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ORIGINAL ARTICLE

Sustained release of basic fibroblast growth factor using gelatin hydrogel improved left ventricular function through the alteration of collagen subtype in a rat chronic myocardial infarction model

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Abstract

Objective Chronic myocardial infarction (CMI) tends to be resistant to treatments possibly due to extensive solid fibrotic scar, hypoxia mediated by poorly vascularized environment, and/or inflammation and apoptosis. Here we aimed to testify the therapeutic effects of sustained release of basic fibroblast growth factor (bFGF) using gelatin hydrogel (GH) in a rat chronic MI model and to elucidate the therapeutic mechanism including the alteration of extracellular matrix component.

Methods CMI model rats are prepared by the permanent ligation of proximal left anterior descending coronary artery. After 4 weeks, GH sheets (GHSs) with bFGF (100 μg) (bFGF group) or with phosphate-buffered saline (Vehicle group) were implanted to the CMI models to evaluate the effect of bFGF–GHS on chronic scar tissue. Sham operation group was also prepared ($n = 5$ for each).

Results 4 weeks after implantation, bFGF–GHS significantly improved cardiac contractile function (fractional shortening: 21.8 ± 1.1 vs 21.5 ± 1.3 vs $29.7 \pm 1.8\%$; $P < 0.001$/fractional area change: 33.0 ± 1.4 vs 34.1 ± 2.3 vs $40.6 \pm 1.8\%$; $P < 0.001$) (Sham vs Vehicle vs bFGF) accompanied with neovascularization. Immunohistochemical studies revealed that bFGF–GHS increased collagen III/I ratio indicating the alteration of solid scar tissue. Quantitative RT-PCR results showed a decrease of collagen I mRNA expression within border MI zone.

Conclusions The implantation of bFGF–GHS altered the collagen subtype of the fibrotic scar more suitable for tissue repair. The treatment of sustained-release bFGF may be promising for ischemic heart disease through chronic pathology.

Keywords Basic fibroblast growth factor · Gelatin hydrogel · Ischemic heart disease · Drug delivery system · Collagen

Introduction

Despite numerous achievements in medical and surgical treatments for cardiac diseases so far, myocardial infarction (MI) remains a major cause of morbidity and mortality worldwide [1]. Cardiac regenerative therapy is a newly emerging therapeutic paradigm for the ischemic disorders of the heart resistant to conventional therapies [2]. However, the therapeutic effects of regenerative cell therapies are rather limited in clinical trials for chronic MI (CMI) [3, 4] possibly due to extensive solid fibrotic scar, hypoxia mediated by poorly vascularized environment, and/or inflammation and apoptosis leading to vast loss of transplanted cells [5]. Considering increasing populations of severe ischemic heart diseases, the improvement of the therapeutic efficiency of regenerative therapy to CMI is anticipated.

Basic fibroblast growth factor (bFGF) is one of the most potent growth factors known to promote stem cell survival and to induce neovascularization [6–9]. Sustained release of bFGF using gelatin hydrogel (GH) extends its clearance rate to weeks and maintains stable concentration in situ [10, 11]. Although the beneficial effect of sustained release of bFGF on rat MI models has been declared [12], the underlying mechanism needs to be further elucidated.

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In the present study, we attempted to validate the functional and myocardial regenerative efficiency of the bFGF-incorporated GH in a rat CMI model and to elucidate the therapeutic mechanism including the alteration of extracellular matrix (ECM) component.

Methods

All protocols were approved by the Kyoto University Animal Experimentation Committee and performed in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to Japanese law and the Guide for the Care and Use of Laboratory Animals prepared by the Institute for Laboratory Animal Research, USA (revised 2011).

Preparation of bFGF-incorporated gelatin hydrogel sheet and gelatin hydrogel microspheres

GH were prepared by chemical crosslinking of gelatin (isoelectric point 5.0, Nitta Gelatin Inc., Osaka, Japan) as described previously [10, 13–16]. For GH sheet (GHS) preparation, 5 wt% aqueous solution of gelatin containing glutaraldehyde was settled in a dish at 4 °C for 12 h to induce crosslinking. The resulting hydrogel sheets were immersed in 0.1 M glycine solution at 37 °C for 1 h to inactive the residual aldehyde groups of glutaraldehyde. Next, the sheets were washed three times with double-distilled water and freeze-dried at − 80 °C followed by ethylene oxide gas sterilization.

