Post-infarct treatment with an erythropoietin–gelatin hydrogel drug delivery system for cardiac repair

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Aims We investigated the effect of an erythropoietin (EPO)–gelatin hydrogel drug delivery system (DDS) applied to the heart on myocardial infarct (MI) size, left ventricular (LV) remodelling and function.

Methods and results Experiments were performed in a rabbit model of MI. The infarct size was reduced, and LV remodelling and function were improved 14 days and 2 months after MI but not at 2 days after MI in the EPO-DDS group. The number of cluster of differentiation 31(CD31)-positive microvessels and the expression of erythropoietin receptor (EPO-R), phosphorylated-Akt (p-Akt), phosphorylated glycogen synthase kinase 3b (p-GSK-3b), phosphorylated extracellular signal-regulated protein kinase (p-ERK), phosphorylated signal transducer and activator of transcription 3 (p-Stat3), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-1 (MMP-1) were significantly increased in the myocardium of the EPO-DDS group.

Conclusion Post-MI treatment with an EPO-DDS improves LV remodelling and function by activating pro-survival signalling, antifibrosis, and angiogenesis without causing any side effect.

Keywords Drug delivery system; Erythropoietin; Akt; VEGF; Angiogenesis; Myocardial infarction

1. Introduction

Erythropoietin (EPO) exerts its haematopoietic effects by stimulating the proliferation of early erythroid precursors and the differentiation of later precursors of the erythroid lineage.¹ The recombinant form of human EPO is now used frequently in the clinic to treat anaemia associated with end-stage renal disease.² Interestingly, recent studies have suggested that EPO also exerts a cardioprotective effect in cases of acute myocardial infarction (MI).³,⁴ With systemic administration, however, the beneficial effects in the context of MI are only observed when large doses of EPO are administered, which are frequently accompanied by polycythaemia and, therefore, the potential for thrombo-embolic complications.⁵,⁶ Thus, in order to use EPO clinically as a cardioprotective agent, one must find a useful way to avoid dangerous side effects such as polycythaemia. To address that issue, we developed a biodegradable gelatin hydrogel patch into which EPO is incorporated and can be administered without inducing polycythaemia. The aims of the present study were to determine whether post-MI treatment with an EPO-containing gelatin hydrogel patch reduces infarct size and improves left ventricular (LV) remodelling and function, and to investigate the local molecular mechanisms underlying the beneficial effects of EPO.

2. Materials and methods

All rabbits used in this study received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication 85-23, revised 1996). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Rabbits were anesthetized with an intravenous injection of sodium pentobarbital (30–40 mg/kg), and additional doses were given when required throughout the experiment. Once anesthetized, the animals were then intubated and ventilated with room air supplemented with a low flow of oxygen using a mechanical ventilator (tidal volume 25–35 mL, respiratory rate 20–30 per minute) (Shimano, model SN-480-5, Tokyo, Japan). Serial blood gas analysis was performed and ventilatory conditions were adjusted to maintain the arterial blood gas within the physiological range. Surgery was performed under sterile conditions. The carotid artery and jugular veins were cannulated to, respectively, monitor peripheral...
arterial pressure and administer drugs. Thereafter, the rabbits were systemically heparinized (500 U/kg), a thoracotomy was performed in the left third intercostal space, and the heart was exposed after excising the pericardium. A 4-0 silk suture on a small-curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle segment of the anterolateral surface of the left ventricle. Both ends of the silk suture were then passed through a small vinyl tube, and the coronary branch was occluded by pulling the suture, which was fixed by clamping the tube with a mosquito haemostat. Myocardial ischaemia was induced for 30 min. Myocardial ischaemia was confirmed by 5T-segment elevation on the ECG and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snares. All rabbits were allowed 20 min after completion of the surgical preparation to reach a steady state before starting the experimental protocol.

2.1 In vivo erythropoietin release ratio from a gelatin hydrogel sheet

Gelatin was prepared from pig skin type I collagen using an acid process and was kindly supplied by Nitta Gelatin (Osaka, Japan). Gelatin hydrogel sheets were made using a previously described process.7,8 Sheets were then freeze-dried and cut into rectangular patches for impregnation with phosphate-buffered saline containing EPO at a concentration of 3000 IU/0.5 mL (Epojin, Chugai Pharmaceutical Co. Ltd, Tokyo, Japan). The prepared hydrogel patches were used immediately after preparation.

