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MAIN TEXT ARTICLE

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PEGylated carboxyhemoglobin bovine (SANGUINATE) ameliorates myocardial infarction in a rat model

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Abstract

Artificial oxygen (O_2) carriers were reported to be protective in ischemia/reperfusion (I/R) in various organs including the heart. In the current study, 20 rats underwent ligation (MI) of the left anterior descending artery, were treated with 10 mL/kg of PEGylated carboxyhemoglobin bovine (SANGUINATE, S+, $n = 10$) or saline (S−, $n = 10$) 10 minutes after MI and daily thereafter for 3 days, and were followed by weekly echocardiography for 4 weeks, when they had left ventricular pressure volume relationship (PVR) analyses followed by necropsy. Echocardiography showed an increase in end-systolic dimension rather than end-diastolic dimension, preserved fractional shortening (36 vs. 26%, $P < .01$), and milder mitral regurgitation in S+ compared with S- rats. PVR revealed a milder increase in end-systolic volume, larger stroke volume (101 vs. 74 μL, $P < .005$) and cardiac output (33.4 vs. 23.8 mL/min, $P = .004$) in S+ rats in actual determination and under a wide range of standardized loading conditions 4 weeks after MI. Excised heart showed significantly limited area of MI (8.9 vs. 13.3%, $P = .028$). The results suggest that SANGUINATE in short-term repeated doses may accelerate weight recovery, preserving the myocardium, mitral competence, and cardiac function after MI. The mechanism of action and optimal treatment for MI remain to be studied.

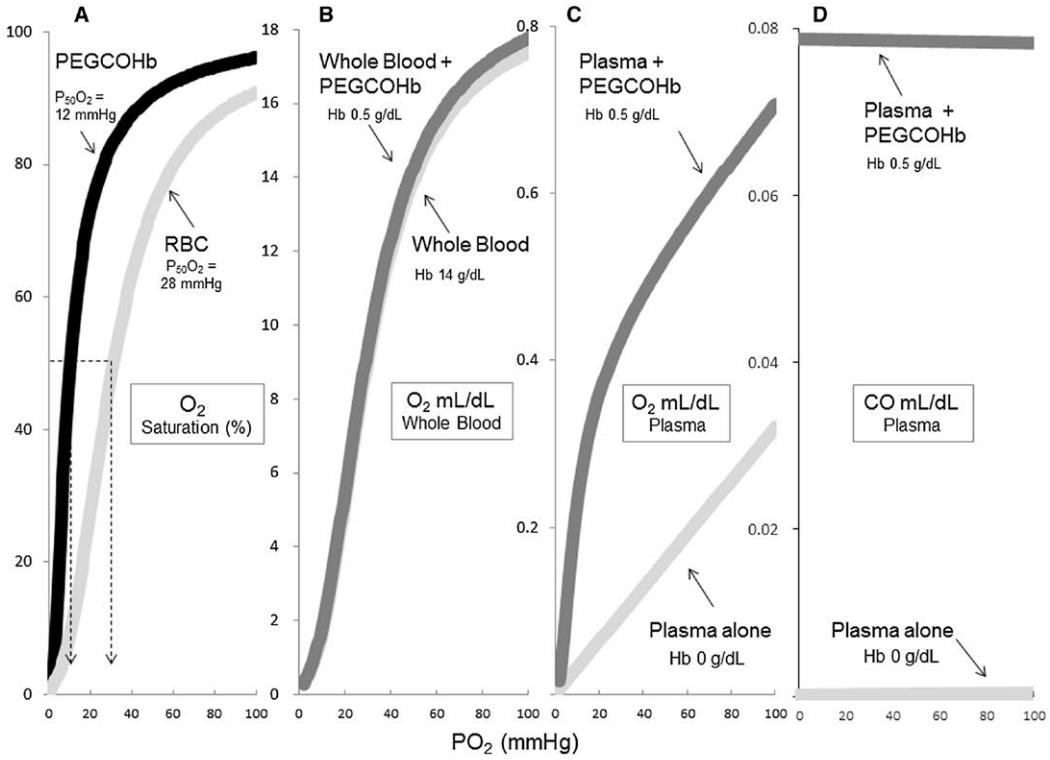
KEYWORDS

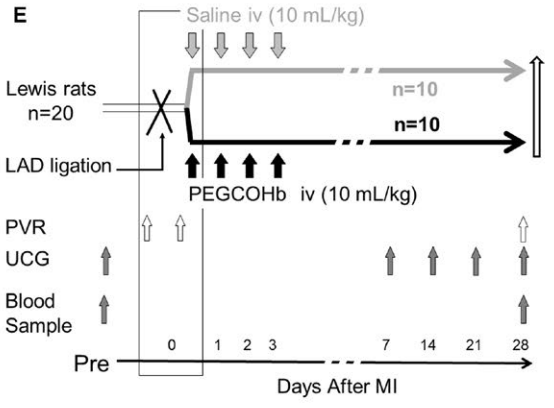
artificial oxygen carrier, carbon monoxide, cardiac function, hemoglobin-based oxygen carriers, mitral regurgitation, myocardial ischemia and reperfusion

1 | INTRODUCTION

Hemoglobin-based oxygen (O_2) carriers (HBOCs) are often made of human (1,2) or bovine (3) hemoglobin (Hb) in an attempt to supplement functions of red blood cells (RBC). As a blood substitute for transfusion, however, Natanson et al. (4) reported increased incidence of myocardial infarction (MI) and death in clinical trials testing various cell-free Hb. Such adverse events were primarily attributable to Hb dissociation (5), causing extravasation, NO scavenging, and vasoconstriction (6), as well as prompt oxidation to free heme, evoking systemic inflammation (7). To avoid such Hb toxicities, PEGylation (8) as well as encapsulation (1), nitrosylation (2), or carboxylation (3) improved Hb stability, safety (9), and efficacy in experimental models of ischemia/reperfusion (I/R) in the brain (10), cochlea, skin and myocardium (11) with or without diabetic

diathesis. Since PEGylated carboxyhemoglobin bovine (Figure 1, SANGUINATE) (3) was reported to be protective of myocardial morphology at I/R (11), functional effect(s) was studied in a rat model in the current study (Figure 1E) by repeated pressure–volume relationship (PVR, Figure 1F,G) (12) analyses for accuracy and weekly echocardiography for functional evolution for 4 weeks after MI. Considering the short intravascular half-life, 10 mL/kg (340 mg of Hb/kg) was given shortly after MI and daily thereafter for 3 days (4 doses in total) to cover hemodynamic consequences occurring after MI.