To incorporate bFGF into the GHS, 1 μg/μl of bFGF solution (100 μl) was dropped onto 1 cm^2 square GHS which was then incubated at an ambient temperature for 1 h to allow bFGF to fully be incorporated into the gelatin hydrogel. The GHSs were designed to slowly release bFGF in animal bodies for approximately 3–4 weeks after treatment which is certified by previous in vitro and in vivo studies [10, 15, 17, 18].

Preparation of CMI model rats

All procedures in vivo (coronary ligation, bFGF–GHS implantation, echocardiogram) were performed under general anesthesia maintained with isoflurane and respiratory control with Harvard-type ventilators. On echocardiogram, the respiration was discontinued temporarily on the timing of data recording to avoid respiratory bias.

Male rats (F344/NJcl-rnu/rnu, CLEA Japan) aged between 12 and 16 weeks were used for generating CMI model. Prior to all surgical operations, the animals were anesthetized with 3.0% isoflurane-mixed air inhalation with a vaporizer, intubated endotracheally, and maintained with 1.5% isoflurane.

MI was induced by permanent ligation of left anterior descending artery as previously described [12, 19, 20]. 4 weeks after MI induction, the rats whose hearts showed less than 25% of left ventricular (LV) fractional shortening (FS) with echocardiogram were assigned to the study.

Implantation of bFGF–GHS

4 weeks after MI induction, the 15 rats assigned for study of sustained release of bFGF were then randomly divided into three groups: bFGF–GHS-treated (bFGF) group, GHS incorporated with phosphate-buffered saline (PBS) group (Vehicle) and Sham group ($n = 5$ for each). We did not add a group of bFGF treatment without GHS because of the already known very short half-life of the bFGF in vivo [21]. In bFGF and Vehicle group, bFGF–GHS of GHS–PBS were placed directly over the infarct area with one suture through thoracotomy. In Sham group, the rats underwent thoracotomy only. The rats were sacrificed 4 weeks after treatment and the hearts were harvested for immunohistochemistry and qPCR analyses.

Assessment of cardiac performance

Transthoracic echocardiogram was performed with the Vivid 7 system (GE Healthcare, Waukesha, WI) and an 11-MHz imaging transducer (GE 10S ultrasound probe, GE Healthcare). Echocardiographic measurements were performed as previously described [22, 23]. Diastolic dimensions of LV (LVDd) were recorded and measured with M-mode examination. Diastolic and systolic area of LV (LVAd, LVAs), diastolic lengths of LV inner circumference (CIRCd) and those of akinetic area in diastole (SCAR) were recorded and measured with B-mode examination, values were calculated as follows:

$$\text{Fractional shortening (FS)}(\%) = (\text{LVDd} - \text{LVDs})/\text{LVDd},$$
$$\text{Fractional area change (FAC)}(\%) = (\text{LVAd} - \text{LVAs})/\text{LVAd},$$
$$\text{Akinetic length (AL)}(\%) = \text{SCAR}/\text{CIRCd}.$$

Histological analysis

Excised hearts were perfusion fixed in 4% paraformaldehyde and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan) and frozen embedded. Five sections with 6 μm thickness were made at 50-μm intervals along the short axis and examined. Sirius red staining was used to observe fibrosis [24]. For immunofluorescent staining, sections were treated with Protein Block Serum Free (DAKO) and incubated for 60 min with primary antibodies

at room temperature. Anti-mouse Alexa 546 or 488 (1:500) and anti-rabbit Alexa 488 (Invitrogen) (1:400) were used as secondary antibodies. To evaluate collagen deposition, anti-collagen I antibody (Mouse monoclonal, Abcam, Cambridge, UK) (90395) and anti-collagen III antibody (Rabbit polyclonal, Abcam, Cambridge, UK) (77778) were used for double staining with collagen I and collagen III. Ten different views were randomly selected within either border MI zone or MI zone (×400, original magnification). The areas of collagen I and collagen III were measured using the Image J software (National Institutes of Health, USA) [25], and the ratio of collagen III/I was calculated. Values corresponding to each rat are an average of five calculated sections for each rat heart. For capillary density (capillary number/mm^2), eight different views of every rat heart within the border MI zone (×400, original magnification) were randomly selected from Von Willebrand factor (vWF)-stained sections, and the number of capillaries was manually counted in each view. For vWF staining, a rabbit polyclonal antibody (DAKO) was used as the primary antibody (1:800). A mouse monoclonal cardiac troponin T (cTnT) antibody (clone 13211, Thermo Fisher Scientific; 1:200) was used for double staining with cTnT and vWF. All immunostained sections were photographed, measured and calculated with Biorevo BZ-9000 (Keyence, Osaka, Japan).

