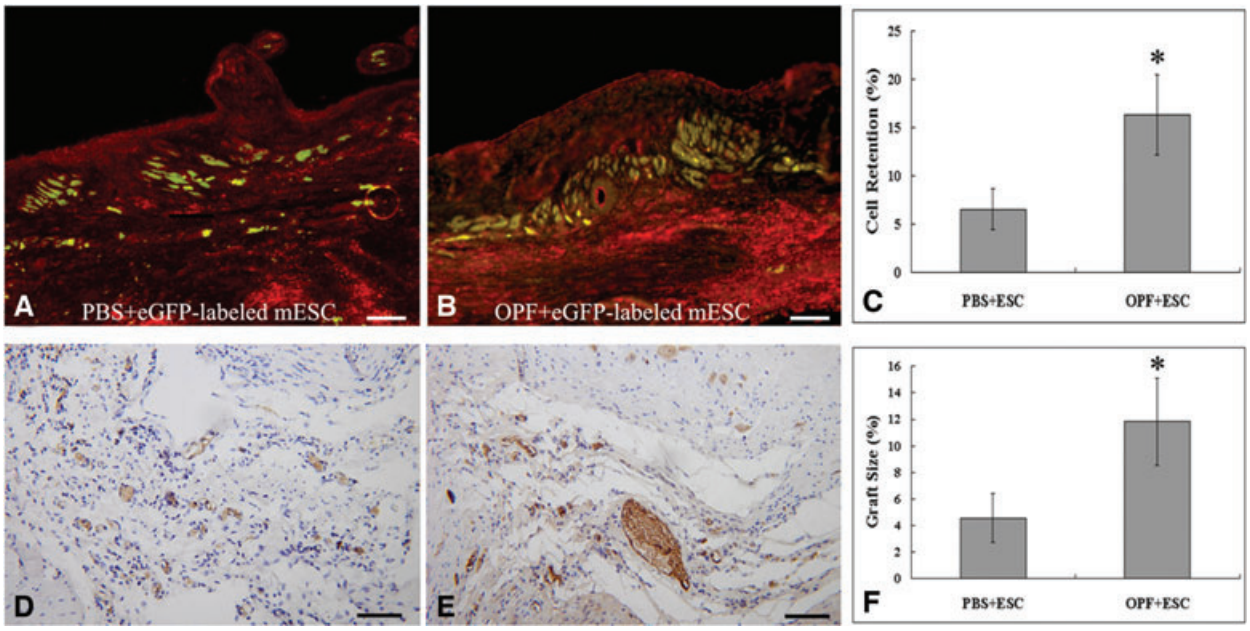

Fig. 2 Cell retention and graft size. (**A**) After 24 hrs of injection, GFP-labelled ES cells injected in PBS covered 6.54 ± 2.10% of the infarcted area. (**B**) The co-injection of GFP-labelled ES encapsulated in OPF hydrogel covered 16.35 ± 4.13%. (**C**) The diagram shows the statistical results. $^*P < 0.01$ compared with PBS + ESC group. (**D**) After 4 weeks of implantation, only 4.56 ± 1.82% of the infarcted areas were covered by the cells co-injected with PBS. (**E**) In contrast, cells encapsulated in OPF hydrogel covered 11.83 ± 3.26% of the infarcted areas. (**F**) The diagram shows the statistical results. $^*P < 0.01$ compared with PBS + ESC group. Scale bars = 50 μm (**A**, **B**) and 10 μm (**D**, **E**).

