Extracorporeal Low-Energy Shock-Wave Therapy Exerts Anti-Inflammatory Effects in a Rat Model of Acute Myocardial Infarction

Yuzuru Abe, PhD; Kenta Ito, MD, PhD; Kiyotaka Hao, MD, PhD; Tomohiko Shindo, MD; Tsuyoshi Ogata, MD; Yuta Kagaya, MD; Ryo Kurosawa, MD; Kensuke Nishimiya, MD, PhD; Kimio Satoh, MD, PhD; Satoshi Miyata, MD; Kazuyoshi Kawakami, MD, PhD; Hiroaki Shimokawa, MD, PhD

Background: It has been previously demonstrated that extracorporeal low-energy shock-wave (SW) therapy ameliorates left ventricular (LV) remodeling through enhanced angiogenesis after acute myocardial infarction (AMI) in pigs in vivo. However, it remains to be examined whether SW therapy also exerts anti-inflammatory effects on AMI.

Methods and Results: AMI was created by ligating the proximal left anterior descending coronary artery in rats. They were randomly assigned to 2 groups: with (SW group) or without (control group) SW therapy (0.1 mJ/mm², 200 shots, 1 Hz to the whole heart at 1, 3 and 5 days after AMI). Four weeks after AMI, SW therapy significantly ameliorated LV remodeling and fibrosis. Histological examinations showed that SW therapy significantly suppressed the infiltration of neutrophils and macrophages at days 3 and 6, in addition to enhanced capillary density in the border area. Molecular examinations demonstrated that SW therapy enhanced the expression of endothelial nitric oxide synthase and suppressed the infiltration of transforming growth factor-β1-positive cells early after AMI. SW therapy also upregulated anti-inflammatory cytokines and downregulated pro-inflammatory cytokines in general.

Conclusions: These results suggest that low-energy SW therapy suppressed post-MI LV remodeling in rats in vivo, which was associated with anti-inflammatory effects in addition to its angiogenic effects, and demonstrated a novel aspect of the therapy for AMI. (Circ J 2014; 78: 2915–2925)

Key Words: Inflammation; Left ventricular remodeling; Macrophages; Myocardial infarction; Shock-wave therapy

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Department of Cardiovascular Medicine (Y.A., K.I., K.H., T.S., Y.K., R.K., K.N., S.M., H.S.), Department of Medical Microbiology, Mycology and Immunology (Y.A., K.K.), Tohoku University Graduate School of Medicine, Sendai, Japan

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Mailing address: Kenta Ito, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryoumachi, Aoba-ku, Sendai 980-8574, Japan. E-mail: ito-kenta@cardio.med.tohoku.ac.jp


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of Health and was performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals at Tohoku University (2011-Idou-179, 2012-Idou-84 and 2013-Idou-37).

Animal Models
Male Sprague-Dawley rats (7–8-week-old, 200–220 g in body weight) were used in the present study. They were anesthetized with inhaled isoflurane (5% for induction and 2% for maintenance), intubated and ventilated by positive pressure through an endotracheal tube attached to a small-animal respirator. The depth of anesthesia was monitored by the tail-pinch reflex test. With a left-sided thoracotomy, the pericardium was opened, and then the left anterior descending coronary artery (LAD) was ligated with a 6-0 silk suture. The chest was closed and the animals were allowed to recover. They were randomly assigned to 2 groups: with (SW group) or without (control group) SW therapy. In addition to the AMI groups, the sham-operated groups (with or without SW therapy) were also made with the same procedure but without the LAD ligation. Animals were excluded from the present study when LV fractional shortening (FS) exceeded 30% at day 1. We stored the heart samples at days 3, 6, and 28 after AMI. Serum cardiac troponin T levels were measured at days 3 and 6 (SRL Inc, Tokyo, Japan). Animals were euthanized by cervical dislocation under anesthesia and overdose with isoflurane.

Extracorporeal SW Therapy
Based on our previous studies,7–11,18 we performed low-energy SW therapy (0.1 mJ/mm²; approximately 10% of the energy used for the lithotripsy treatment, 200 shots, 1 Hz, to the whole heart) using a specially designed SW generator equipped with an echocardiographic probe (Storz Medical AG, Kreuzlingen, Switzerland) under anesthesia with 2% isoflurane. The SW group was subjected to SW therapy 3 times in the first week (1, 3 and 5 days after AMI), whereas the control group underwent the same procedures 3 times including anesthesia but without SW treatment.