Reverse-transcription polymerase chain reaction

Total RNA was extracted from border MI zone of hearts and reverse transcribed using the Invitrogen SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Eugene, OR, USA). Quantitative reverse-transcription polymerase chain reaction (qPCR) was performed using Taqman Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA, USA) and StepOnePlus system (Applied Biosystems). Gene-specific primers for rat collagen I (Taqman, Col1a1, Rn01463848_m1), collagen III (Taqman, Col3a1, Rn01437681_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Taqman, Gapdh, Rn01775763_g1) were purchased from Applied Biosystems (Foster City, CA). Relative mRNA expression levels were normalized to the amount of GAPDH mRNA.

Statistical analysis

The values are presented as mean standard deviation. Statistical analyses were performed with IBM SPSS statistics 23 software (IBM Corp. Armonk, NY, USA). Comparisons between two groups were made with the unpaired *t* test. Other intergroup comparisons were made with one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was assumed when the *P* value was less than 0.05.

Results

bFGF–GHS treatment improved cardiac function on echocardiogram

GHSs with 100 μg bFGF (bFGF group) or with PBS (Vehicle group) were placed to cover the ischemic scar of CMI model rats 4 weeks after MI induction. We examined cardiac function with echocardiogram for 4 weeks (Fig. 1). The parameters of LV systolic function, FS and FAC (Fig. 1a, b) were significantly higher in bFGF group compared to those in Sham group or Vehicle group at 4 weeks after treatment (FS: 21.8 ± 1.1 vs 21.5 ± 1.3 vs 29.7 ± 1.8%; $P < 0.001$/FAC: 33.0 ± 1.4 vs 34.1 ± 2.3 vs 40.6 ± 1.8%; $P < 0.001$) (Sham vs Vehicle vs bFGF). The percentage of akinetic endocardial length in the whole LV endocardial circumference

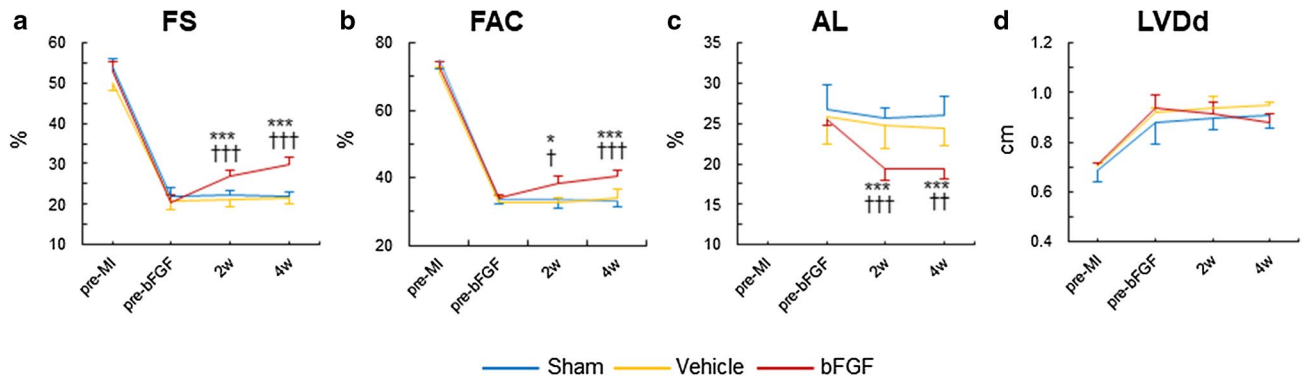

Fig. 1 Echocardiograms of cardiac function after bFGF–GHS treatment on rat chronic MI. **a, b** The left ventricular contractile function assessed by FS (**a**) and FAC (**b**) at 2 and 4 weeks after treatment. **c** Akinetic length assessed by AL at 2 and 4 weeks after treatment. **d** The diameter of left ventricle assessed by LVDd. *$P < 0.05$, ***$P < 0.001$ (bFGF vs Sham). $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ (bFGF vs Vehicle). $n = 5$ for each. *FAC* fractional area changes, *FS* fractional shortening, *AL* akinetic length, *LVDd* diastolic diameter of left ventricle, *pre-MI* before induction of myocardial infarction, *pre-bFGF* before bFGF–GHS (gelatin hydrogel sheet) treatment