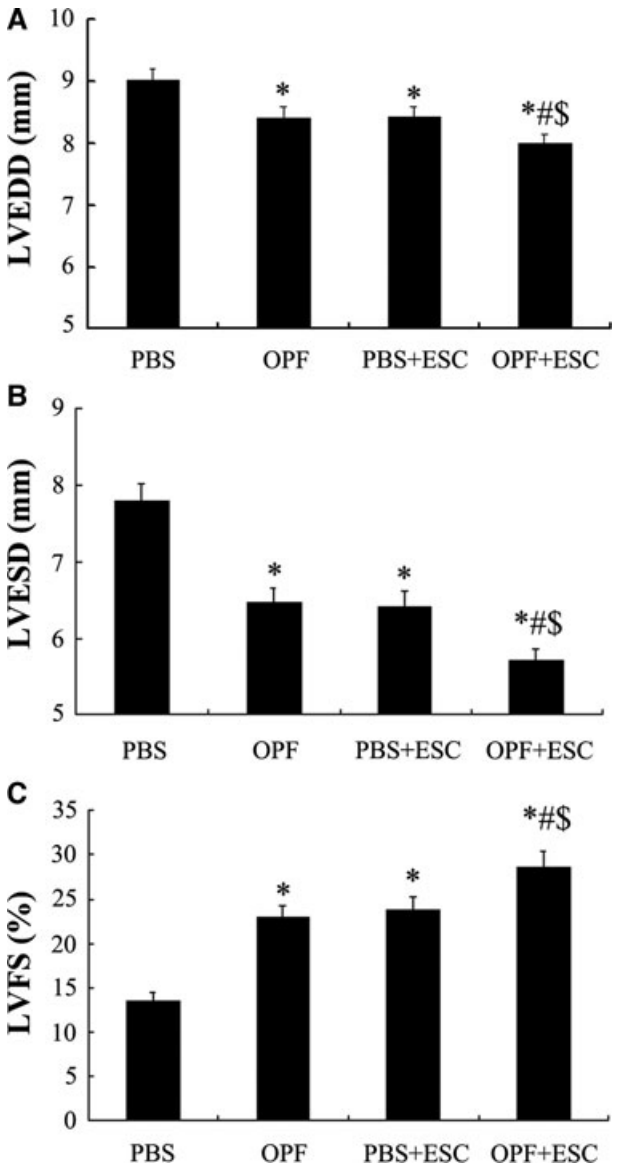

Fig. 3 Echocardiography. (**A**) LVEDD, (**B**) LVESD and (**C**) LVFS. Heart function is better in the rats which were treated with OPF-only, PBS + ESC, and OPF + ESC compared with that using the control (PBS-only). The OPF + ESC group showed a significantly higher heart function compared with OPF-only group and PBS + ESC group. $^{*}P < 0.01$ compared with PBS-only, $^{\#}P < 0.01$ compared with OPF-only, $^{\$}P < 0.01$ compared with PBS + ESC.

3.7% (Fig. 4B). Similarly, the infarct size was reduced to 34.6 ± 3.3% when mESCs were co-injected with PBS (Fig. 4C). Co-transplantation of mESCs with OPF hydrogel reduced the infarct size to 23.8 ± 2.3% (Fig. 4D). All treatments have significantly reduced infarct sizes compared to the PBS-control group ($P < 0.01$, respectively). The co-transplantation of mESCs with OPF hydrogel significantly reduced the infarct size compared with either the OPF-only or the PBS + ESC group ($P < 0.01$). No significant difference was observed between the OPF-only and the PBS + ESC groups ($P > 0.05$; Fig. 4E).

Collagen deposition

There was much less collagen deposition in the infarcted myocardium in the OPF + ESC, PBS + ESC and OPF groups compared with that in the PBS group. Quantitative analysis showed that the percentage of fibrotic area was significantly lower in OPF + ESC (36.05 ± 2.87%), PBS + ESC (48.58 ± 4.92%) and OPF (51.14 ± 5.25%) groups than that in the PBS group (71.24 ± 6.21%) 4 weeks after injection ($P < 0.01$). The OPF + ESC group exhibited significantly less collagen deposition than the OPF-only and PBS + ESC groups ($P < 0.01$). There was no significant difference between the OPF-only group and the PBS + ESC group ($P > 0.05$; Fig. 4F).

Effects of OPF hydrogel and mESCs on MMP expression

In this study, Western blotting showed that all treatments had significantly reduced MMP2 and MMP9 protein levels compared to the PBS control group ($P < 0.01$, respectively). The co-transplantation of mESCs with OPF hydrogel significantly reduced the MMP2 and MMP9 protein levels compared with either the OPF-only or PBS + ESC group ($P < 0.01$; Fig. 5A). No significant difference was observed between the OPF-only and PBS + ESC groups ($P > 0.05$; Fig. 5B).