Echocardiography
In order to follow up the time-course of LV function and remodeling after AMI, we performed transthoracic echocardiography (Aplio 80; Toshiba Medical Systems, Tochigi, Japan) at days 1, 7, 14, 21 and 28 under inhalation anesthesia with 2% isoflurane.

Histopathological Analysis
Excised hearts were fixed with 4% paraformaldehyde for histological and immunohistochemical examination. After fixation, the tissue specimens were embedded in paraffin and sliced to 3 μm in thickness. The sections were used for hematoxylin-eosin, Masson-trichrome, immunohistochemical stainings for CD31 (anti-CD31, 1:400; Abcam, Cambridge, UK), neutrophils (anti-granulocyte, 1:400; Abcam), macrophages (anti-ED-1, 1:800; Abcam), M2 macrophages (anti-CD206, 1:100; Santa Cruz, TX, USA) and TGF-β1 (anti-TGF-β1, 1:200; Abcam). Immunodetection was accomplished using a Histofine Kit (Nichirei, Tokyo, Japan). The extent of LV fibrosis was calculated using the following formula: fibrotic area/(LV free wall+interventricular septum)×100 (%) The number of immune-positive cells was counted in the infarcted, border and remote areas, where 10 random fields were examined in each sample at a ×400 magnification in a blinded manner.

Statistical Analysis
Continuous results are expressed as mean±SD. We adopted 2-way repeated-measures ANOVA to compare longitudinal data. We also utilized the Student’s-t test followed by Bonferroni type multiple comparisons and 2-way ANOVA with Tukey’s honest significant difference (HSD) multiple comparison test to compare mean values. To test ordered alternative hypotheses among groups, we used the Jonckheere-Terpstra trend test. P-values <0.05 were considered to be statistically significant.

Results
Effects of SW Therapy on Cardiac Function After AMI
There was no difference in serum cardiac troponin T levels between the control and the SW groups (day 3, 0.58±0.51 vs. 0.63±0.49 mg/ml, P=0.81, n=10; day 6, 0.19±0.26 vs. 0.08±
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0.05 ng/ml, P=0.28, n=9), suggesting that the MI size was comparable between the 2 groups. In the control group, LV contractile function, when evaluated by FS, was progressively decreased by day 28 (day 1, 18.8±3.9% vs. day 28, 14.2±3.9%, P<0.01), which was significantly ameliorated in the SW group (day 1, 19.7±4.1% vs. day 28, 18.1±2.6%, P=NS; P<0.05 by 2-way repeated measured ANOVA; Figure 1A). Similarly, LV end-diastolic dimension (LVDd) and LV end-systolic di-

**Figure 1.** Shock-wave (SW) therapy ameliorates left ventricular (LV) function after acute myocardial infarction. LV function was serially evaluated by echocardiography. (A) LV fractional shortening (FS). (B) LV end-diastolic dimension (LVDd). (C) LV end-systolic dimension (LVDs). Results are expressed as mean±SEM.

**Figure 2.** Shock-wave (SW) therapy ameliorates left ventricular (LV) fibrosis after acute myocardial infarction. (A, B) Masson-trichrome staining in the control (A) and the SW group (B). Fibrotic area was stained in blue. (C) LV fibrosis on day 28. Fibrotic area was quantified as a percentage of the total LV area. Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.
Infiltration of Inflammatory Cells

The number of neutrophils and macrophages was examined at days 3 and 6 after AMI. Infiltration of neutrophils was detected in the infarcted, border and remote area at day 3 in the control group, which was significantly ameliorated in the infarcted area by the SW group (Figures 4A–C). In contrast, no neutrophils were detected in either the infarcted, border or remote area at day 6 (Figure 4D). Infiltration of macrophages was noted at both day 3 and day 6 in the control group, which was also significantly attenuated in the infarcted area by the SW group (Figures 5C–D). Although SW therapy ameliorated macrophage infiltration, infiltration of M2 macrophages was enhanced by SW therapy, suggesting the polarity shift of the macrophage phenotype from M1 to M2 (Figures 5E, F).

Expression of eNOS and VEGF

Messenger RNA expression of eNOS and VEGF was examined at days 3, 6 and 28. There was no difference in the expression of eNOS between the control and the SW groups at the observed time points (Figures 6A–C). The expression of VEGF was low but higher in the control group than in the SW group at day 3. The expression of VEGF in the border area

LV Fibrosis

LV fibrotic area was quantified as a percentage of the total LV area. The extent of LV fibrosis at day 28 was significantly attenuated in the SW group compared with the control group (12.4±3.9 vs. 18.5±4.7%, P<0.05; Figure 2).