(AL) (Fig. 1c) was significantly smaller in the bFGF group than those in the Sham group or Vehicle group at 4 weeks after treatment (26.1 ± 2.3 vs 24.4 ± 2.0 vs 19.3 ± 1.2%; $P < 0.001$). Although LVDd showed no significant difference among all of the three groups, the extent of dilation exhibited a tendency of decrease in bFGF group (Fig. 1d).

bFGF–GHS treatment attenuated left ventricular remodeling and induced neovascularization at the border zone of MI

Histological analyses were performed 4 weeks after bFGF–GHS treatment (Fig. 2). Sirius red staining exhibited that treatment that showed a tendency of limited scar extension limited the extent of fibrosis (Fig. 2a, b) (MI length/total length: 40.9 ± 9.0 vs 31.4 ± 7.0%, $P = 0.098$) (Sham vs bFGF) and significantly limited thinning of the infarct wall at 4 weeks after treatment (Fig. 2a, c) (483 ± 157 vs 1026 ± 243 μm, $P < 0.01$), indicating that bFGF–GHS treatment ameliorated LV remodeling. Capillaries stained with vWF showed a higher capillary density within border MI zone in the bFGF group compared with that in the sham group (Fig. 2d, e) (6.4 ± 2.3 vs 12.4 ± 3.2 /mm^2, $P < 0.001$).

bFGF–GHS altered collagen subtype at MI zone

We next examined the ECM components within ischemic area by immunostaining for collagen I and collagen III. Quantitative estimation showed that collagen III/I ratio in both border zone and MI zone was significantly higher in bFGF group (Fig. 3a, b) (border zone: 0.65 ± 0.42 vs 1.30 ± 0.31%, $P < 0.05$/MI zone: 0.98 ± 0.46 vs 1.76 ± 0.81, $P < 0.05$) (Sham vs bFGF), indicating that bFGF–GHS treatment regulated and altered collagen I and collagen III deposition more suitable for tissue repair. We further detected the mRNA expression of collagen I and collagen III using qPCR (Fig. 3c, d). The collagen I mRNA level in border MI zone was significantly lower in bFGF group compared to Sham group (1.00 ± 1.87 vs 0.53 ± 0.14, $P < 0.01$), whereas the amount of collagen III mRNA showed no significant difference between those two groups (1.00 ± 1.90 vs 1.41 ± 0.25).

Discussion

In the present study, we testified the effects of sustained release of bFGF on CMI and further showed the underlying mechanisms of the alteration of collagen subtype at the scar region. Our results show that the treatment of sustained release of bFGF may be a promising treatment for ischemic heart diseases in chronic phase.

Recent studies of cardiac regeneration using stem cells derived from various sources to models of acute or subacute MI demonstrated fair improvement of cardiac performance as well as a decent survival rate [22, 26, 27]. However, when transplanted to CMI models, both beneficial effects and engraftment were limited [28]. As the heart is post-mitotic and one of the least regenerative organs in

