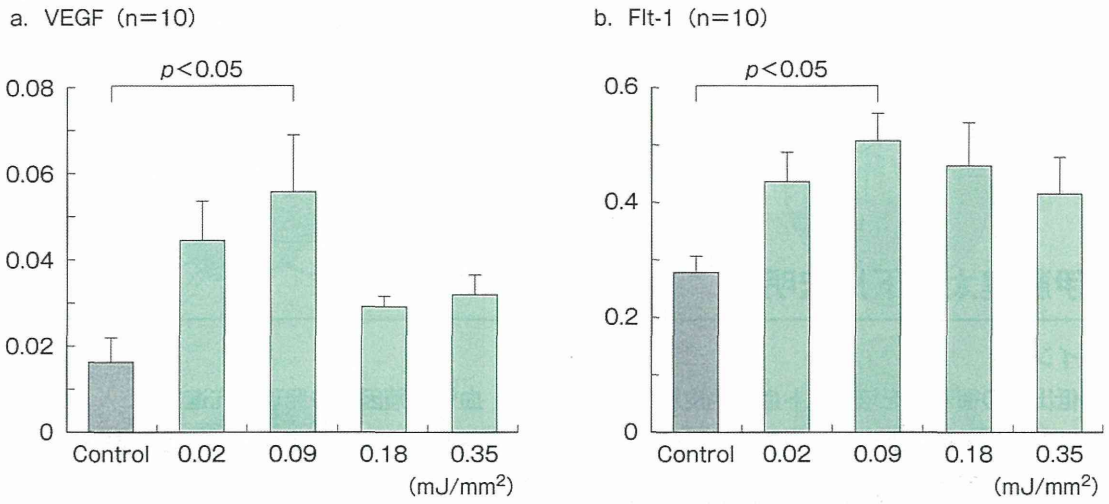

図 1　衝撃波による血管増殖因子と受容体の発現亢進（文献 3 より引用）
培養ヒト臍帯静脈内皮細胞に衝撃波を照射すると，vascular endothelial growth factor（VEGF）と Flt-1 の発現が亢進した．その効果は，結石破砕治療に用いる約 10 分の 1 という弱い出力時（0.09 mJ/mm^2）に最大であった．

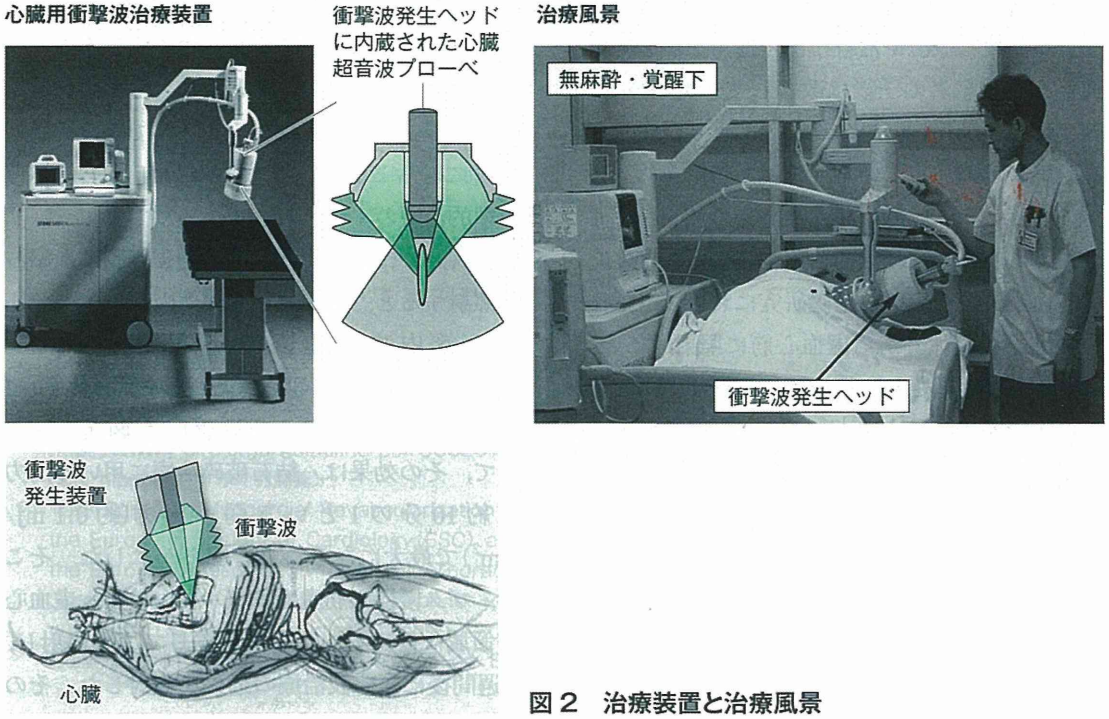

図 2　治療装置と治療風景

度と冠血流の有意な増加，それに伴う左室壁運動の改善を認めた[3]．一方，衝撃波治療中および治療後 3 日間に不整脈の増加や突然死を認めず，組織学的検討においても出血などの組織損傷は認めなかった．

