

Pioglitazone-incorporated microspheres targeting macrophage polarization alleviates cardiac dysfunction after myocardial infarction

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30 **Visual abstract**

31 key question: Do pioglitazone loaded microspheres hold beneficial effect on remodeling after
32 myocardial infarction? (101 characters)

33

34 Key finding: Pioglitazone loaded microspheres promoted macrophage polarization and inhibited left
35 ventricular remodeling. (109 characters)

36

37 Take home message: Drug delivery system-based macrophage polarization potentially suppresses
38 the inflammation and alleviates left ventricular remodeling. (135 characters)

39

Abstract

Objectives: Excessive and chronic inflammation after myocardial infarction is associated with left ventricular remodeling and impaired cardiac function. Among inflammatory cells, macrophages play a critical role to polarize pro-inflammatory M1 or reparative M2 subtype. Pioglitazone is reported to regulate macrophage polarization to M2 subtype. Here, we aimed to validate the therapeutic effects and the mechanisms of pioglitazone utilizing a drug delivery system.

Methods: Poly L-lactic-co-glycolic acid microspheres incorporating pioglitazone (PGZ-MS) were prepared. To validate the therapeutic potential of PGZ-MS, Sprague-Dawley rats were subjected to permanent left coronary artery ligation to induce myocardial infarction. Placebo-MS (100 µg) or PGZ-MS (100 µg) was injected to the infarct region just after induction. Cardiac function and size were assessed by echocardiography. At 28-day after surgery, rats were sacrificed, and excised hearts were evaluated histologically.

Results: Sustained release of pioglitazone from PGZ-MS was confirmed in vitro. PGZ-MS significantly rehabilitated cardiac dysfunction after myocardial infarction (fractional shortening: MI vs MI+Placebo-MS vs MI+PGZ-MS, 24.4 ± 1.1 vs 24.3 ± 1.6 vs $32.2 \pm 1.4\%$; $P = 0.0035$) with reverse remodeling. Immunohistochemical analyses revealed that PGZ-MS enhanced macrophage

57 polarization (ratio of M2 subtype: 0.39 ± 0.03 vs 0.42 ± 0.02 vs 0.54 ± 0.02 ; $P = 0.0004$) and attenuated
58 apoptosis of cardiomyocytes in the ischemic border zone.

59 **Conclusion:** We confirmed macrophage polarization by sustained release of pioglitazone which
60 resulted in amelioration of adverse left ventricular remodeling and cardiac dysfunction. Drug delivery
61 system-based macrophage polarization might serve as a promising strategy in cardiac regenerative
62 therapy for ischemic heart disease. (250 words)

63

64 **Key words**

65 Biomaterial; Macrophage polarization; Myocardial Infarction; Apoptosis; Drug delivery system

66

67 **Abbreviations and Acronyms**

68 AP-1: activator protein-1; BMDMs: Bone Marrow Derived Macrophages; DDS: Drug Delivery
 69 System ; iNOS: inducible nitric oxide synthase; IL-4: interleukin 4; IL-10: interleukin 10; LV: left
 70 ventricle; MAPK: mitogen activator protein kinase; MI : myocardial infarction ; MS: microspheres,
 71 NF- κ B: nuclear factor-kappa B; PGZ: pioglitazone ; PLGA: poly L-lactic-co-glycolic acid ; PPAR- γ :
 72 Peroxisome Proliferator-activated receptor- γ ; STAT-1: signal transducer and activator of
 73 transcription-1; TRIB:Tribbles homolog; VEGF: vascular endothelial cell growth factor

74

75 **Introduction**

76

77 Despite recent advances in medicine, heart failure is increasing and regarded as an urgent issue in
 78 worldwide healthcare[1]. Myocardial infarction (MI) is one of the highest causes of heart failure, and
 79 post-MI left ventricular (LV) remodeling is underlying the progression of heart failure[2]. Even though
 80 inflammatory processes are essential for the repair processes after MI, excessive and prolonged
 81 inflammation could be harmful for the optimal healing process[3]. Activities of inflammatory molecules
 82 are genetically, epigenetically, or environmentally regulated after MI which results in the extent of
 83 subsequent LV remodeling[3].