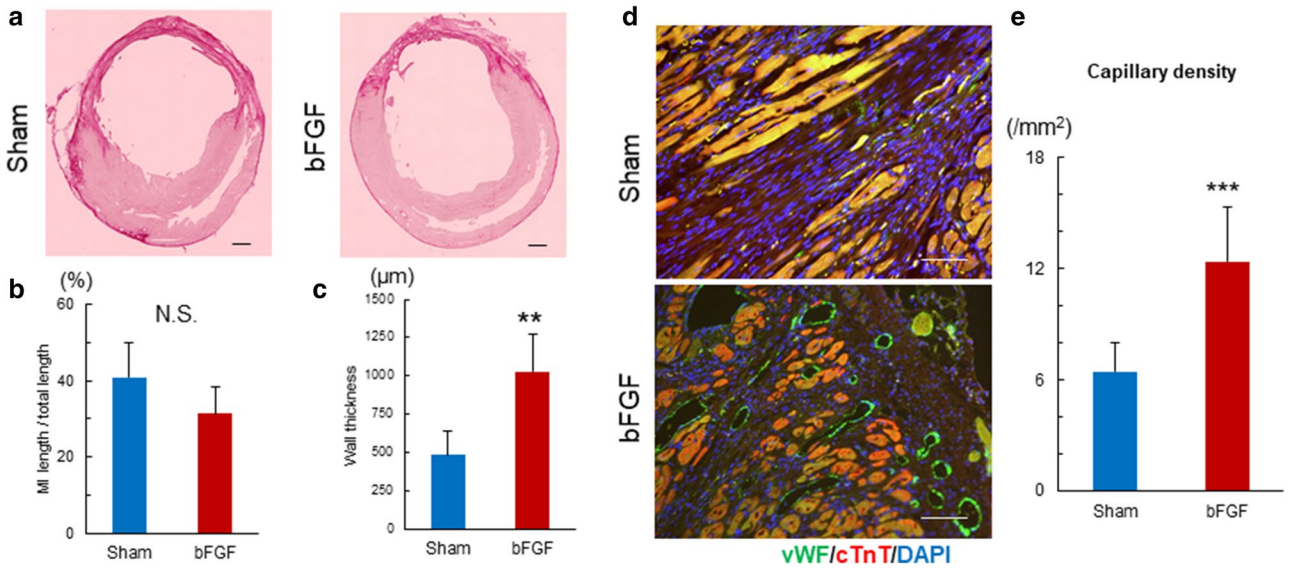

Fig. 2 Histological examination of host myocardium for fibrosis and capillary formation 4 weeks after bFGF–GHS treatment. **a–c** Extent of fibrosis and thickness of the infarct wall. Representative section of Sirius red staining are shown in **a**; scale bar 1000 μm. **b** The ration of MI length to total length. **c** Wall thickness. **d, e** Capillary formation within border MI zone. Photomicrographs of double staining for vWF (green) and cTnT (red) are shown in **d**; scale bar 100 μm. **e** The capillary density in the border MI zone. *N.S.* not significant, $^{**}P < 0.01$, $^{***}P < 0.001$. $n = 5$. *cTnT* cardiac troponin T, *DAPI* 4′, 6-diamidino-2-phenylindole, *vWF* von Willebrand factor