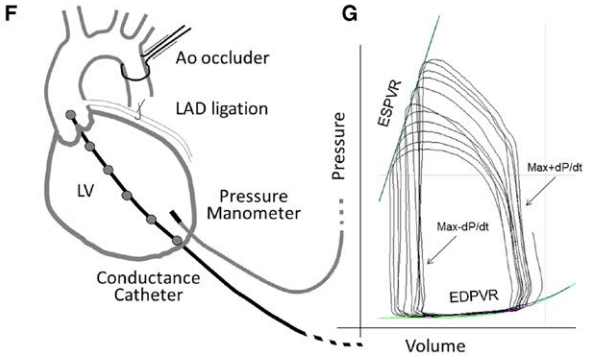


FIGURE 1 Experimental materials and protocols. O_2 dissociation curve (SO_2) for RBC and SANGUINATE (A), O_2 contents in the whole blood (B), plasma O_2 contents (C), and plasma CO contents (D) are plotted theoretically based on the values presented in the figures and calculations below: O_2 content = $0.0032 \cdot PO_2$ (mm Hg) + $SO_2 \cdot Hb_{RBC}$ + $SO_2 \cdot Hb_{Sang}$The contents of CO as a function of PO_2 were calculated based on:

$$COHb + O_2 \leftrightarrows O_2Hb + CO \quad (1)$$

$$P_{50} = COHb \cdot PO_2 / O_2Hb \cdot PCO = P_{50}O_2 / P_{50}CO \quad (2)$$

$$COHb = Hb_{total} P_{50} \cdot PCO / (PO_2 + P_{50} \cdot PCO) \quad (3)$$

where HbRBC = Hb in RBC, HbSang = Hb in SANGUINATE, $P_{50}O_2$ = 12 mm Hg (SANGUINATE) and 30 mm Hg (RBC), $P_{50}CO$ = 0.0013 mm Hg, Hb_{total} = 0.5 g/dL. PCO was assumed to be 1.74-mm Hg based on the normal level of endogenous COHb in RBC. Using equation (3), theoretical curves were drawn as shown (D). Although SANGUINATE fails to increase Hb in whole blood (B) very much, it significantly increases each of Hb (B), O_2 (C), and CO contents (D) in the plasma (E). Twenty Lewis rats had LAD ligation and were treated either with SANGUINATE (closed arrows) or saline (10 mL/kg, gray arrows) 10 minutes after MI on Day 0 and daily thereafter for 3 days. With conductance catheter and pressure manometer in LV (F), changing the afterload by constricting the aortic sling helped define ESPVR as a line and EDPVR as a three-dimensional curve (G) to characterize the particular LV to compare with different ones. In each of these pressure–volume loops, isovolumic contraction defines max positive dP/dt and isovolumic relaxation defines max negative dP/dt.

2 | MATERIALS AND METHODS

All experiments were approved by the Institutional Review Board of Tokai University. The animals received humane care as required by the institutional guidelines for animal care and treatment in experimental investigations according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

2.1 | Animals

Lewis rats were purchased from Charles River Co. Ltd., (Yokohama, Japan) at the age of 5 weeks. They were acclimated for a week before undergoing experiments in an animal cage under air conditioning at 26 ± 0.5°C with water and food ad libitum.

2.2 | PEGylated carboxyhemoglobin bovine (SANGUINATE)

SANGUINATE was supplied by Prolong Pharmaceuticals, LLC, (South Plainfield, NJ, USA). Its pharmacokinetics and characteristics were reported elsewhere (3,9). SANGUINATE is bovine hemoglobin bound to carbon monoxide (CO) and modified with polyethylene glycol (PEG), resulting in an adduct with higher O_2-affinity ($P_{50}O_2$ = 12 mm Hg, Figure 1A) compared to that of RBC ($P_{50}O_2$ = 30 mm Hg). The solution contains 3.4 ± 0.1 g/dL of bovine Hb with a high fraction of COHb (90.3 ± 3.6%) and a low rate of metHb (2.6 ± 0.7%). Because of the large amount of Hb in whole blood as RBC (Figure 1B), 10 mL/kg of SANGUINATE administration constitutes only 3.5% of Hb in whole blood, while it represents most of plasma Hb (Figure 1C), as there is little natural Hb in plasma. Once infused into the blood stream, it releases CO and starts binding and releasing O_2 as well as CO based on the surrounding PO_2 and endogenous CO levels (Figure 1D). It is removed from circulation and is presumed to be metabolized by the reticuloendothelial system as RBC with an intravascular half-life of 12 hours in rats (3,9).

2.3 | Experimental procedures

Rats were anesthetized with sevoflurane at 3%, orally intubated, and ventilated at 15 mL/kg of tidal volume with ambient air at a ventilation rate of 60 per minute with no end-expiratory pressure. An indwelling catheter was placed in the tail vein and saline was administered at 3 mL/h. A rectal probe was placed to monitor body temperature, which was maintained at 36°C with a water blanket, MEDI-Therm II (Gaymer Industries Inc., Orchard Park, NY, USA). The chest was entered at the 4th intercostal space, sevoflurane was reduced to 2%, and a catheter-tip manometer (Millar Instruments, Houston, TX, USA) and a conductance catheter (Unique Medical Co. Ltd, Tokyo, Japan) were placed through the left ventricular (LV) apex to record the LV PVR (12) (Figure 1E,F); this recording included LV blood sampling for volume calibration and blood gas analyses (ABL825, Radiometer Co. Ltd., Copenhagen, Denmark), aortic constriction as afterload change to develop end-systolic PVR (ESPVR, line), and end-diastolic PVR (EDPVR as three-dimensional curve). In a representative pressure-volume loop, stroke volume (SV), stroke work (SW), pressure–volume area (PVA), max positive dP/dt, and max negative dP/dt were determined (Figure 1G). After recording control PVR, the left anterior descending artery (LAD) was ligated, and PVR recording was repeated 3 minutes later. After these recordings, the chest was closed in 3 layers and SANGUINATE

(S+) or saline (S-) at 10 mL/kg was started 10 minutes after MI at a rate of 0.5 mL/min, anesthesia was terminated, and the animal was extubated and returned to the cage. Seven other rats were treated in the same way except for the creation of MI to serve as a non-MI control. The same protocol was followed except for functional studies in 14 rats treated with SANGUINATE ($n = 7$) or saline ($n = 7$), and they were sacrificed on Day 5 to study the distribution of SANGUINATE.