Capillary Density

Capillary density was examined with CD31 staining at day 28. Although capillary density in the infarcted and remote areas was comparable between the 2 groups, it was significantly higher in the SW group than in the control groups (1,183±70 vs. 949±247/mm², P<0.05; Figure 3).

Figure 3. Shock-wave (SW) therapy enhances capillary density after acute myocardial infarction. The number of CD31-positive cells on day 28 was counted at ×400 magnification. Scale bars represent 50 μm. Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.
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In comparisons between the SW and the control groups at different observation days with Bonferroni correction, the levels of pro-inflammatory cytokines (IL-1α, IL-4, IL-6, IL-12p70, IL-13, IL-17 and IFN-γ) were significantly suppressed at day 6 in the SW groups compared with the control group, although the levels of IL-1β at day 3 were higher in the SW group than in the control group (Figure 8). By Tukey’s HSD multiple comparison, the levels of pro-inflammatory cytokines (IL-1α, IL-4, IL-6, IL-12p70, IL-13, IL-17 and IFN-γ) increased with time from day 3 to day 6 in the control group while those increases in the cytokine levels were blunted in the SW group. In all cytokines, except for TNF-α, there were significantly decreasing trends detected in the SW group by the Jonckheere-Terpstra trend test with 1 and 2-sided alternatives, although there were no significant differences in the control group (Figure 8).

Discussion

In the present study, we demonstrated that low-energy SW therapy exerts anti-inflammatory effects in a rat model of AMI in addition to its angiogenic effects. To the best of our knowledge, this is the first study that demonstrates the anti-inflammatory effects of SW therapy in the healing process after AMI.
Suppression of Post-MI Inflammatory Responses by SW Therapy

Inflammatory cells play important roles in myocardial tissue repair after AMI. We have previously demonstrated that SW therapy ameliorates post-MI LV remodeling and that it also enhances eNOS activity, capillary density and myocardial blood flow in pigs in vivo. In the present study, we confirmed in rats our previous findings found with pigs, and further examined the effects of SW therapy on inflammatory responses because inflammation is critically important in the healing process after AMI.

Effects of Extracorporeal Low-Energy SW Therapy on Post-MI Hearts in Rats

We have previously demonstrated that SW therapy ameliorates post-MI LV remodeling and that it also enhances eNOS activity, capillary density and myocardial blood flow in pigs in vivo. In the present study, we confirmed in rats our previous findings found with pigs, and further examined the effects of SW therapy on inflammatory responses because inflammation is critically important in the healing process after AMI.

Figure 5. Shock-wave (SW) therapy suppresses macrophage infiltration after acute myocardial infarction. (A,B) Immunohistochemical staining of macrophages in the control group (A) and the SW group (B) on day 3. Scale bars represent 50 μm. (C–F) The number of infiltrated macrophages or M2 macrophages in the control and the SW group on day 3 (C,E) and day 6 (D,F). The number of inflammatory cells was counted at ×400 magnification. Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.
Shock-wave (SW) therapy enhances endothelial nitric oxide synthase (eNOS) activity after acute myocardial infarction. (A–C) mRNA expression levels of eNOS on day 3 (A), day 6 (B) and day 28 (C). (D–F) The expression levels of vascular endothelial growth factor (VEGF) on day 3 (D), day 6 (E) and day 28 (F). (G–I) The ratio of phosphorylated eNOS (phospho-eNOS) to total-eNOS, a marker of eNOS activity, on day 3 (G), day 6 (H) and day 28 (I). (J–L) Protein levels of VEGF (J–L) on day 3 (J), day 6 (K) and day 28 (L). Results are expressed as mean±SEM. *P<0.05 for the control vs. the SW group.
Attenuation of LV Fibrosis After AMI by SW Therapy

TGF-β1, which is known to promote LV fibrosis, is released from fibroblasts and infiltrated macrophages after myocardial injury, and excessive infiltration of macrophages might promote LV fibrosis and thus deteriorate LV function.15–17

In the present study, SW therapy attenuated macrophage infiltration, TGF-β1 expression and LV fibrosis. These results suggest that the anti-fibrotic effects of SW therapy are related to the suppression of macrophage infiltration and TGF-β1 expression. However, it remains to be examined whether the reduced expression of TGF-β1 is attributed to the reduction of macrophage infiltration and TGF-β1 production from macrophages and other cells.