Degradation of OPF in infarcted heart

Haematoxylin and eosin staining showed that there was a significant amount of OPF hydrogels in the infarcted area 24 hrs post-transplantation (Fig. 6A), while little was left 4 weeks post-transplantation (Fig. 6B). The OPF hydrogel degraded completely in 6 weeks (Fig. 6C). No signs of prolonged inflammatory response were observed in 4–6 weeks.

Differentiation of mESCs encapsulated in OPF hydrogel in infarcted heart

The cTnT immunofluorescence staining of eGFP-labelled mESCs encapsulated in OPF hydrogel after 4 weeks of transplantation showed that both eGFP-positive and cTnT-positive cells exist in the infarcted areas (Fig. 7D). Some eGFP-positive cells were found to be vWF or α-SMA, suggesting that they formed small blood vessels within the infarcted zone (Fig. 7H and L).

Neovasculature formation

Neovasculature formation potential was assessed in the sections immunohistochemically stained with vWF antibody. Because

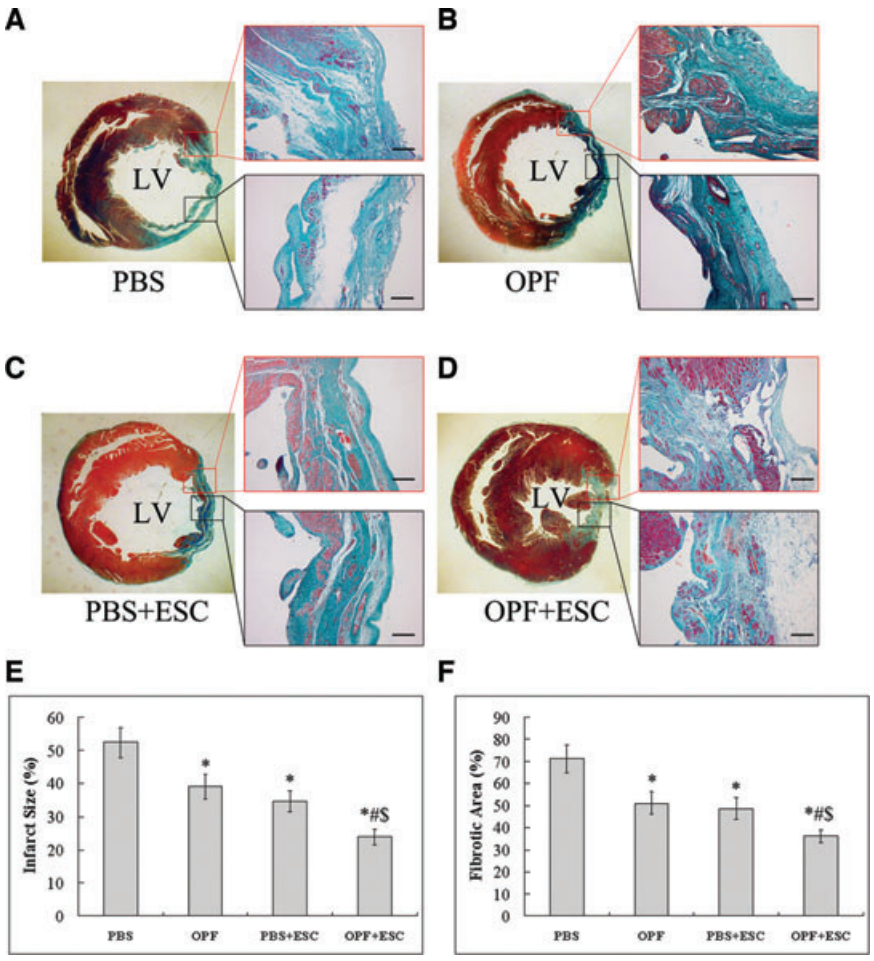

Fig. 4 Infarct size and collagen deposition. (**A**–**D**) Masson trichrome staining of representative sections showing the infarcted wall of the four groups. (**A**) PBS-only; (**B**) OPF-only; (**C**) PBS + ESC; (**D**) OPF + ESC. Photomicrographs show two cases of representative myocardial sections stained with Masson trichrome in the individual group. Scale bars = 200 μm. (**E**) The diagram shows the statistical results of infarct size. (**F**) The diagram shows the statistical results of fibrotic area. $^{*}P < 0.01$ compared with PBS-only, $^{\#}P < 0.01$ compared with OPF-only, $^{\$}P < 0.01$ compared with PBS + ESC.