2.4 | Medication

After the first dose of SANGUINATE or saline administration after MI on Day 0, the same solution was administered daily at roughly 24-hour intervals for 3 more days, 4 doses in total (Figure 1E), under sevoflurane 3% anesthesia via an indwelling catheter in the tail vein at a rate of 0.5 mL/min.

2.5 | Echocardiography

Echocardiography (Aloka Co., Ltd., Tokyo, Japan) using a 3.5-MHz probe determined the LV end-diastolic dimension (LVDD), end-systolic dimension (LVDS), severity of mitral regurgitation (MR), height of E-wave and A-wave, and the size of the left atrium and ascending aorta. These measurements were repeated before infusion (0 weeks) and then weekly following MI until 4 weeks later (4 weeks). The severity of MR was defined by the size of regurgitation flow reaching the left atrial wall (severe, score 3), 2/3 of the left atrium (moderate, score 2), and 1/3 of the left atrium (mild, score 1). Fractional shortening (%FS) was defined as (LVDD-LVDS)/LVDD × 100.

2.6 | Repeated PVR, macroscopic measurements

Four weeks after MI, the rats were anesthetized, intubated, and the chest was opened through the same incision to record repeated PVR and obtain blood samples. Finally, the heart was excised and perfused from the aorta with 20 mL of 4% paraformaldehyde after saline flush. The heart was macroscopically measured for maximal short axis circumference and LV luminal volume by weight difference with repeated saline filling and evacuation. The heart was preserved overnight in 4% paraformaldehyde, sliced into 4 cross-sectional planes (Figure 5A), and stained with hematoxylin and eosin. The cross-sectional slices were assembled (Figure 5A), and the fibrotic area was determined using an automated graphical analyzer (ImageJ, National Institutes of Health, Bethesda, MD, USA) to assess the area of fibrosis (Figure 5B) by an anatomist (MY) blinded to grouping information.

2.7 | Statistics

All data were expressed as mean ± SD. Statistical analysis was performed with GraphPad Prism for Windows, version 6.0 (GraphPad Software, San Diego, CA, USA). Differences among the treatment groups were examined by Kruskal–Wallis test and Mann–Whitney U test. Correlations between echocardiographic and PVR-derived values were evaluated by Spearman's correlation coefficients. P value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Growth

Body weight of the rats recovered to the preoperative level by 3.4 ± 1.4 days in S+ rats and 5.0 ± 2.6 days in S- rats (Figure 2A). The S+ rats grew comparably to non-MI rats and had significantly heavier body weight than S- rats on Days 3 through 6 (Figure 2A,*). Such differences disappeared after the first week, and all rats grew steadily to 185% of their preoperative level by Day 28 (Figure 2B). There was no difference between groups in LV blood samples on Day 0 and Day 28, including plasma glucose, Hb content, and COHb (Figure 2C), which showed no change in the LV blood COHb fraction before and after LAD ligation on Day 0 before SANGUINATE, nor between the treatment groups on Day 28 (Figure 2C). Blood sampling from 14 additional rats on Day 5, 2 days after the last dose, showed that the COHb fraction was highest in LV and lowest in the pulmonary artery without difference between the treatment groups, when plasma Hb, derived from SANGUINATE, remained at 77 ± 16 mg/dL in LV blood (Figure 2D).

3.2 | Echocardiography

While LVDD (Figure 3A) showed a significant difference between rats with and without MI (#) at the first week, it became a significant difference between the treatment groups (*) 4 weeks after MI. Rats with MI regardless of treatment showed increased LVDS (Figure 3B) and significant decrease of %FS (Figure 3C) at the first week and throughout the observation period compared to rats without MI (#). LVDS increased and %FS decreased steadily thereafter only in the S- rats, showing significant differences 3 and 2 weeks later compared to S+ rats (*), respectively. The severity of MR (Figure 3D) became aggravated in the same way as LVDS (Figure 3E), which was significantly correlated with %FS (Figure 3F). As a result, S- rats developed severe MR 4 weeks after MI, which suppressed %FS significantly lower compared to S+ rats, which on average had MR at a mild level. There was no other significant difference among the variables between the groups.