84 Among various cell types involved in the repair process, macrophages act as one of the key
85 cell lineages[4]. Macrophages consist of two major components, tissue resident macrophages and
86 monocyte-derived macrophages[5]. Tissue resident macrophages reside in the heart to maintain
87 homeostasis[6]. However, their homeostatic role in post-MI inflammatory reaction is immediately
88 replaced by monocyte-derived macrophages infiltrated from vessels[6]. Infiltrated macrophages
89 possess remarkable plasticity which can modulate their phenotype in response to environmental cues,
90 resulting in the generation of continuum with multiple biological functions. In post-MI healing process,
91 inflammatory macrophages (M1 macrophages) initially accumulate in the infarct zone which manifest
92 phagocytic activity and express high levels of inflammatory cytokines, thus facilitating removal of
93 debris. Subsequently, these inflammatory macrophages transform to reparative subtype (M2
94 macrophages) which initiate the settlement of inflammation and infarct healing via increased
95 expression of anti-inflammatory genes. Such macrophage subtype shift is called macrophage
96 polarization[7]. Appropriate and timely switch of macrophage polarization limits excessive
97 inflammation and contributes to optimal post-MI healing. Therefore, macrophage polarization is a
98 potential target of drug discovery for heart failure.

99 Pioglitazone (PGZ), known as a treatment drug for diabetes mellitus, recently recognized as
100 a cardioprotective agent, is reported to regulate macrophage polarization[8, 9]. It is reported that PGZ
101 suppresses the activity of inducible nitric oxide synthase (iNOS), a molecule known as a potent

inducer of inflammatory macrophages[10] which results in cardiac protection previously validated by a rat myocardial ischemia reperfusion injury model[11]. By virtue of accumulated knowledge on macrophages polarization thereafter, PGZ is currently used experimentally as a regulator of macrophage polarization towards M2 state[12, 13].

To efficiently deliver therapeutic drugs toward the target organ, drug delivery systems (DDS) are as important as the treatment drugs themselves [14, 15] . Poly (L-lactic-co-glycolic acid) (PLGA), a copolymer of poly (L- lactic acid) (PLA) and poly (glycolic acid) (PGA), has been intensively investigated as a carrier of drugs with sufficient results[16-18]. The hydrolysis of PLGA incorporated with therapeutic drugs in the body results in sustained release of the drug securing long-term therapeutic efficiency. The biocompatibility, low toxicity, drug encapsuling, and cell permeability of PLGA are suitable for the DDS[16-18].

This study investigated the effect of intramyocardial injection of PGZ-incorporated PLGA microspheres (PGZ-MS) in terms of improved LV remodeling after MI through optimized macrophage polarization and the alleviation of cardiac dysfunction and cell death in a rat MI model.

Materials and Methods

119 **Preparation of Pioglitazone PLGA microspheres**

120 Five milligrams of Pioglitazone hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was
121 dissolved in 3 ml of dichloromethane stirring 1 hr at 50 °C. One hundred milligrams of poly (L-lactic
122 (LA) -co-glycolic acid (GA)) (PLGA) (average molecular weight 20,000, LA and GA component ratio
123 1: 1, FUJI FILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in the pioglitazone
124 solution. The resulting solution was poured into polyvinyl alcohol (PVA) aqueous solution (PGZ PLGA:
125 3 ml of resulting solution in 300 ml of 0.3 wt.% PVA aqueous solution). For the preparation of PGZ
126 PLGA microspheres (PGZ-MS), the emulsion of PGZ/PLGA solution in PVA solution was stirred at
127 370 rpm at 25 °C overnight in the flask until dichloromethane was completely evaporated. Immediately
128 after termination of stirring, microspheres settled in the flask. They were aspirated with Pasteur pipette
129 and collected into tubes, then washed several times with double-distilled water. Finally, they were
130 freeze-dried to obtain PGZ-MS. Placebo-MS were prepared by pouring PLGA dichloromethane
131 solution without PGZ into PVA aqueous solution as the same manufacturing process with that of PGZ-
132 MS.