collateral arterioles often bordered the scar post-MI, vessels within the infarcted area were counted to assess the effect of neovasculature by different treatments. The arteriole/venule densities in the central area of MI 4 weeks after implantation were as follows: 86.6 ± 12.8/mm^2 in PBS-only group, 118.2 ± 23.6/mm^2 in OPF-only group, 219.4 ± 29.7/mm^2 in PBS + ESC group and 293.8 ± 36.5/mm^2 in OPF + ESC group. The arteriole/venule density in scar areas increased significantly in the PBS + ESC (Fig. 8C) and OPF + ESC groups (Fig. 8D) as compared to the PBS-only (Fig. 8A) and OPF-only groups (Fig. 8B; $P < 0.01$). The arteriole/venule density was found to increase most in the OPF + ESC group ($P < 0.01$; Fig. 8E).

Discussion

After MI, both the cardiomyocytes and the extracellular matrix are pathologically modified. Although methods exist to transplant stem cells to the ischaemic myocardium, the cells are generally retained in inadequate numbers and with insufficient viability for therapeutic effect. This appears to be the main problem affecting the therapeutic effects of MI by stem cell transplantation [30]. One strategy to improve retention and viability of transplanted cells is the use of injectable biomaterials to deliver cells directly into the infarcted wall to provide a temporarily favourable environment for cell retention and cell survival [10]. In this work, we investigated the encapsulation of mESCs within the injectable OPF hydrogel for the treatment of MI. We demonstrated that (1) cell retention was increased using OPF for delivery of mESCs; (2) fibrosis is minimized with OPF alone or with mESCs; (3) MMP2 and MMP9 may play a role in minimizing ventricular remodelling, as the levels of both MMPs are reduced in all treatment groups and most notably in the OPF + mESCs group; (4) heart function (LVFS) improved at 4 weeks in all treatment groups.

The cytocompatibility of OPF hydrogels for cell encapsulation has been demonstrated previously using different cell types, including marrow mesenchymal stem cells [31, 32], bovine chondrocytes [26] and rat fibroblasts [25]. Previous studies also

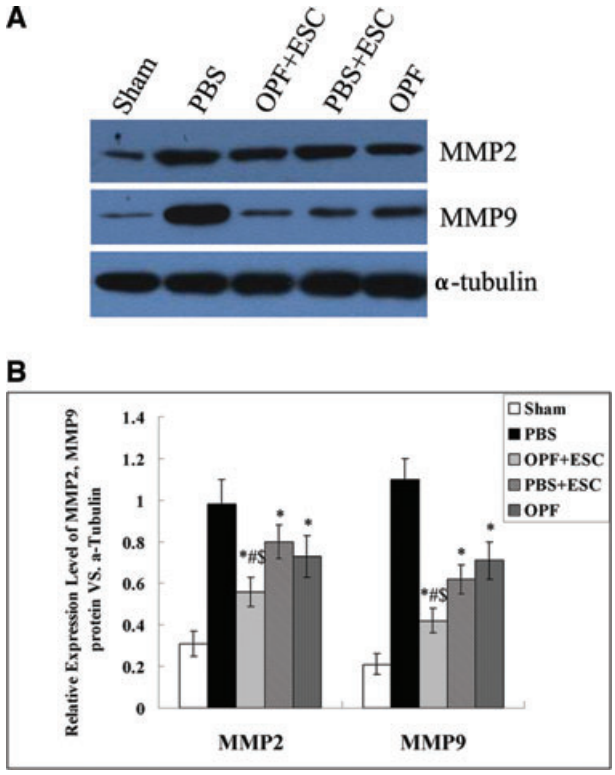

Fig. 5 Effects of OPF hydrogel and mESCs on MMP expression. (**A**) Western blotting analysis of MMP-2 and MMP-9 protein levels. (**B**) Semi-quantitative assay of MMP-2 and MMP-9 protein expression by optical density value. $^{*}P < 0.01$ compared with PBS-only, $^{\#}P < 0.01$ compared with OPF-only, $^{\$}P < 0.01$ compared with PBS + ESC.

showed that MSCs retained their capacity for osteogenic and chondrogenic differentiation when cultured *in vitro* within the OPF hydrogels [27, 32]. However, whether encapsulated embryonic stem cells within OPF hydrogel could retain their viability and the differentiation ability have not been investigated. Our results also showed that with ascorbic acid induction, mESCs in OPF 10K hydrogel maintained the ability of differentiating into cardiomyocytes and other cell types of all three lineages *in vitro*, indicating good cytocompatibility of OPF hydrogels with ESCs.