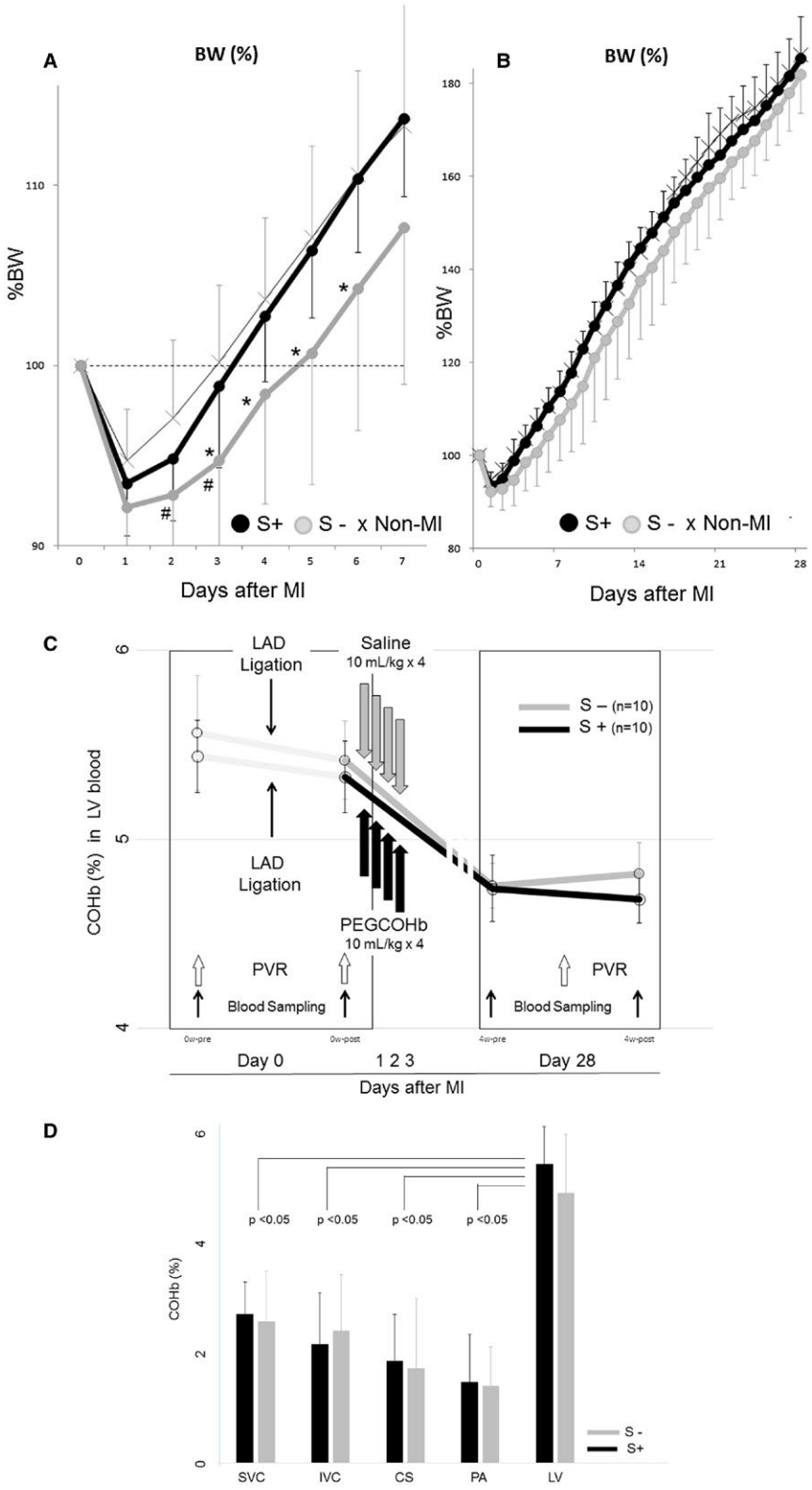


FIGURE 2 Body weight and COHb changes. Body weight of the rats recovered to preoperative levels by 3.4 ± 1.4 days in S+ rats (closed circles) and 5.0 ± 2.6 days in S- rats (gray circles, A). The S+ rats grew in comparison to control rats without MI (×) and had significantly heavier body weight (*) than S- rats on Days 3 through 6. The differences disappeared after the first week as they grew steadily to 185% of preoperative level on Day 28 (B). There was no difference in the fraction of COHb (C) and no change in arterial COHb before and after LAD ligation on Day 0 before SANGUINATE and between the treatment groups on Day 28. Blood sampling from 14 additional rats on Day 5 (D, 2 days after last dose at 10 mL/kg) showed that the COHb fraction was highest in LV and lowest in the pulmonary artery (PA), without difference between treatment groups, suggesting that pulmonary circulation is the source of endogenous CO as well as exogenous CO. Repeated administration of SANGUINATE failed to change CO homeostasis 2 days after the last dose, when plasma Hb or SANGUINATE remained at 77 ± 16 mg/dL in LV blood. Significant differences (A) are depicted by hash symbols (#) against non-MI control rats and by asterisks (*) between treatment with SANGUINATE and saline. CS, coronary sinus; IVC, inferior vena cava; PA, pulmonary artery; SVC, superior vena cava

3.3 | Pressure volume relationship analyses

PVR on Day 0 revealed that MI was induced at the same level in both groups (Figure 4). At the end of week 4, actual SV (SV_{actual}, Figure 4A) and EF (EF_{actual}, SV/EDV × 100, Figure 4B) differed significantly between the treatment groups (*). Recorded EDPVR and ESPVR (Supplement A) were used to determine EDV, ESV, SV, and EF at standardized loading conditions; preload varied between 5, 10, and 15 mm Hg with afterload changing from 70- to 100-mm Hg at 10-mm Hg increments. Under any of these loading conditions, S+ rats had significantly larger SV as well as EF than