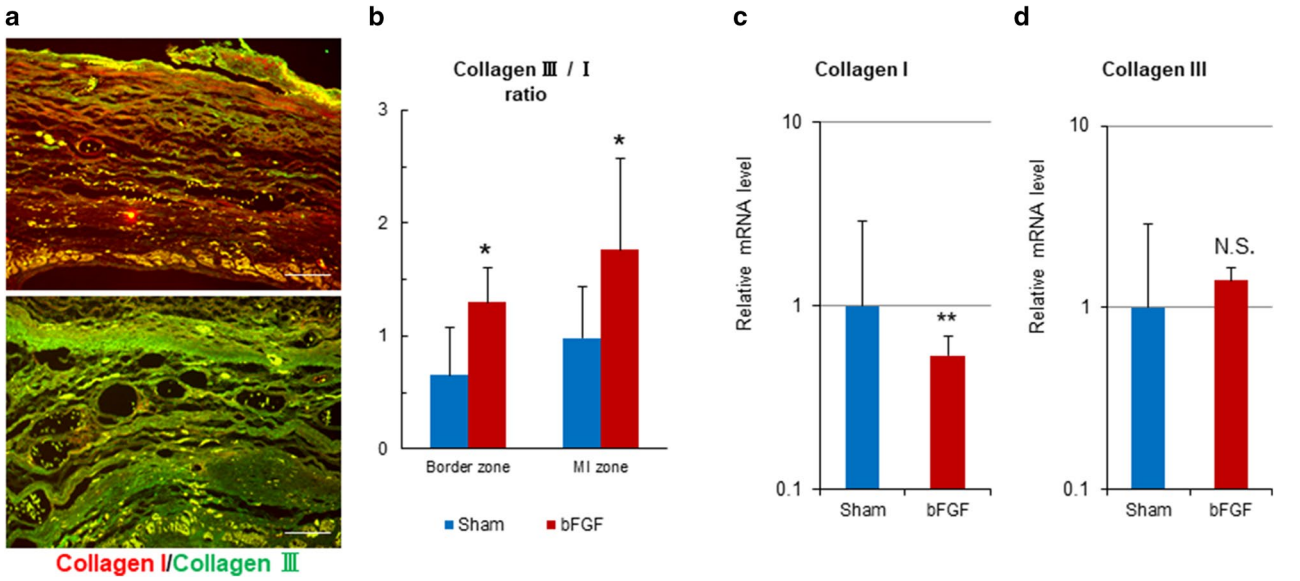

Fig. 3 Histological examination and qPCR analyses of host myocardium for collagen subtypes 4 weeks after bFGF–GHS treatment. **a, b** Collagen I and collagen III distribution evaluated by immunohistochemical analysis. Photomicrographs of double staining for collagen I (red) and collagen III (green) are shown in **a**; scale bar 100 μm. **b** Collagen III/I ratio in border and central MI zone. **c, d** Relative mRNA expression level of collagen I and collagen III within border MI area measured by qPCR. Collagen I expression was decreased in bFGF group than in Sham group. *N.S.* not significant, $*P<0.05$, $**P<0.01$. $n=5$. qPCR, quantitative real-time polymerase chain reaction

the body, recurrent MI often leads to the development of chronic heart failure [29]. More like the situation of clinical patients, myocardium is often irreversibly damaged and fibrotic scar is stiffly formed in the infarct zone on CMI models. Towards further clinical application, the obstacle needs to be surmounted.

In the present study, we took a drug delivery method to ameliorate the pathological condition of the recipient heart. The delivery approach for bFGF using biodegradable GH prolonged the half-life period of bFGF to a few weeks and avoided repeated administration [30]. bFGF-incorporated GH has been studied in several animal studies and clinical researches and the safety and efficacy were proven. Moreover, GH could provide stable local concentration of bFGF which avoids multiple interventions and is less invasive for the recipients [11, 14, 31].

Deposition of ECM plays a pivotal role in LV remodeling after MI, which results in fibrotic scar formation thus severely limiting cardiac contractile function [32]. Collagen I and collagen III are the major components of ECM [33, 34]. Collagen I was shown to be thick fiber which provides tensile strength while collagen III was shown to be thin fiber with resilience [35]. Accompanied with the development of myocardial fibrosis, cardiac stiffness advances due to the unbalanced deposition of collagen I and collagen III. In the present study, bFGF–GHS treatment downregulated collagen I expression, thereby elevated the ratio of collagen III to collagen I. Relatively large volume of collagen III, which is composed of loose reticular fiber, provides better capacity for cardiac motion and more space for nutrient vessels ingrowth. In accordance with former studies [12], bFGF–GHS also abundantly increased regional myocardial blood flow by inducing angiogenesis. The mutual promotion of those two effects further improved LV contractile function. The precise biological mechanisms of the alteration of collagen subtype mediated by bFGF–GHS and the alteration of other ECMs such as elastin, glycosaminoglycans, and fibronectin should be investigated in future studies. It is also required to evaluate the involvement of macrophages and the subtypes (M1/M2) in the present bFGF–GHS treatment which is recently reported to serve as a pivotal role in tissue repair [36], and the roles of various growth factors potentially related to the tissue repair.

Considering the surgical application of the present technology, the combination therapy of coronary artery bypass grafting and the implantation of bFGF–GHS would be a possible strategy. According to the results of the present study, surgical revascularization for viable region and concomitant sustained release of bFGF for chronically ischemic region aiming for neovascularization may theoretically enhance the therapeutic effects of the surgery. Before clinical application of the bFGF–GHS treatment, we should conduct further preclinical studies with sufficient number of animals and longer period of observation period to evaluate the dose-dependent therapeutic effects and safety which is not conducted in the present study.

Conclusion

The treatment of CMI heart with sustained-release bFGF effectively attenuated cardiac remodeling and prompted angiogenesis through the alteration of collagen subtype. This therapeutic strategy would be a promising option to enhance the efficacy of regenerative therapy for ischemic heart diseases.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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