We initially carried out a preliminary experiment to determine how long gelatin hydrogel containing EPO would release the drug. We measured the cumulative release of EPO after 0, 1, 5, 7, and 14 days and found that gelatin hydrogel patches containing EPO released EPO continuously for 14 days (Figure 1).

2.2 Measurement of cardiac tissue erythropoietin content

The amount of EPO in the cardiac tissue in the risk and non-risk areas was measured by ELISA (Mitsubishi Kagaku Bio-Chemical Laboratories Inc., Tokyo, Japan) 24 h after injection of EPO or application of gelatin hydrogel patch with and without EPO to the heart.

2.3 Protocol

The subjects were 120 rabbits subjected to 30 min of ischaemia followed by reperfusion, as described above. Rabbits in the EPO-systemic group (n = 30) were subcutaneously injected with 1500 IU/kg of recombinant human EPO (Epojin, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) in 0.5 mL of saline once a day for 5 days (Totally 7500 IU/kg of EPO) starting immediately after infarction. The saline group (n = 30) received the same volume of saline alone. In the EPO-DDS (drug delivery system) group (n = 30), a gelatin hydrogel patch containing 1500 IU/kg EPO was applied to the surface of the risk area of the heart immediately after infarction. In the DDS group (n = 30), a gelatin hydrogel patch only without EPO was applied to the surface of the risk area of the heart immediately after infarction. Rabbits from the Saline, Systemic, DDS, EPO-DDS, groups were sacrificed 2 days, 14 days, and 2 months post-MI (n = 10 in each of group at 2 days, 14 days, and 2 months post-MI, respectively).

2.4 Physiological studies

Echocardiography (SSD2000, Aloka Co. Ltd) was performed on day 14 post-MI and 2 months, and ejection fraction, fractional shortening, and LV end-diastolic and end-systolic dimensions were obtained. Arterial blood pressure and heart rate were also measured via a catheter introduced in the carotid artery. After echocardiography, a micro-manometer-tipped catheter (SPR 407, Millar Instruments) was inserted into the left ventricle to record ±dP/dt. All measurements were made by two persons blinded to the treatment.

2.5 Blood sampling

Blood samples (0.3 mL each) were collected from an ear vein before and 14 days after infarction for peripheral blood cell counts. Peripheral blood cell count was performed using an automatic cell count analyzer (Sysmex XE-2100, Sysmex Corporation, Nagoya, Japan).

2.6 Infarct size and histological analysis

To measure the risk area, excised hearts were mounted on a Langendorff apparatus, and Evans blue dye (4 C) was injected via the aorta for 1 min after reocclusion of the coronary branch. The left ventricle was then weighed and sectioned into seven transverse slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of triphenyl tetrazolium chloride (TTC) for 10 min at 37 C to visualize the infarct area, and photographed. The areas of the ischaemic regions and the infarcted myocardium were traced on each LV slice, multiplied by the weight of the slice, and expressed as a percentage of the risk region or the entire left ventricle. Each slice was then fixed in 10% buffered formalin for 4 h, embedded in paraffin, and cut into 4-µm-thick sections with a microtome. These sections were stained with haematoxylin-eosin, Masson-Trichrome, and Sirius red.

2.7 Immunohistochemistry

We used an indirect immunoperoxidase method for immunohistochemical staining of cardiac sections on day 14 post-MI. The primary antibodies used were monoclonal mouse anti-human CD31, an endothelial cell marker (1:100 dilution; Dako), and monoclonal mouse anti-human MMP-1 antibody (1:400 dilution; Daiichi Fine Chemical Co. Ltd, F-67), each of which cross-reacts with...
2.8 Western blot analysis