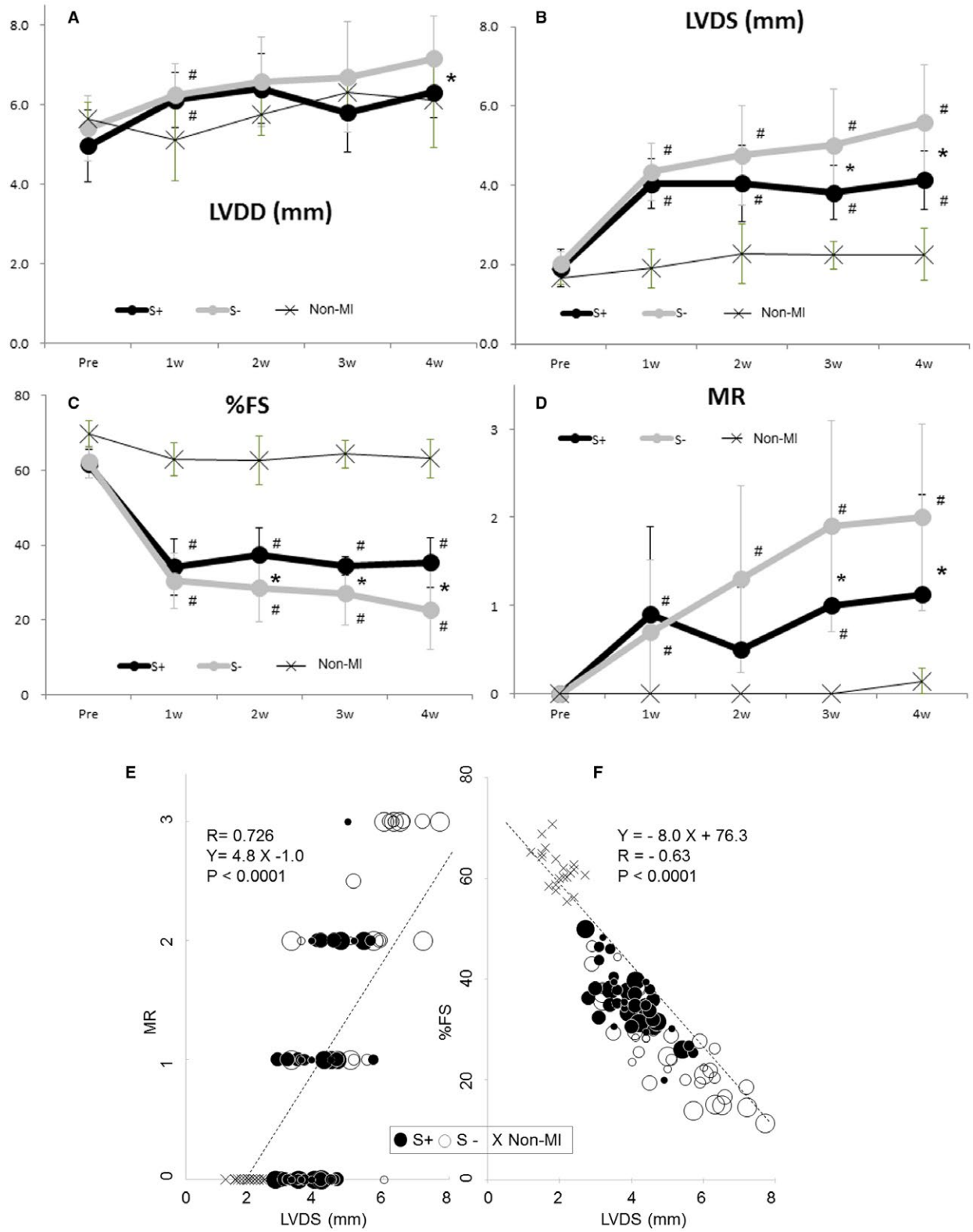


FIGURE 3 Echocardiographic study. Echocardiography recorded before and after MI showed development of MR and increased LVDS, which largely determined %FS. S- rats had significantly increased LVDD (A) 4 weeks after MI compared to S+ rats with a similar value to non-MI control rats. While LVDS (B) increased to the same level at the first week in rats with MI regardless of treatment, it increased further in S- rats, making %FS (C) reduced in the S- rats 4 weeks after MI compared to the S+ group ($P < .05$, A). The severity of MR (D) increased in the same way as LVDS (E), which was linearly correlated with %FS (F). Closed symbols depict S+ rats and open symbols indicate S- rats for Pre-MI determination (X), 1 week (small circles), 2 weeks (middle circles), 3 weeks (large circles), and 4 weeks after MI (extra-large circles)

the S- group (Supplement B, Figure 4A,B). When the individual arterial elastance (Ea) on Day 0 was applied 4 weeks later in each animal (Supplement C), SV was significantly larger than that of S- rats ($SV_{10/Ea}$, $EF_{10/Ea}$, $P < .001$, Figure 4A,B). While the actual values of SW and PVA (Figure 4C) started at low levels after MI, both values remained the same in S- rats compared to S+ rats, which showed them to have improved significantly 4 weeks later ($P < .01$, **). While max positive dP/dt and max negative dP/dt started at similarly depressed levels on Day 0, both clearly improved in S+ rats, and their values tended to be better compared to S- rats 4 weeks later (Figure 4D, $P < .1$, #).