Mechanisms for the Inhibitory Effects of SW Therapy on LV Remodeling After AMI

We and others have previously demonstrated angiogenic effects of low-energy SW therapy in several animal models,7,10–12,14,16,21–25 as well as in humans.8,9,26–32 In the present study, we have demonstrated that SW therapy attenuates inflammatory responses and LV fibrosis in a rat model of AMI.

Figure 7. Shock-wave (SW) therapy suppresses transforming growth factor (TGF)-β1 expression after acute myocardial infarction. (A–C) The mRNA expression levels of TGF-β1 on day 3 (A), day 6 (B) and day 28 (C). (D–F) Immunohistochemical staining of TGF-β1-positive cells on day 3 (D), day 6 (E) and day 28 (F). Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.
unknown whether different levels, numbers and protocols of SW therapy could be more effective than that used for the present study. Second, in the present study, we had to apply SW to the whole rat heart due to the focus size of the SW machine, whereas we were able to selectively apply SW to the border area in our previous studies in pigs.10,11 Interestingly, however, in the present study, the SW therapy increased capillary density only in the border area. Thus, SW therapy might enhance angiogenesis and exert anti-inflammatory effects mainly in the border area in an AMI model even when the SW was applied to the whole heart. The detailed molecular mechanisms for the different effects of SW therapy between ischemic and non-ischemic areas remain to be examined. Third, the detailed molecular mechanisms of the anti-inflammatory effects of SW therapy also remain to be elucidated in future studies. Fourth, in the present study, we did not show whether the anti-inflammatory action mediates the beneficial effects of SW therapy. To clarify this issue, an additional approach such as gene deletion or selective inhibition of candidate molecules might provide further insights into the effects of SW therapy. Finally, in the present study, we focused on neutrophils and macrophages as inflammatory cells; however, other types of cells, such as fibroblasts, myofibroblasts, natural killer T cells and regulatory T cells, have been

Figure 8. Shock-wave (SW) therapy upregulates anti-inflammatory cytokines and downregulates pro-inflammatory cytokines after acute myocardial infarction. The production of inflammation-related cytokines in left ventricular homogenates from the border zone. Results are expressed as mean±SEM. *P<0.05, **P<0.01; among different observation days in each group by Tukey’s honest significant difference multiple comparison test. †P<0.05, ‡P<0.01; the control group vs. the SW group by Bonferroni type multiple comparisons.

These results suggest that SW therapy ameliorates post-MI LV remodeling not only through angiogenesis but also through suppression of inflammatory responses and LV fibrosis (Figure S2). The low-energy SW therapy, when applied to ischemic tissues, has been reported to enhance the expression of stromal-derived factor 1, a key regulator of stem cell migration to the site of tissue injury during the process of tissue repair.33–37 In addition, SW therapy has also been reported to promote migration and differentiation of bone marrow-derived mononuclear cells (BMDMC).38,39 Furthermore, macrophages could modulate the activity of stem cells.40 In the present study, we also showed that macrophage infiltration was ameliorated by SW therapy. Thus, SW therapy might directly and/or indirectly affect the function of stem cells, such as BMDMCs, residential cardiac stem cells, and multilineage-differentiating stress-enduring (Muse) cells.38,39,41,42 Additional studies are needed to clarify the contribution of stem cells to the beneficial effects of SW therapy.

Study Limitations
Several limitations should be mentioned for the present study. First, in the present study, we chose the condition of SW therapy (e.g., energy levels, number of shots) based on our previous studies7–11,18 and did not test other therapeutic conditions. It is

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binding to the inflammatory state and LV remodeling after AMI. Also, we did not examine the effects of SW therapy on functional aspects of inflammatory cells. Further studies are needed to clarify these issues.

Conclusions
In the present study, we demonstrated that low-energy SW therapy suppresses post-MI LV remodeling in rats in vivo, which is associated with anti-inflammatory effects in addition to its angiogenic effects, thus demonstrating a novel aspect of the therapy for AMI (Figure S2). Because SW therapy is non-invasive and safe, it could be a novel option for the prevention of LV remodeling after AMI in humans.

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**Supplementary Files**

**Supplementary File 1**

**Figure S1.** No effects of shock-wave (SW) therapy on left ventricular (LV) function in the sham-operated group.

**Figure S2.** Summary of the present findings and proposed mechanisms of the inhibitory effects of shock-wave (SW) therapy on post-myocardial infarction (MI) left ventricular (LV) remodeling.

Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-14-0230