The strength of injectable biomaterials is the improvement of cell retention as well as the survival of the transplanted cells. In this study, we found that the 24 hrs retention of mESCs and 4-week graft size increased significantly when the mESCs were encapsulated in OPF hydrogels. The cell encapsulation was performed using a thermal radical initiation system (APS/TEMED), which enables crosslinking *in situ*. Previous studies demonstrated the times to onset and completion of gelation for the OPF 10K hydrogels were within 4 and 15 min. at 37°C [26]. This ability of *in situ* gelation facilitates retention of cells at the injection site. Another reason for the increased 4-week graft size was related to the increased arteriole/venule densities. Results showed that the injection of OPF hydrogel together with mESCs significantly increased the arteriole/venule densities within the infarcted scars. An increase in blood supply would provide an optimal microenvironment for the cells to survive [33].

One of the most important findings of this study is that the injection of OPF hydrogel alone inhibited infarcted ventricle remodelling, significantly reduced the infarcted size and improved the cardiac function after MI. Progressive thinning and enlargement of the infarct zone occurred during the early stage of MI [34]. The OPF hydrogel alone had not increased the angiogenesis compared to PBS alone. Although this did not translate into significant differences in FS between the PBS and OPF hydrogel groups, these results suggest that the structural and mechanical support for the injured LV provided by OPF hydrogel contributed more than angiogenesis to the improved LV function. This is in agreement with others who have suggested that biomaterials can decrease infarct size and improve cardiac function in the absence of cells and growth factors. Recent experiments in small animals suggested that direct injection of biomaterials into the infarcted areas could act to internally check the expansion of myocardium to limit the LV remodelling [11–17]. Compared with biologically derived materials such as fibrin, collagen, alginates, chitosan and self-assembling peptides which can provide a platform to increase the delivery and/or support of cells or cytokines, the OPF hydrogel can provide additional advantages because it is inert (unlikely to induce inflammatory response), easy to manufacture (and therefore, more cost-efficient than the biologically derived materials), and it is synthetic, avoiding the possible pathogen transfer.

Preserving collagen homeostasis is likely to ameliorate heart failure [35]. Inhibition of MMP activities could prevent progressive left ventricle remodelling and improved heart function in an animal model of heart failure [36]. Studies also indicated that MSC transplantation reduced left ventricle remodelling through decreased expression of MMP2 and MMP9 in the infarcted hearts [37, 38]. In our study, we found that all treatments have significantly

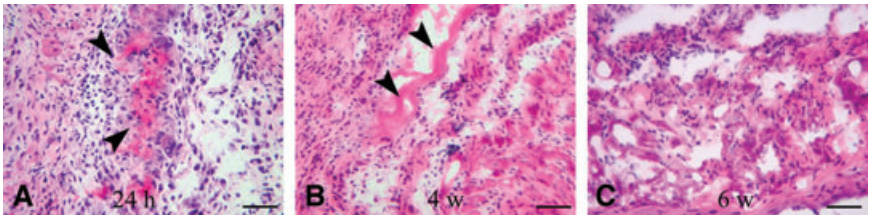

Fig. 6 Degradation of OPF hydrogel in the infarcted heart. Representative haematoxylin and eosin staining images of OPF hydrogel in the infarcted heart after 24 hrs (**A**), 4 weeks (**B**) and 6 weeks (**C**) of transplantation. Arrowheads indicate the OPF hydrogels. Scale bars = 100 μm.