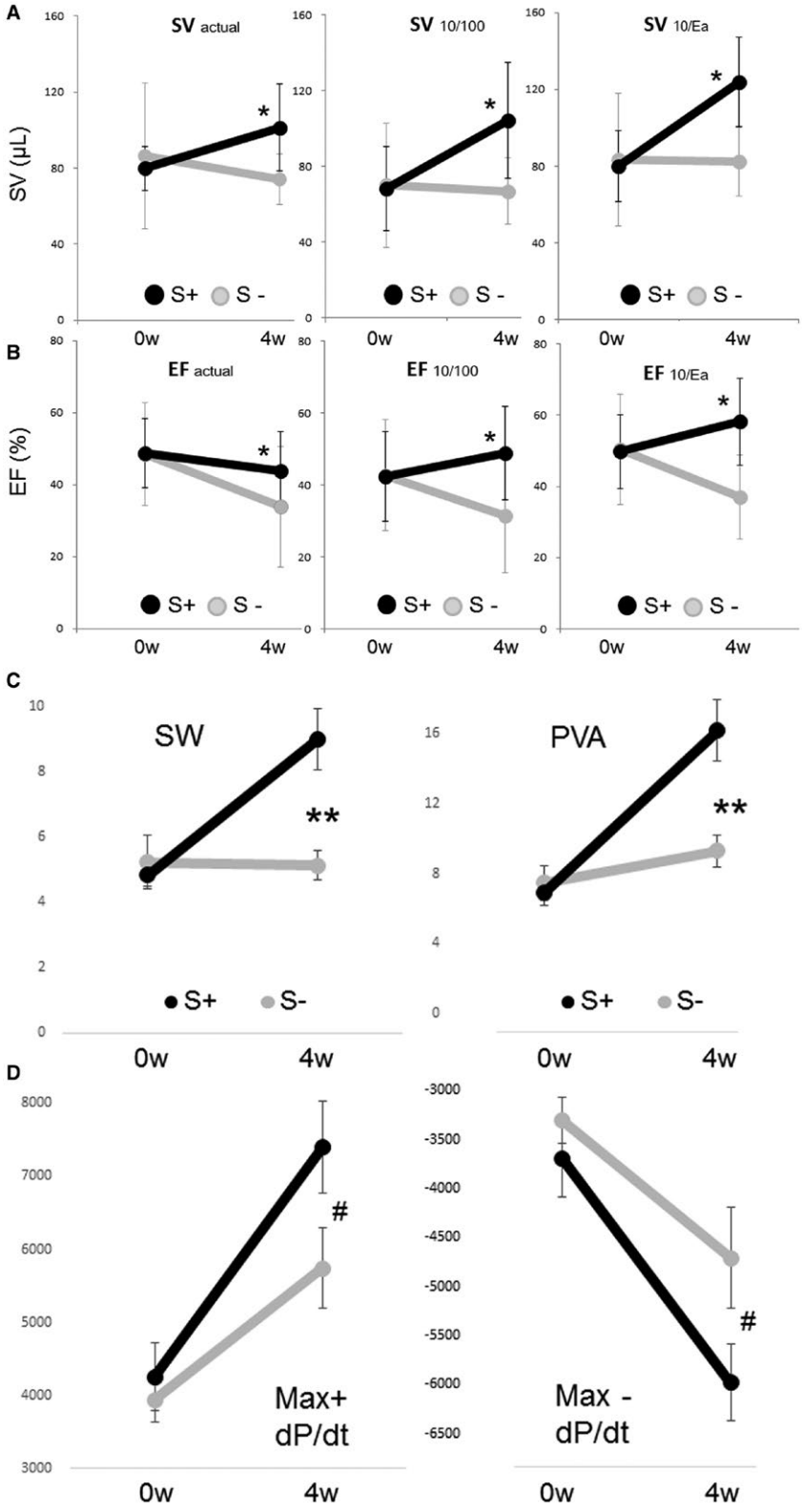


FIGURE 4 PVR. PVR showed increased SV (A) and ejection fraction (EF, B) in the S+ group in the actual determination (left). Differences in LV function between treatment groups and changes after MI were compared by SV under standardized loading conditions (Supplement A), preload, and afterload at 10/100 mm Hg (middle) and against the same arterial elastance (right). In any of these conditions, SV was larger in S+ rats compared to S- rats with a similar LVEDV, making EF larger in S+ rats. The SW as well as PVA (C) were similarly depressed in both groups on Day 0 and remained so 4 weeks later in S- rats in contrast to S+ rats, which had improved values, showing a significant difference (**, $P < .01$). A similar tendency was observed in the max positive dP/dt and max negative dP/dt (D), the indices of LV contraction and relaxation, which were improved in S+ rats, although the differences failed to reach statistical significance (#, $P < .1$). Supplement A. LV functions were recorded as ESPVR (one-dimensional line) and EDPVR (three-dimensional curve) by changing the afterload by constricting the aortic sling (Figure 1F) and changing the preload by acute volume load. Two LVs, LV1 and LV2, were compared at the same EDP (line 1, blue) and the same ESP (line 2, red); intersection between EDP and EDPVR defines EVD and intersection between ESP and ESPVR depicts ESV. The difference between EDV and ESV is defined as SV for each LV. Supplement B. By changing EDP (5, 10, and 15 mm Hg) and ESP (70, 80, 90, and 100 mm Hg), the respective EDV and ESV were defined to allow calculation of SV and EF for each LV. SV and EF were calculated for S+ rats (red) and S- rats (blue) before (0w, fine line) and after MI (4w, thick line), showing significant differences in SV as well as in EF between the treatment groups under a wide range of preloads and afterloads. Loading conditions encircled by a box (preload at 10-mm Hg and afterload at 100-mm Hg) were presented as $SV_{10/100}$ and $EF_{10/100}$ (Figure 4A, middle). Supplement C. By changing EDP (5, 10, and 15 mm Hg) and setting the arterial elastance (Ea) at each ESP (70, 80, 90, and 100 mm Hg) after MI, individual Ea was used for the Ea 4 weeks later. The intersection between EDPVR (black) and EDP (blue) defines EDV and the intersection between ESPVR (black) and ESP (red) determines ESV for Day 0. These data define Ea on Day 0 after MI (LV1), which was used to define Ea 4 weeks later (LV2) to detect ESV for LV2 as the intersection with ESPVR. Setting EDP at 10-mm Hg and ESP at 100-mm Hg on Day 0 was used to define Ea, which allowed calculation of SV and EF for each LV as presented as $SV_{10/Ea}$ and $EF_{10/Ea}$ (Figure 4A, right). Supplement D. Immunohistochemical staining for bovine Hb of the infarcted myocardium 5 days after MI where myoglobin was not present. Bovine Hb was heavily stained over all the infarcted area in S+ rats, although no such reaction was detected 4 weeks after MI

3.4 | Pathohistological study

Macroscopic measurements of LV luminal volume and the maximal short axis circumference tended to be larger in S- rats than in S+ rats, but failed to reach statistical significance. The microscopic short axis cross section of the myocardium typically revealed shrunken papillary muscles in S- rats (open arrow, Figure 5A). Each of the 4 cross-sectional slices was assessed for LV intact wall and fibrosis (dotted line, Figure 5B) and summed for each slice (Figure 5C). The total wall area tended to be larger in S+ rats than in S- rats without statistical significance (Figure 5D, # $P = .083$). The fibrotic area was larger in each slice and as a whole (S+: 18.8 ± 6.4 mm^2, S-: 25.7 ± 5.3 mm^2, * $P = .028$), which was 8.9% in S+ and 13.3% in S- rats (Figure 5D, ** $P = .01$).