133

134 **Characterization of PGZ-MS and the in vitro drug release profile**

135 The size of PGZ-MS was measured by optical microscopy (BZ-X810, KEYENCE, Osaka, Japan).
136 pioglitazone concentration was determined by high performance liquid chromatography (HPLC;
137 Prominence, Shimadzu Corp., Kyoto, Japan) equipped with two pumps, an auto sampler, and an
138 ultraviolet (UV) detector. Mobile Phase was prepared with 0.1M ammonium acetate, acetonitrile and
139 acetic acid (50:50:1). The sample solution was composed of mobile phase and methanol (2:3). Based
140 on the UV absorbance peak area at 266 nm of multiple pioglitazone solutions with determined
141 properties, a calibration curve was prepared. The calibration curve was used to calculate PGZ amount
142 incorporated into microspheres. The pioglitazone release profile was evaluated in vitro. PGZ-MS were
143 incubated in a tube containing 1.0 ml, 1% sodium lauryl sulfate phosphate-buffered saline (PBS)
144 solution at 37 °C. PBS was collected at different time intervals and the concentration of PGZ in the
145 PBS was determined by HPLC. The solution was prepared with collected samples and methanol (2:3).
146 The release profile was calculated with following equation; (cumulative amount of release PGZ
147 amount / estimated total amount of PGZ, which was calculated with previously measured loading
148 amount).

149

150 **Preparation of murine bone marrow-derived and peritoneal macrophage**

151 All animal experiments for in vitro tests were carried out in accordance with procedures approved by
152 the Animal Experimentation Committee of Institute for Frontier Life and Medical Sciences, Kyoto

University. Murine bone marrow macrophages (BMDMs) or peritoneal macrophages (PMs) were harvested from the bone marrows or the peritoneal cavity respectively of 7-week-old female mice (C57BL/6n CLEA Japan, Tokyo, Japan) according to the previous reported protocol[19] with slight modifications. In brief, bone marrow cells were collected from the femurs and tibias of mice by flushing with ice-cold PBS using a 5 ml syringe with a 27-gauge needle. After removal of red blood cells with red blood cell lysis buffer (4.15 g of NH_4Cl , 0.5 g of NaHCO_3 , and 0.0186g of EDTA were dissolved in double-distilled water), the bone marrow cells suspension was cultured in Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher Scientific, MA, USA) supplemented with 20 vol% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT, USA), 50ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF) (BioLegend, San Diego, CA, USA), and 1 wt.% penicillin-streptomycin. After 6 days of culture, the cells were used as BMDMs in subsequent experiments. BMDMs are used in experiments of the evaluation for IL-10 secretion (Fig. 2B).

PMs were harvested as follows. After 5 ml of ice-cold PBS injection into the peritoneal cavity using a 10ml syringe with 26-gauge needle, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. The fluid was collected as much as possible and deposit in tubes. The collected cell suspension was cultured in DMEM (Low Glucose) (FUJI FILM Wako Pure Chemical Industries, Ltd.) supplemented with 10 vol% FBS and 1 wt.% penicillin-streptomycin. After 24 hours of culture, the medium was replaced, and the attached cells were used as PMs in subsequent

experiments.[20] Murine PMs were stimulated with 20 ng/ml of lipopolysaccharide (LPS) for 2 hrs, then they were treated with PGZ, Placebo-MS and PGZ-MS, respectively. PMs are used in experiments of the evaluation for IL-10 kinetics (Fig. 2A).

Evaluation for cytokine secretion from BMDMs and PMs by ELISA

Harvested bone-marrow cells (1×10^6 cells/well) were seeded and cultured on conventional 6-well multi-dish culture plates (Corning Inc., Kennebunk, ME, USA) to differentiate to BMDMs as mentioned above. After 7-day culture, medium was replaced with medium only, medium with PGZ dissolved in dimethyl sulfoxide, medium with Placebo-MS and medium with PGZ-MS and they were cultured for 48 hrs. Then, the culture supernatants were collected to evaluate the level of secreted interleukin-10 (IL-10) by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, MN, USA).

Harvested peritoneal cells (3×10^5 cells/well) were seeded and cultured on conventional 12-well multi-dish culture plates (Corning) for 24 hours to leave PMs as mentioned above. Then, they were stimulated with 20 ng/ml LPS contained medium for 2 h. Subsequently, medium was changed with medium, medium with PGZ dissolved in dimethyl sulfoxide, medium with Placebo-MS and medium with PGZ-MS and they were cultured. The culture supernatants at 3 independent time points (2, 6, 24 hours) were collected to evaluate the level of secreted IL-10 by ELISA.