以上の結果から，低出力の衝撃波を用いた体

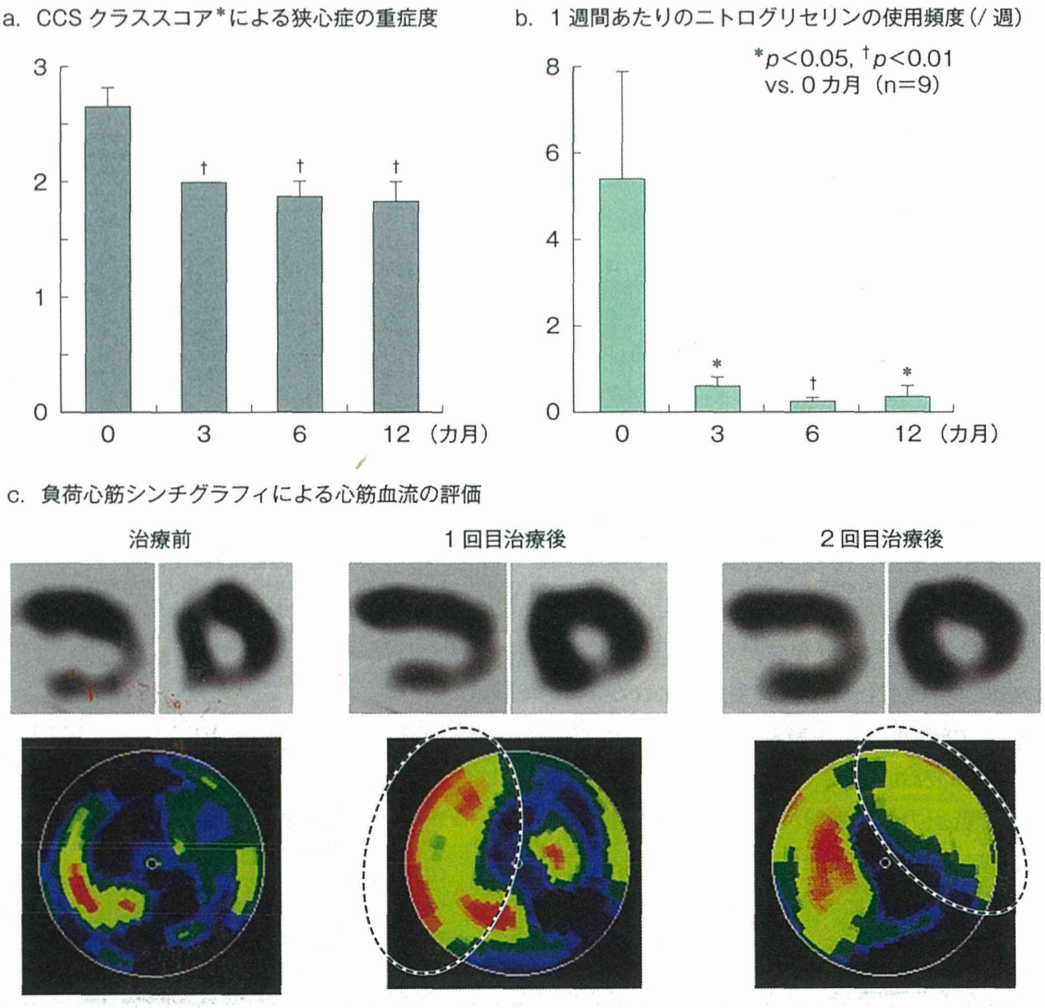

図 3　狭心症に対する低出力体外衝撃波治療（第 1 次臨床試験）（文献 1 より引用）
重症狭心症症例において，低出力体外衝撃波治療により自覚症状の改善を認めた（a，b）．また，衝撃波を照射した領域（破線で囲まれた領域）でのみ血流の改善を認めた（c）．
*CCS クラススコア：カナダ心血管疾患協会（Canadian Cardiovascular Society）による狭心症重症度分類．
【クラス 1】日常生活では狭心発作を起こさない．
【クラス 2】日常の身体活動はわずかながら制限される．
【クラス 3】日常活動は著しく制限される．
【クラス 4】いかなる動作も症状なしにはできない．