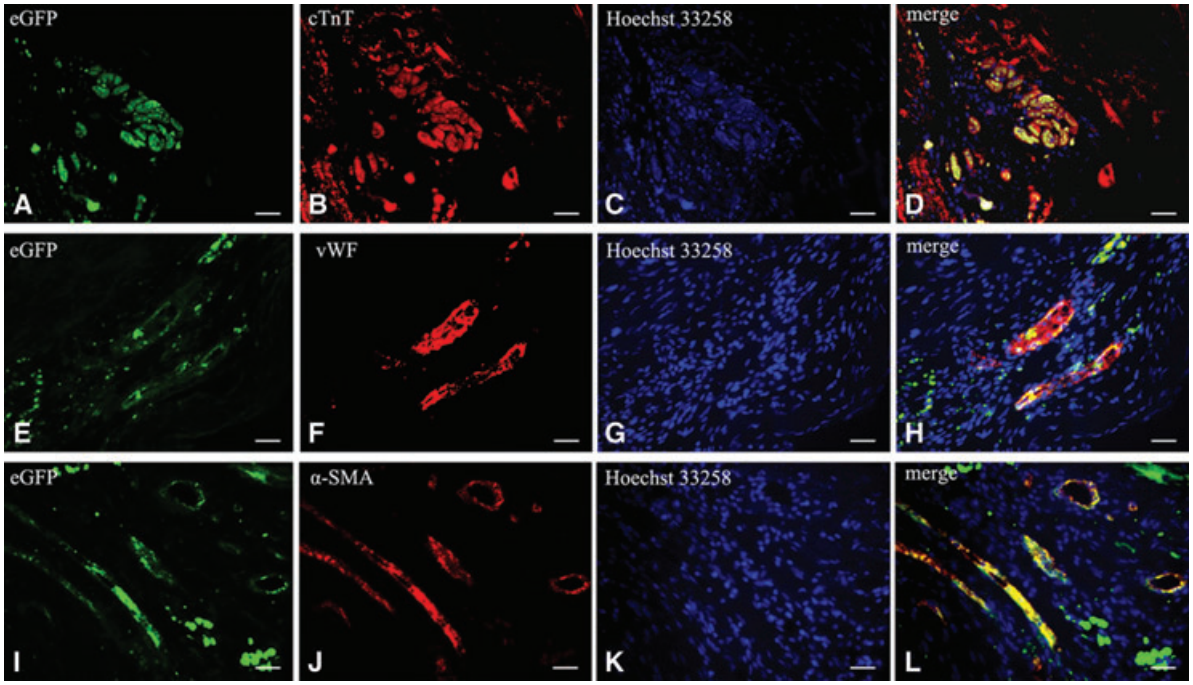

Fig. 7 Differentiation of mESCs encapsulated in OPF hydrogel in the infarcted heart 4 weeks after transplantation. Representative immunofluorescence images of eGFP-labelled cells immunostained with antibodies against cTnT (**A–D**), vWF (**E–H**) and SMA (**I–L**). Scale bars = 30 μm.

reduced the MMP2 and MMP9 protein levels compared to the PBS control group and most notably in the OPF + mESCs group. These changes could contribute to the degradation of collagen in the infarcted areas, and inhibit the formation of poorly cross-linked collagens, which could result in attenuation of cardiac fibrosis and improvement of heart function.

Because ESCs are pluripotent stem cells, the possibility of inducing tumour formation has to be evaluated when the cells