188

189 **Rat myocardial infarction model and study design**

190 The experimental design is shown in Fig. 3A. All animal procedures were approved by the Kyoto
191 University Animal Experimentation Committee (#MedKyo21198) and performed in accordance with
192 the guidelines for Animal Experiments of Kyoto University, which conforms to Japanese law and the
193 Guide for the Care and Use of Laboratory Animals prepared by the Institute for Laboratory Animal
194 Research, USA (revised 2011).

195 Wild-type female rats of 11-17 weeks (Sprague-Dawley, CLEA Japan., Tokyo, Japan) were
196 used in this study. All rats were kept in the same room in a light- controlled environment with a 12:12
197 h light–dark cycle and with free access to standard rat chow and water. All 30 rats were subjected to
198 permanent left coronary artery ligation, and 6 rats died during or immediately after the operation.
199 During the operation, myocardial infarction (MI) was confirmed by pallor and PBS or PBS diluted
200 Placebo-MS or PBS diluted pioglitazone MS (100µg/100µl) was injected using a 1ml syringe with 30-
201 gauge needle intramuscularly into the left ventricular apex area, assumed infarct area, where a purse-
202 string suture was previously placed and that was tied to avoid the leakage of administrated solutions
203 (PBS;n=9, Placebo-MS;n=11, PGZ-MS;n=10). We determined the dose of pioglitazone MS in
204 accordance with a previous report[21].

205

206 **Echocardiography**

207 Transthoracic echocardiography was performed with the Vivid 7 system (GE Healthcare, Waukesha,
208 WI) and an 11-MHz imaging transducer (GE 10S ultrasound probe, GE Healthcare).
209 Echocardiographic measurements were performed as previously described. Diastolic dimensions of
210 LV in end-diastolic and end-systolic phase (LVDd / LVDs) were recorded and measured with M-mode
211 examination. Fractional shortening (FS) was calculated with the equation: $FS(\%) = \frac{LVDd - LVDs}{LVDd}$.
212 Images were acquired prior to MI surgery and at day 7, day 14 and day 28 post-MI.

213

214 **Statistical analysis**

215 The values are presented as mean $\pm$ SEM. Statistical analyses were performed with Prism 9 (Graph
216 Pad Inc., La jolla, CA, USA). Comparisons between groups were made with the non-parametric
217 Kruskal–Wallis test followed by Dunn’s post hoc multiple comparison test. A p-value of <0.05 was
218 assumed statistically significant. All p-value may not be interpreted as confirmatory but rather
219 descriptive.

220

Results

Preparation and pharmacological kinetics of PGZ-MS

Microscopic appearance of PGZ-MS is shown in Fig. 1A. The diameter of PGZ-MS was 41.0 ± 7.2 μm and the loading amount of PGZ was 29.9 ± 1.9 $\mu\text{g}/\text{mg}$ MS (Fig. 1B). The release profile of PGZ in vitro is shown in Fig. 1C. PGZ-MS released 1.8 ± 0.3 % of incorporated PGZ within 24 hours and 29.5 ± 1.2 % within 14 days indicating sustained release of PGZ in vitro.

PGZ-MS augmented IL-10 release from macrophages and increased M2 macrophages in vitro

To evaluate the effects of PGZ-MS on the release of IL-10, a representative anti-inflammatory cytokine from macrophages, we treated mouse macrophages with PGZ-MS and quantified IL-10 level of the culture supernatant. The level of IL-10 gradually increased in accordance with the treatment duration until 24 hours (Fig. 2A). We compared IL-10 levels of supernatant after 48 hours treatment with PGZ, Placebo-MS, and PGZ-MS, respectively and confirmed that IL-10 level was significantly higher after treatment with PGZ-MS compared to those with other treatment groups (Control vs PGZ vs Placebo-MS vs PGZ-MS: 9.47 ± 1.85 vs 1.51 ± 1.69 vs 10.02 ± 1.15 vs 36.16 ± 4.74 pg/ml / PGZ vs PGZ-MS, $P < 0.001$; Placebo-MS vs PGZ-MS, $P < 0.001$) (Fig. 2B). We performed

immunocytochemical analysis for cultured mouse macrophages and found that the PGZ-MS treatment increased CD163 (M2 macrophage marker)-positive macrophages among all macrophages compared to those without PGZ-MS treatment (Fig. 2C).