Western blot analysis was carried out using lysates from heart tissues on day 2 and day 14 post-MI. Proteins were separated and transferred to membranes using standard protocols, after which they were probed with antibodies against EPO receptor (EPOR) (H-194; Santa Cruz), vascular endothelial growth factor (VEGF) (RDI-VEGFabm-12; Fitzgerald), Bcl-2 (sc-7382; Santa Cruz), and Bcl-xL (sc-8392; Santa Cruz). The phosphorylation (activation) of Akt, signal transducer and activator of transcription 3 (Stat3), and extracellular signal-regulated protein kinase (ERK) were assessed using antibodies against Akt, phosphorylated (p)-Akt, Stat3, p-Stat3, ERK, and p-ERK (all from cell signalling), and phosphorylated glycogen synthase kinase-3β (p-GSK-3β) (Santa Cruz Biotechnology, Inc.). The blots were visualized by means of chemiluminescence (ECL, Amersham) and the signals were quantified by densitometry. α-Tubulin (analysed with an antibody from Santa Cruz) served as the loading control.

2.9 Statistical analysis

All values are presented as means ± SEM. Differences among the Saline, EPO-DDS, DDS, and EPO-systemic groups were assessed by ANOVA with a Bonferroni correction. Values of P < 0.05 were considered significant.

3. Results

3.1 Measurement of the erythropoietin content of cardiac tissue

The levels of EPO in the cardiac tissue in risk and non-risk areas 24 h after injection of EPO or application of a gelatin hydrogel patch, with or without EPO, to the heart are shown in Table 1. EPO was only detected in hearts treated with EPO-containing gelatin hydrogel patches, and levels were significantly higher in the infarct areas than in the remote areas of the heart.

3.2 Physiological studies

Echocardiography and cardiac catheterization carried out 14 days and 2 months post-MI revealed that the LV end-systolic and end-diastolic dimensions were significantly smaller, while the LV ejection fraction, fractional shortening, and ±dp/dt were significantly larger in the EPO-DDS group than in the Saline, DDS, or EPO-systemic group (Figure 2).

<table>
<thead>
<tr>
<th>Heart (mIU/mg)</th>
<th>Serum (mIU/mg)</th>
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<tr>
<td>Infarct areaa</td>
<td>Remote area</td>
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<tr>
<td>Systemic</td>
<td>&lt;0.08</td>
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<tr>
<td>DDS-EPO</td>
<td>371 ± 182.4</td>
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<tr>
<td>DDS</td>
<td>371 ± 182.4</td>
</tr>
<tr>
<td>EPO-systemic</td>
<td>371 ± 182.4</td>
</tr>
<tr>
<td>Saline</td>
<td>99.6 ± 5.7</td>
</tr>
<tr>
<td>EPO-DDS</td>
<td>255 ± 68.9</td>
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</tbody>
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3.3 Peripheral blood cell count

There were no significant differences among the groups with respect to the white blood cell and thrombocyte counts made on days 0 and 14 post-MI. On the other hand, by 14 days post-MI, erythrocyte counts and haematocrit had increased significantly from 558 ± 79 × 10^12 to 677 ± 28 × 10^12 per µL and from 37.2 ± 1.6 to 44.0 ± 2.3%, respectively, in the EPO-systemic group, but not in the saline group (from 575 ± 14 × 10^12 to 538 ± 20 × 10^12 per µL and from 38.0 ± 0.7 to 36.1 ± 1.4%, respectively) or in the EPO-DDS group (from 567 ± 13 × 10^12 to 568 ± 26 × 10^12 per µL and from 37.8 ± 0.9 to 37.8 ± 1.4%, respectively) or DDS group (from 560 ± 13 × 10^12 to 574 ± 28 × 10^12 per µL and from 37.1 ± 1.3 to 38.3 ± 1.2%) at day 14 post-MI.

3.4 Infarct size

On day 2 post-MI, the infarct size as a percentage of the area at risk was not different statistically among the Saline, EPO-systemic, DDS, and EPO-DDS groups; however, the infarct size in the EPO-DDS group tended to reduce when compared with the saline group (P < 0.10) (Figure 2F). The infarct size in the EPO-DDS group was significantly smaller than in the Saline, EPO-systemic, or DDS group on 14 days (Figure 2F). The infarct size on 2 months post-MI was also smaller in the EPO-DDS group than in the Saline, EPO-systemic, or DDS group (Figure 2F).