4 | DISCUSSION

While histochemical effects of SANGUINATE had been studied in a murine model (11), functional aspects were explored in the rat. Weekly echocardiography showed an early increase in LVDS over LVDD, an associated decrease in %FS, and the development of MR in both MI rats, regardless of treatment, for the first week. Thereafter, only S- rats had increased LVDS, aggravated MR, and further %FS reduction, making significant differences compared to S+ rats at 2–3 weeks and later following MI. Although PVR allows load-independent analyses of LV function (12), a conductance catheter could not precisely define parallel conductance, making the absolute LV volume determination difficult. Therefore, SV was compared under standardized loading conditions and against fixed arterial elastance. Under any of these conditions, SV was significantly better preserved in S+ rats. The functional differences emerging late after MI (>1 week) might be derived from deterioration in mitral competence, myocardial contractility as detected by max positive dP/dt, and relaxation determined by max negative dP/dt; all these functional variables remained depressed in S- rats in contrast to S+ rats, which had values recovering to normal ranges. In accordance with these changes, the area of LV wall fibrosis was reduced in each cross section, suggesting the presence of collateral perfusion from neighboring tissues and/or shortened diffusion distance (13) of O_2 or CO, to reduce myocardial stress, necrosis, apoptosis, and eventual fibrosis 4 weeks later in S+ rats. The limitations of the current study include the lack of direct evidence regarding mechanism(s) of action. Nonetheless, the functional evolution, early dysfunction regardless of treatment followed by further deterioration in untreated rats, may serve as an indirect evidence suggesting suppression of ventricular remodeling (14) by repeated administration of SANGUINATE. In this regard, it would become important to study early (<1 week) changes in myocardial-eluting enzymes, RNA and cytokine production to explore the mechanism(s) of action as well as to determine the optimal timing and dosage to maximize the benefits of SANGUINATE.

Although the first-generation cell-free Hbs were modified through cross-linking, pyridoxylation or polymerization, these modifications turned out to be insufficient to prevent dissociation (5) to release free Hb, which caused vasoconstriction (6) and systemic inflammation (7) associated with

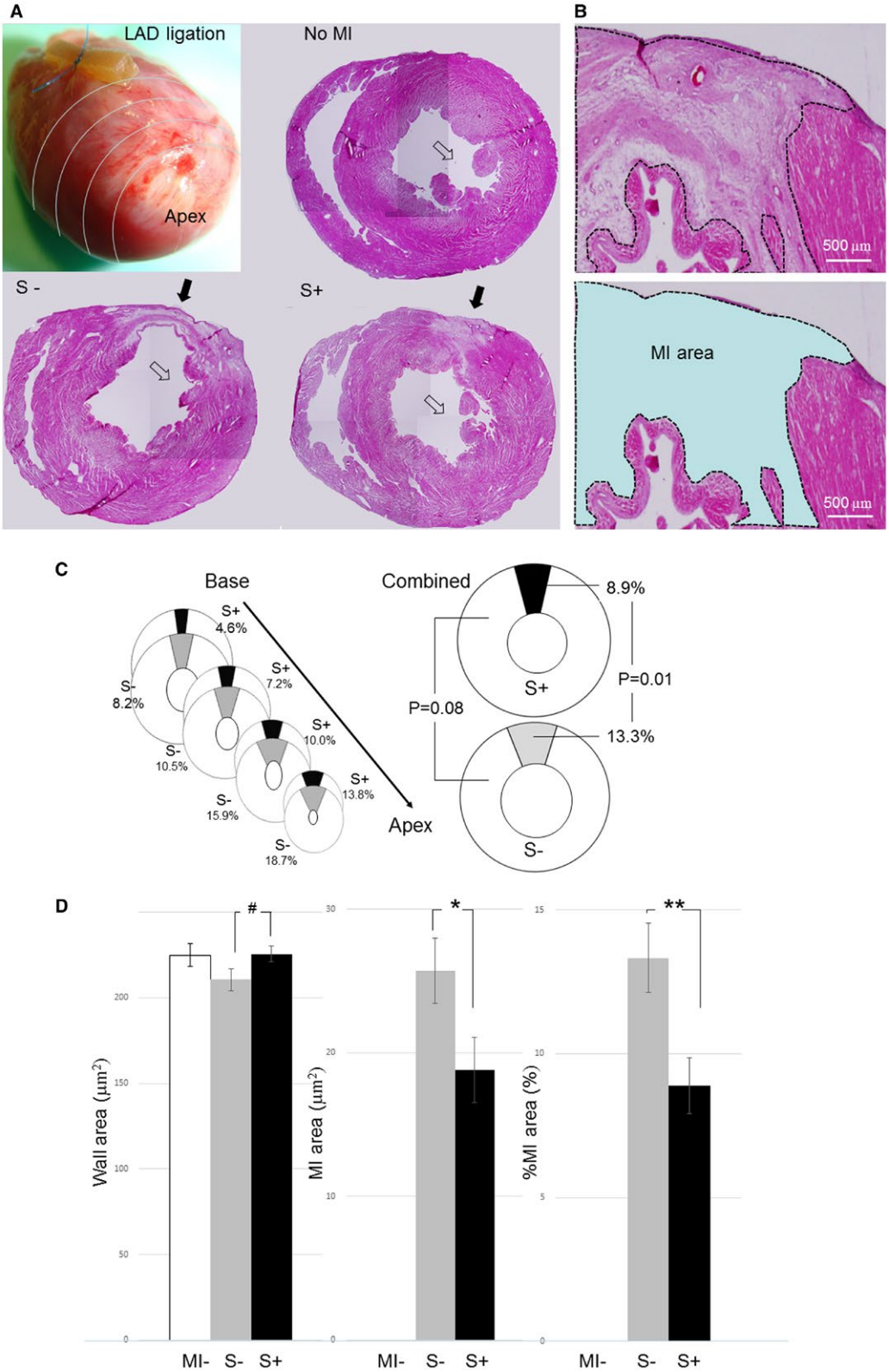


FIGURE 5 Histopathological study. The LV cavity volume determined by saline-filled method showed a tendency toward dilated LV cavity ($P = .104$) in S- rats (data not shown). There was no apparent correlation between the severity of MR and LV cavity volume 4 weeks after MI. Macroscopically, the area of MI appeared larger and the LV wall seemed thinner (solid arrow) with shrinkage or disappearance of the papillary muscles (open arrows) in some of the S- rats in cross-sectional planes (A). Each of 4 cross-sections was graphically analyzed by ImageJ (NIH system) to determine fibrosis and intact myocardium (B), and they were then summed for the fibrosis and intact myocardium (C). The area of fibrosis tended to be limited in each of the cross sections, which became significant in the summed areas in S+ rats (*, $P = .028$). While the fibrotic area (D) as well as its fraction to the LV wall (%MI, **, $P = .01$) were significantly limited in S+ rats, the summed LV wall area tended to be larger (#, $P = .083$) in S+ rats, which was equivalent to rats without MI, suggesting that the myocardial wall thickness was preserved in S+ rats [Color figure can be viewed at wileyonlinelibrary.com]