PGZ-MS treatment rehabilitated post-MI cardiac dysfunction

We evaluated cardiac function after intramyocardial injection of PGZ-MS in a rat MI model by echocardiography (Fig. 3A). In this study, 9, 11 and 10 rats were assigned to MI or MI+ Placebo-MS group or MI+PGZ-MS group, respectively. 1, 2 and 3 rats in each group died during or immediately after the procedure. In addition, we screened 1 case in MI group and 2 cases in Placebo-MS group from the evaluation on cardiac function, because in those cases we could not find MI lesion histologically and judged them as technically or anatomically incompetent cases. After all, 7 each case was enrolled and examined. Fractional shortening (FS; indicating contractile function of LV) in MI group was significantly lower at 28 days after MI induction which indicates successful induction of MI (Pre vs Day28: 52.2 ± 2.2 vs 24.4 ± 1.1 %, $P < 0.001$) (Fig. 3B). We confirmed that FS was significantly higher in PGZ-MS group compared to those in other groups (MI vs MI+ Placebo-MS vs MI+PGZ-MS: 24.4 ± 1.1 vs 24.3 ± 1.6 vs 32.2 ± 1.4 %, $P = 0.004$). On the other hand, there was no significant difference in end-systolic LV diameter (LVDs) at 28 days after treatment even though LVDs

255 after PGZ-MS treatment tended to be smaller compared to those in other groups (0.57 ± 0.03 vs 0.57
 256 ± 0.03 vs 0.48 ± 0.03 cm, $P = 0.09$) (Fig. 3C).

257

258 **PGZ-MS treatment decreased post-MI adverse LV remodeling**

259 We histologically evaluated the fibrosis of LV at 28 days after the treatment by Sirius Red staining
 260 (Fig. 4). Even though the treatment with PGZ-MS did not significantly reduce the infarct size compared
 261 to those in other groups, the PGZ-MS treatment significantly increased the wall thickness of infarct
 262 region of LV, indicating the prevention of post-MI adverse LV remodeling after PGZ-MS treatment
 263 (infarct size; MI vs MI+ Placebo-MS vs MI+PGZ-MS ; 0.49 ± 0.01 vs 0.45 ± 0.08 , 0.40 ± 0.05 , $P =$
 264 0.19 , thickness; MI vs MI+ Placebo-MS vs MI+PGZ-MS: 0.69 ± 0.12 vs 0.71 ± 0.13 vs 1.06 ± 0.09
 265 cm, $P = 0.03$) (Fig. 4B).

266

267 **PGZ-MS treatment enhanced macrophage polarization towards anti-inflammatory phenotype**
 268 **and attenuated apoptosis of cardiomyocytes**

269 To evaluate macrophage polarization at ischemic border zone, we evaluated the subtype of
 270 accumulated macrophages at 28 days after treatment by immunochemical staining (Fig. 5A,B). We
 271 found that the polarization ratio ($M2/M1+M2$) was significantly higher in PGZ-MS group compared to

those in other groups (MI vs MI+ Placebo-MS vs MI+PGZ-MS: 0.39 ± 0.03 vs 0.42 ± 0.02 vs 0.54 ± 0.02 ; $P < 0.001$)(Fig. 5B). Next, we evaluated cardiomyocytes apoptosis at ischemic border zone after treatment by TUNEL staining and found that PGZ-MS treatment significantly diminished cardiomyocytes apoptosis (2.6 ± 0.2 vs 1.9 ± 0.2 vs 1.2 ± 0.2 cells/hpf, $P = 0.002$) (Fig. 5C,D).

Discussion

In the present study, we confirmed that the intramyocardial injection of PGZ-MS augmented anti-inflammatory macrophages through regulation of macrophage polarization, which resulted in the reduction of cardiomyocytes apoptosis at ischemic border zone and adverse LV remodeling, and alleviated cardiac dysfunction after MI.