3.5 Histological analysis

Fibrotic areas that stained positive with Sirius red or Masson Trichrome were significantly smaller in the EPO-DDS group than in the Saline, EPO-systemic group, or DDS group on 14 days post-MI (Figure 3).

3.6 Western blot analysis

Western blot analysis revealed that myocardial expression of EPOR was markedly stronger in hearts in the EPO-DDS group than in the EPO-systemic or Saline group on day 2 post-MI (Figure 4). Receptor-associated Janus family tyrosine kinase (Jak)/Stat, PI3K/Akt, and mitogen-activated protein kinase (MAPK)/ERK are all known to be downstream mediators of EPOR signalling in cardiac cells, both in vitro and in vivo. In the infarct border zone, levels of Stat3, Akt, ERK, and GSK-3β activation, as indicated by the presence of the phosphorylated forms (p-Akt, p-GSK-3β, p-Stat3, and p-ERK) were significantly higher in hearts from the EPO-DDS group than in those from the EPO-systemic or Saline group on day 2 post-MI (Figure 4). In addition, expression of ProMMP-1, VEGF, and Bcl-2, but not Bcl-xL, was upregulated in hearts from the EPO-DDS group, but not in those from the EPO-systemic or Saline group on day 2 post-MI (Figure 5A and C). On day 14 post-MI, the expressions of EPO-R, p-Akt, p-ERK, and pro-MMP-1 were upregulated (Figure 5B) but not VEGF, Bcl-2, Bcl-xL, p-Stat3, or p-GSK3β (data are not shown).

3.7 Immunohistochemistry

On day 14 post-MI, the density of the CD31-positive microvessels (capillary density) was clearly greater in the infarct border zone in hearts from the EPO-DDS group than in the EPO-systemic or Saline group (Figure 6). However, the...
three groups did not differ with respect to the densities of CD31-positive microvessels in the remote uninfarcted area or in the infarcted area. In addition, expression of proMMP-1 was upregulated in the border zone and uninfarcted areas in the EPO-DDS group, but not in the EPO-systemic or Saline group (Figure 6).

4. Discussion

The present study demonstrated that EPO-gelatin hydrogel patches, but not systemic administration of EPO, reduced MI size and improved LV remodelling and function with no adverse side effects such as polycythaemia by activating...
prosurvival signalling and exerting antifibrotic and angiogenic effects.

There have been some reports demonstrating that pre- and post-ischaemic treatment with EPO has a cardioprotective effect such as an infarct size-reducing effect in rats.\textsuperscript{3,9–11} Others reported that EPO administered at reperfusion did not show an infarct size-reducing effect in sheep and pigs.\textsuperscript{12,13} However, there has been only one report investigating the effect of EPO on the infarct size in a rabbit model of MI.\textsuperscript{4} In rabbits, Parsa et al.\textsuperscript{4} reported that EPO administered even at the time of reperfusion at doses of 1000 and 5000 IU/kg significantly reduced the MI size, although EPO did not improve cardiac function. Therefore, in the present study, we administered EPO after reperfusion at a dose of 1500 IU/kg for 5 days (7500 IU/kg in totally), a similar dose of EPO used by Parsa et al. as a systemic EPO group. As a result, inconsistent with the report by Parsa et al.,\textsuperscript{4} the MI size was not reduced in the present study. On the contrary, EPO gelatin hydrogel patch which contains only 1500 IU/kg of EPO, one-fifth of the dose administered for systemic EPO treatment, reduced the infarct size in the present study.

### 4.1 Release of erythropoietin

In the present study, gelatin hydrogel patch containing EPO released EPO continuously during the entire 14 days of the
experiments (Figure 1). Notably, the levels of EPO in the cardiac tissue was significantly increased 24 h after applying the gelatin-hydrogel patch to the heart (Table 1), indicating that EPO was effectively released from the hydrogel patch into the myocardial tissue. At the same time, there was no increase in serum EPO levels and no induction of erythropoiesis. Thus, an EPO-containing hydrogel patch affixed to the heart exerts effects selectively on the heart.

4.2 Expression of erythropoietin receptors and their downstream signal transduction

It was previously reported that EPORs are expressed in the heart.