increased risk of MI and death (4). Thus, HBOCs of later generations have been modified specifically to prevent such toxicities by PEGylation (8), encapsulation (LEH) (1), *S*-nitrosylation (2), or carboxylation of Hb (3). It has also been demonstrated in experiments that PEGylation of HBOCs alone was not enough to afford protection, as *S*-nitrosylation of PEGylated Hb (2) or carboxylation of MP4 (PEGylated human hemoglobin) (15) was necessary to yield protection of ischemic brain (2) or myocardium (15). The latter report in particular provides an indirect evidence to support the therapeutic contribution of CO in the current study as reported of CO (16) and CO-releasing molecules (17) in cytoprotection or suppressed apoptosis, vasoactivity, redox control, and anti-inflammation. Due to the extremely high affinity to Hb, it is supposed that blood CO content (Figure 1D) does not change with blood PO_2 but rather with the endogenous CO level. While depletion of CO has been reported to induce heme oxygenase-1 (HO-1) to maintain CO homeostasis (18), exogenous CO is considered to suppress induction of HO-1. Since hemolysis-derived free heme induces HO-1 and CO to stabilize Hb and to suppress the vicious cycle (5–7,17), CO is considered to protect against in vivo models of cerebral malaria (7,16), reactive O_2 species from I/R injury (16,17) and hemolytic events in a patient with sickle cell disease (19).

In the current study, the LV blood COHb fraction remained at around 5% with no change before and after LAD ligation on Days 0 and 28 (Figure 2C). Furthermore, the COHb fraction was comparable regardless of the treatment with SANGUINATE 2 days after the final dose, when it remained at 77 ± 16 mg/dL (Figure 2C) in the plasma of LV blood. The COHb fraction was significantly higher than that in SVC, IVC, CS, or PA. These observations suggest that exogenous CO from SANGUINATE was no longer influential 2 days after the last dose at 10 mL/kg, and that pulmonary circulation is the source of endogenous CO. Thus, intravenous administration of SANGUINATE may first unload CO as a CO donor (16,17) and then stay in plasma as an artificial O_2 as well as CO carrier to deliver both exogenous O_2 and endogenous CO at the highest levels after pulmonary circulation. These results may support a concept that a small amount of stable plasma HBOCs, such as LEH (2,10) or SANGUINATE (3,9,11), have an advantage in flowing with plasma and effecting a shorter diffusion distance (13) of O_2 and CO to hypoxic tissues over a much larger amount of Hb in RBC.

As to the structure, MP4 (15) used human hemoglobin (6), which is not stable and dissociates into subunits, while bovine Hb (20) does not. Therefore, MP4 was cross-linked and then modified by covalent attachment of PEG at the cysteine residues, while SANGUINATE uses bovine Hb, which does not require cross-linking and is modified at the lysine residues. Furthermore, carboxylation was necessary for MP4 to be protective of the ischemic myocardium (15). While MP4 has extremely high O_2-affinity, or very low $P_{50}O_2$ at 5 mm Hg (15), SANGUINATE, *S*-nitrosylated Hb (2), has $P_{50}O_2$ at 12 mm Hg as in LEH with high O_2-affinity (10), with a significant protection of the organ and function at I/R injury (10,11). These differences in O_2-affinity and the human (5) or bovine (20) source of Hb may make significant differences in the cerebral I/R (9) and in the myocardial morphology (11), as well as in function as observed in the current study.

5 | CONCLUSION

In this in vivo study, repeated treatment with SANGUINATE (10 mL/kg) after myocardial infraction accelerated initial weight gain, maintained mitral competence, and preserved myocardial histology and cardiac function. While the total doses in the present study amounted to 40 mL/kg to cover myocardial I/R injury so as to obtain maximal protection, the mechanism(s) of action must still be explored, which may be able to define optimal treatment, dose and timing with SANGUINATE for myocardial I/R. This work should prove informative for designing studies to evaluate safety, appropriate dosing and potential efficacy in experimental as well as in clinical settings.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Supple A LV functions were recorded as ESPVR (one-dimensional line) and EDPVR (three -dimensional curve) by changing the afterload by constricting the aortic sling (Fig. 1B) and changing the preload by acute volume load. Two LVs, LV1 and LV2, were compared at the same EDP (line 1, blue) and the same ESP (line 2, red); intersection between EDP and EDPVR defines EVD and intersection between ESP and ESPVR depicts ESV. The difference between EDV and ESV is defined as SV for each LV.

Supple B By changing EDP (5, 10 and 15 mmHg) and ESP (70, 80, 90 and 100 mmHg), the respective EDV and ESV were defined to allow calculation of SV and EF for each LV. SV and EF were calculated for S+ rats (red) and S- rats (blue) before (0w, fine line) and after MI (4w, thick line), showing significant differences in SV as well as in EF between the treatment groups under a wide range of preloads and afterloads. Loading conditions encircled by a box (preload at 10 mmHg and afterload at 100 mmHg) were presented as SV10/100 and EF10/100 (Fig. 4, middle).

Supple C By changing EDP (5, 10 and 15 mmHg) and setting the arterial elastance (Ea) at each ESP (70, 80, 90 and 100 mmHg) after MI, individual Ea was used for the Ea four weeks later. The intersection between EDPVR (black) and EDP (blue) defines EDV and the intersection between ESPVR (black) and ESP (red) determines ESV for Day 0. These data define Ea on Day 0 after MI (LV1), which was used to define Ea four weeks later (LV2) to detect ESV for LV2 as the intersection with ESPVR. Setting EDP at 10 mmHg and ESP at 100 mmHg on Day 0 was used to define Ea, which allowed calculation of SV and EF for each LV as presented as SV10/Ea and EF10/Ea (Fig. 4, right).

Supple D Immunohistochemical staining for bovine Hb of the infarcted myocardium five days after MI where myoglobin was not present. Bovine Hb was heavily stained over all the infarcted area in S+ rats, although no such reaction was detected 4 weeks after MI.

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