Macrophage polarization is considered to affect the progression of LV remodeling after MI. A previous study reported that the administration of interleukin-4 (IL-4), a regulator of macrophage polarization toward anti-inflammatory M2 state lessened post-MI LV remodeling accompanied with decreased cardiomyocytes apoptosis and increased neovascularization in $TRIB1^{-/-}$ mice exhibiting a selective depletion of M2 macrophages[22]. Another study reported that IL-10 released from anti-inflammatory macrophages inhibited cardiomyocytes apoptosis through the inhibition of p38-MAPK

289 pathway and consequently reduced LV remodeling in a mouse MI model[23]. In the present study,
290 echocardiography showed restored cardiac function in PGZ-MS group along with maintained LV wall
291 thickness indicating the prevention of adverse LV remodeling. It would be possible that PGZ-MS in
292 the present study contributed to the accumulation of anti-inflammatory M2 macrophages which
293 supplemented anti-inflammatory cytokines such as IL-10 in ischemic border zone and suppressed
294 cardiomyocytes apoptosis, and consequently mitigated adverse LV remodeling after MI.

295 PGZ is a thiazolidinedione which is broadly used in clinical settings as a treatment drug for
296 diabetes mellitus[24]. PGZ works as a ligand of PPAR γ , an intranuclear receptor which promotes
297 differentiation of adipocytes and the replacement of hypertrophic adipocytes toward normal
298 adipocytes. This results in the acceleration of glucose intake of skeletal muscle, liver and fat tissue,
299 and gluconeogenesis suppression leading to a reduction in blood glucose level[25]. PPAR γ is
300 abundantly expressed in macrophages and suppresses NF- κ B, AP-1, and STAT1 signal transduction
301 pathways which results in the attenuation of M1 macrophage induction and inflammatory gene
302 expression[7, 26]. PGZ-incorporated nanoparticles are also reported to prevent plaque destabilization
303 and rupture in ApoE^{-/-} Mice through polarity shift of macrophage mediated by the regulation of PPAR γ
304 [27]. PGZ is also known to suppresses the activity of iNOS which induces inflammatory
305 macrophages[10]. It might be possible that PGZ-MS regulated macrophage polarization through
306 PPAR γ and iNOS-based molecular mechanisms in this study.

In the present study, we showed an excellent therapeutic potential of PGZ-MS through a DDS based on PLGA. DDS utilizing PLGA facilitates prolonged treatment in damaged tissue. In a rat MI model, intramyocardial administration of vascular endothelial cell growth factor (VEGF)-incorporated PLGA microparticles was associated with a significant increase of capillary density in the infarct and peri-infarct area, compared to that of VEGF administration group without PLGA[28]. It was also reported that PLGA microspheres capable of PGZ release modified macrophage function[29]. We observed beneficial effects of intramyocardial injection of PGZ-MS on LV remodeling process after MI which would confer sustained macrophage polarization in vivo. Considering that the balance between inflammatory and anti-inflammatory reaction after acute myocardial injury during 1-4 weeks after injury would be one of the keys to set the trajectory on healing process, the DDS-based sustained effect should be maintained over 1 week as conducted in the present study[30]. The serum level of glucose should be evaluated to examine the extent of systemic effect of PGZ which can be caused even in local administration of PGZ-MS to the heart in our future pre-clinical and clinical studies.

The route of the administration of PGZ-MS would be crucial for the clinical implementation of the therapeutic strategy. In the present study, we found that local administration of PGZ-MS to the ischemic region in acute phase of the ischemia is efficient for the functional restoration which may indicate that PGZ-MS can be administrated by transcatheter intracoronary approach in acute phase of MI at the timing of primary percutaneous coronary intervention. For the clinical implementation of

the intracoronary approach, we should validate the efficiency of the delivery of PZG-MS to the ischemic region by experiments using large animal models, and the safety of the intracoronary administration of PGZ-MS whether it may cause embolism of coronary microvessels or not. Epicardial administration of the PGZ-MS concomitant with surgical interventions such as coronary artery bypass grafting in acute phase of MI would be another option[31].