Our western blot analysis confirmed those earlier findings and further showed that the application of an EPO-containing hydrogel patch significantly upregulates cardiac expression of EPOR. Moreover, activation of STAT, Akt, and ERK, three molecules downstream of EPOR that
Figure 5  (A) Western blot analysis of myocardial VEGF, Bcl-XL, and Bcl-2 expression in the indicated group on day 2 post-MI. *P < 0.05 vs. Saline, **P < 0.01 vs. Saline, #P < 0.05 vs. Systemic, ##P < 0.01 vs. Systemic. (B) Western blot analysis of myocardial EPOR, Akt, p-Akt, ERK, p-ERK expression in the indicated groups on day 14 post-MI. **P < 0.01 vs. Saline, ##P < 0.01 vs. Systemic. (C) Levels of pro-MMP-1 measured on day 2 post-MI. Note the upregulated expression of pro-MMP-1 in the EPO-DDS group. Pro-MMP 1 was expressed in cardiomyocytes surrounding infarcted area. **P < 0.01 vs. Saline, ##P < 0.01 vs. Systemic.
mediate prosurvival signalling, was upregulated in ischaemic areas of hearts receiving the EPO-containing patch DDS group, but not in the hearts of animals receiving EPO systemically. This suggests that EPO-DDS stimulated the EPOR and its downstream signalling.

4.3 Underlying mechanism for the EPO-DDS-mediated reduction of infarct size and improvement of left ventricular remodelling and function

We found that the infarct size on 2 days post-MI was not different statistically among the Saline, EPO-systemic, DDS, and EPO-DDS groups, but the infarct size in the EPO-DDS group tended to reduce when compared with the saline group and the infarct size on 14 days post-MI and that on 2 months post-MI were significantly smaller in the EPO-DDS group than in the EPO-systemic, DDS, or Saline group. This suggests that the infarct size-reducing effect of EPO-DDS treatment post-MI is not an acute effect on the heart but a subacute effect that occurred during the first 14 days post-MI.

We observed significant phosphorylation of Stat3, Akt, and ERK in the ischaemic myocardium on day 2 post-MI in the EPO-DDS group. In haematopoietic and cardiac cells, EPOR signalling can stimulate the PI3K/Akt, JAK/STAT, and...
ERK/MAPK signalling pathways. JAK-STAT pathway has been reported to play an important role in cardiac ischaemic pre-conditioning, thereby reducing the ischaemic damage to the heart. Furthermore, JAK-STAT activation has been shown to prevent apoptosis in cultured myocytes. Activation of Akt has also been reported to play a role in cardiac ischaemic pre-conditioning, thereby reducing the MI size. Ischaemic post-conditioning effect is also a cardioprotective mechanism, which is mediated by Akt and ERK signalling, thereby protecting the heart against ischaemia and reperfusion injury. As mentioned earlier, Stat3, Akt, and ERK are all pro-survival signals. In the present study, on day 2 post-MI, Stat3, Akt, and ERK were all activated in the ischaemic area of the myocardium and these pro-survival signals may have contributed to the cardioprotective effects. On day 14 post-MI, Akt and ERK were still activated but not Stat3, which may suggest that activation of Stat3 plays an important role in the early phase post-MI but activation of Akt and ERK lasts as long as 14 days post-MI.

GSK-3β is usually always activated and the phosphorylation of GSK-3β inhibits the activation of GSK-3β. It has been reported that one of the signalling pathways of ischaemic pre-conditioning is the activation of PI3-kinase and Akt, and the down stream target of Akt is GSK-3β. It has been reported that hypoxic pre-conditioning activates PKC, which phosphorylates and inhibits GSK-3β and leads to cardioprotection of adult cardiomyocyte in vitro. In the present study, treatment with EPO-DDS upregulated the phosphorylation of GSK-3β in the ischaemic area of the myocardium on day 2 post-MI but not on day 14 post-MI. This suggests that phosphorylation of GSK-3β also contributes to the beneficial effect of EPO-DDS in the early phase post-MI. GSK-3β is known to be a common target of converging cell-protective signals provoked by different trigger mechanisms. The mechanism by which phospho-GSK-3β protects the myocardium remains to be unclear, but a possible explanation is the suppression of permeability transition pores that open during reperfusion. Furthermore, recent report demonstrated that phosphorylation of GSK-3β promotes angiogenesis and inhibits apoptosis through the induction of VEGF and Bcl-2 in rat ischaemic pre-conditioned myocardium. Indeed, in the present study, the expression of both VEGF and Bcl-2 was upregulated in the ischaemic area of the myocardium in the EPO-DDS group on day 2 post-MI. On day 14 post-MI, either VEGF or Bcl-2 was not upregulated. This may suggest that the expression of Bcl-2 to inhibit apoptosis plays a role in the early phase post-MI and VEGF also plays a role in promoting angiogenesis in the early phase post-MI.