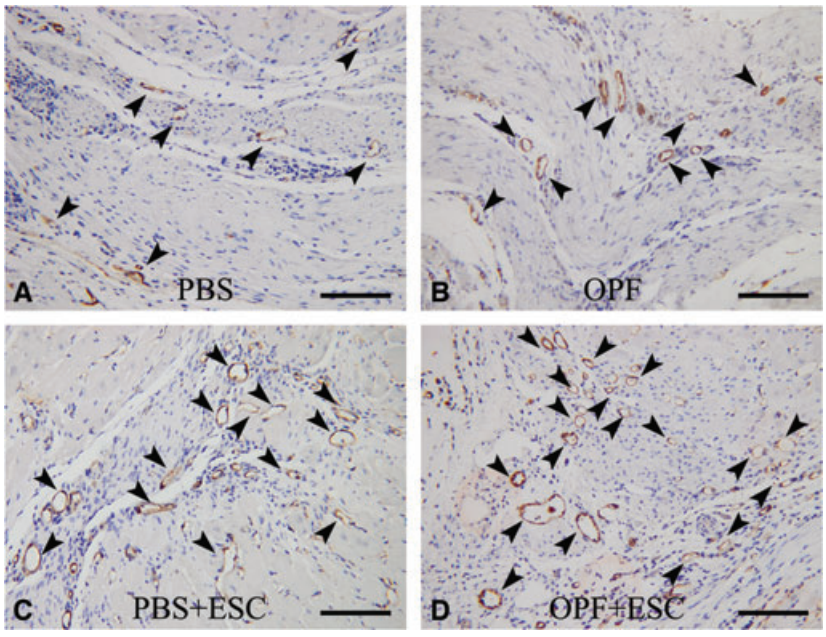

Fig. 8 Arteriole/venule density (vWF stain) in the myocardial infarction sites after 4 weeks of injection. (**A**) The arteriole/ venule density is low in PBS-only group within infarct area. (**B**) The group injected with OPF exhibited higher but not significant arteriole density than the one receiving PBS, failing to improve arteriole density. (**C**) ESCs cells injected in PBS improved the arteriole/ venule density. (**D**) mESCs injected in OPF hydrogel improved arteriole/venule density significantly. The diagram shows the statistical results. $^{*}P <$ 0.01 compared with PBS-only, $^{\#}P < 0.01$ compared with OPF-only, $^{\$}P < 0.01$ compared with PBS + ESC. Scale bars = 100 μm. Black arrows indicate the arterioles/venules in the infarcted area.

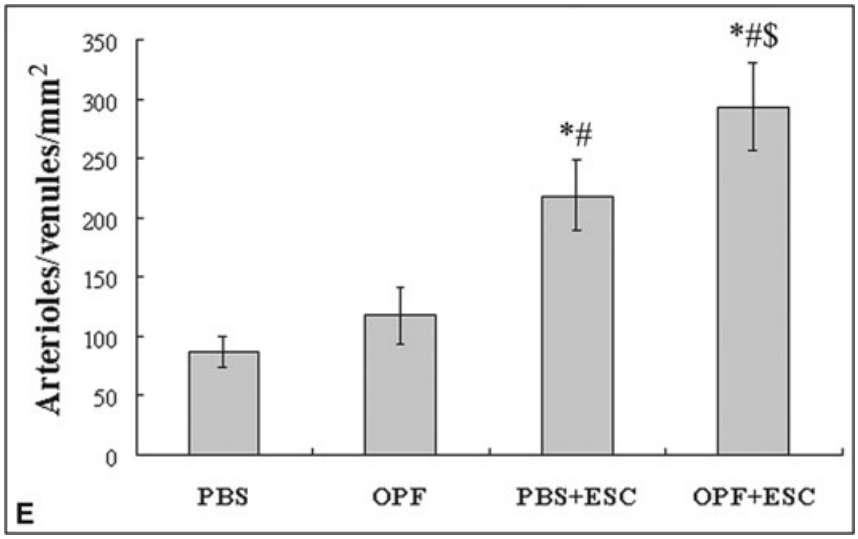

Fig. 8 Continued.

are transplanted *in vivo*. In this study, we carefully tested the samples morphologically and histologically, no tumour formation was observed in the histopathological sections of infarcted myocardium 4–6 weeks post-transplantation of mESCs. Nonetheless, the possible formation of tumour post-transplantation cannot be neglected [39]. Further investigations may also need to verify whether the OPF scaffold formed in the infarcted myocardium could prevent tumours from forming. Moreover, the differentiation of ESCs to specific cell types would be a better solution to avoid tumour formation.

The study has limitations. Firstly, the beneficial effects in terms of cardiac function and LV remodelling were only evaluated in the 4-week period following injection. The architectural and functional changes need to be examined for a longer duration. Secondly, the small animal model employed is limited. Because the hydrogel volumes injected and the volume of the infarcted LV wall are markedly different in the clinical setting, a larger animal model is needed to effectively evaluate the prospect of this approach. Although there are limitations to this study, it provides important initial data for continued development of the technique on the road to clinical translation and that limitations to the present model will be addressed in continued studies toward clinical translation.

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Conflict of interest

The authors declare no conflicts of interest.

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