There are several limitations in the present study. 1) it might be possible that the efficiency of intramyocardial injection of drugs varied among the recipients considering rather thin LV wall of rats (approximately 2-3 mm) which may affect to the results of the study. Further experiments using larger animal models and consequent clinical trials would be anticipated to address this limitation. 2) It is reported that there are variants in the anatomy of rat coronary arteries [32] which might result in inconsistent quality of MI model even with strictly consistent surgical procedures. It was impossible to check the extent of MI using echocardiography or other modalities such as angiogram before treatment (just after coronary artery ligation) because of the nature of the present study which required treatment just after the induction of MI. 3) The release kinetics of pioglitazone has not been confirmed in vivo because measuring the pioglitazone concentration in the myocardium, on each time point, is not feasible. Therefore, how extensive pioglitazone would spread in the tissue and how long it would be retained within the targeted area are still unclear. However, considering the nature of PLGA and

the result of in vitro test we assumed that PGZ-MS gradually released pioglitazone even in vivo, during the relevant period[28].

Conclusions

In the present study, we showed the possibility that PGZ-MS could realize sustained release of PGZ and validated that intramyocardial administration of PGZ-MS resulted in sustained effect of macrophage polarization and inhibited LV remodeling after MI accompanied with the amelioration of cardiac dysfunction and cardiac cell apoptosis. The strategy of DDS-based macrophage polarization might be an option of cardiac regenerative therapy for ischemic heart diseases in the future.

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Conflict of Interest

The authors declare that they have no financial relationship with the organization that sponsored the research.

Author's contributions

Y.K., H.M. and Y.T. designed the study. Y.K., T.K., JI.J., K.Murata, T.T. and H.M. conducted the experiments. Y.K., H.M. and Y.T. wrote the manuscript. T.K., JI.J. and Y.T. designed the biomaterial. T.I., K.Minatoya, H.M. and Y.T. supervised the research.

374 **Data Availability Statement**

375

376 The datasets generated during and/or analysed during the current study are available from the
377 corresponding author on reasonable request.

378

Figure legends

Figure 1: Characterization of PLGA microspheres incorporating pioglitazone.

(A) Representative image of PGZ PLGA microspheres (PGZ-MS). (B) Release profile of PGZ from PGZ-MS. (C) Characterization of PLGA-MS. PGZ, pioglitazone. PLGA, poly (L-lactic-co-glycolic acid); MS, microsphere. Scale bar = 100 μ m in (A).

Figure 2: In vitro quantitative analysis of IL-10 and immunocytochemistry of macrophages.

(A) Secreted IL-10 in each group at 3 different timepoints from mouse macrophages. (B) Quantification of IL-10 in each group at 48 hours from the treatment. N=6 per group. (C) Representative immunostaining of F4/80 (all macrophages) and CD163 (M2 macrophages) for untreated control (top) and PGZ-MS-treated group (bottom). NS, not significant. *P<0.05, **P<0.01, ***P<0.001. Scale bars = 100 μ m in (C).

Figure 3: PGZ-MS administration improved post-MI cardiac function.

394 (A) Experimental design. (B)(C) Echocardiography. The left ventricular function and size assessed by
395 fractional shortening (FS) (B) and end-systolic diameter of left ventricle (LVDs) (C) at pre-myocardial
396 infarction (Pre MI), 7, 14- and 28-days after the treatment (Day 7, 14 and 28). *P<0.05.

397

398 Figure 4: Macrophage polarization with PGZ-MS alleviates apoptosis in infarct border zone.

399 (A) Representative Sirius Red staining. (B) Infarct size (left) and wall thickness (right) in each group.
400 N=6-7 per group. NS, not significant. *P<0.05. Scale bars = 500 μ m in (A).

401

402 Figure 5: Macrophage polarization and the attenuation of apoptosis through PGZ-MS treatment in
403 infarct border zone.

404 (A) Representative immunostaining of all macrophages and M2 macrophages (brown). Insets (black
405 squares) indicate magnified view of selected region (blue squares). (B) Polarization ratio for M2
406 macrophages in each group. (C) Representative TUNEL staining (brown) and HE staining. Arrow
407 heads: TUNEL-positive cells. (D) TUNEL-positive apoptotic cells per high power field (hpf) in each
408 group. N=6-7 per group. NS, not significant. **P<0.01. Scale bars = 50 μ m in (A) and (C). TUNEL,
409 TdT-mediated digoxigenin (biotin)-dUTP nick end labeling.

410

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