It has also been reported that EPO stimulates neovascularization. In the present study, we confirmed that the density of CD31-positive microvessels is increased in the EPO-DDS group and that myocardial expression of VEGF is upregulated. VEGF is known to increase capillary density, and the EPOR system reportedly plays an important role in angiogenesis induced in response to hindlimb ischaemia through upregulation of VEGF/VEGF receptor system, leading to both enhanced neovascularization and recruitment of endothelial progenitor cells and bone marrow-derived angiogenic cells in mice. Administration of exogenous EPO also reportedly augments the proliferation of stem/progenitor cells in bone marrow and induces mobilization and proliferation of endothelial progenitor cells, which ultimately contribute to neovascularization. In the present study, however, the EPO-containing gelatin hydrogel patch increased cardiac levels of EPO in the infarcted area but had little or no effect on serum EPO levels (Table 1). This suggests the effect of the EPO hydrogel patch is an entirely local one that enhances angiogenesis through neovascularization.

Apoptosis is governed by a number of regulatory proteins capable of either proapoptotic or prosurvival signalling. Among those is Bcl-2, which reportedly acts to inhibit apoptosis by interfering with the release of cytochrome c from mitochondria and suppressing the actions of Apaf-1. Bcl-2 has also been shown to be an antioxidant and to protect against necrosis. In the present study, EPO-DDS significantly enhanced expression of Bcl-2 in the ischaemic area and this may be involved in the protection against MI.

On day 14 post-MI, infarct size and LV systolic and diastolic dimensions were all smaller in the EPO-DDS group than in the EPO-systemic or Saline group. Moreover, the structural benefits seen in the EPO-DDS group were accompanied by improved LV function. In the present study, fibrotic areas revealed by Masson-Trichrome and Sirius red staining within the infarcted area were smaller in the EPO-DDS group than in the EPO-systemic or saline group, and expression of the collagenase MMP-1 was upregulated in the infarct border zone in the EPO-DDS group on 2 days and 14 days post-MI. Fibrosis after infarction is thought to protect against structural fragility. In post-infarction heart failure models in which MMPs are typically upregulated, MMP inhibitors exert beneficial effects on both cardiac structure and function. We suggest that collagen degradation catalysed by MMPs may have a protective effect on the heart. On the other hand, it is well known that the volume of reactive granulation and/or scar tissue after skin injury, burn, or surgery frequently becomes excessive, resulting in hypertrophic scarring. In the heart, excessive fibrosis accelerates cardiac remodelling and diminishes cardiac function, as is seen in ischaemic and dilated cardiomyopathies. In such cases, an increase in MMP expression may exert a protective effect via proteolysis of excessive collagen. Consistent with this idea are the earlier findings that post-MI increases in MMP-1 induced by hepatocyte growth factor exerted a beneficial effect on the heart via its anti-fibrotic action, and an increase in MMP-1 induced by G-CSF reduced the size of fibrotic areas in infarcted hearts. Furthermore, mice with cardiomyocyte-restricted deletion of Stat3 are reportedly susceptible to inflammation-induced heart damage and show a dramatic increase in cardiac fibrosis. Thus, the observed activation of Stat3 in the EPO-DDS group also may be involved in the reduction in myocardial fibrosis.
improved LV remodelling and function with no adverse side effects (e.g. erythropoietin) through activation of prosurvival mediators such as Stat3, Akt, ERK, GSK-3β, and Bcl-2, and by exerting antiinfective and angiogenic effects through activation MMP-1 and VEGF.

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