外衝撃波治療は，安全で有効な血管新生療法であると考えられた．

臨床応用：狭心症症例に対する低出力体外衝撃波治療

基礎研究で得られた良好な結果をもとに，われわれは重症狭心症症例を対象に低出力体外衝撃波治療の臨床試験を行ってきた．ガイドラインに沿った十分な薬物治療下でも狭心症発作を有し，かつ経皮的冠動脈インターベンション（percutaneous coronary intervention：PCI）や冠動脈バイパス術（coronary artery bypass graft：CABG）による治療が困難な労作性狭心症患者（いわゆる no-option 症例）を対象とした．対象例は，CABG 後慢性期にバイパスグラフ

トが閉塞した症例やびまん性冠動脈病変症例が多かった．ただし，血管新生療法の特性上，悪性腫瘍併存症例や過去5年以内に悪性腫瘍の手術を受けている場合は除外している．

衝撃波発生ヘッドを患者の胸壁に当てて，装置に内蔵された心臓超音波プローベで観察しながら虚血領域に照準を合わせ，衝撃波を照射した(**図2**)．1カ所につき200発の衝撃波を虚血領域の約40カ所に照射する治療を，隔日で計3回行った．2003年から重症狭心症患者9人を対象に実施した第1次臨床試験では，全例で狭心症症状が軽減，ニトログリセリンの使用量が減少し，その効果は1年以上にわたって持続した(**図3a，b**)[1]．また，負荷心筋シンチグラフィで評価した心筋血流も，衝撃波を照射した部位においてのみ改善を認めた(**図3c**)[1]．一方，治療に伴う合併症や副作用は認めなかった．さらに，2005年から実施した第2次臨床試験では，衝撃波治療とプラセボ治療との比較を行ったが，低出力体外衝撃波治療後には，狭心症の重症度，ニトログリセリンの使用頻度，6分間歩行距離が有意に改善し，MRIで測定した左室1回拍出量，左室駆出率も有意に増加した[2]．これらの効果はプラセボ治療後では認められなかった．

以上の良好な結果により，狭心症に対する低出力体外衝撃波治療は，2010年7月に先進医療に承認され，現在，東北大学病院を含めた国内数施設で治療を行っている．費用は，3日間の治療で265,500円である．すでに世界で5,000例以上の狭心症患者に対して治療が行われているが，重篤な合併症の報告はない．本治療法で用いる衝撃波の出力は弱いため麻酔や鎮静薬の投与は不要であること，また，体外から衝撃波を照射する非侵襲的な治療法であることから，重症例や高齢者にとっても肉体的負担が少ないという点で優れている．

おわりに

われわれは虚血性心疾患に加えて，ウサギ下肢虚血モデル，ラットリンパ浮腫モデル，マウス難治性皮膚潰瘍モデルにおいても，低出力体外衝撃波治療の有効性・安全性を確認している．また，間欠性跛行を有する下肢末梢動脈疾患症例を対象とした臨床試験においても，最大歩行距離と末梢循環の改善を認めている[6]．整形外科領域では，炎症性疾患や難治性骨折の治療にも衝撃波治療が応用されており，今後，幅広い疾患への応用が期待される．

文献

1) Fukumoto Y, et al : Extracorporeal cardiac shock wave therapy ameliorates myocardial ischemia in patients with severe coronary artery disease. Coron Artery Dis 17 : 63-70, 2006
2) Kikuchi Y, et al : Double-blind and placebo-controlled study of the effectiveness and safety of extracorporeal cardiac shock wave therapy for severe angina pectoris. Circ J 74 : 589-591, 2010
3) Nishida T, et al : Extracorporeal cardiac shock wave therapy markedly ameliorates ischemia-induced myocardial dysfunction in pigs *in vivo*. Circulation 110 : 3055-3061, 2004
4) 伊藤健太，下川宏明：体外衝撃波を用いた非侵襲性血管新生治療．日内会誌 99 : 2846-2852, 2010
5) Ito K, et al : Extracorporeal shock wave therapy for ischemic cardiovascular disorders. Am J Cardiovasc Drugs 11 : 295-302, 2011
6) Serizawa F, et al : Extracorporeal shock wave therapy ameliorates walking ability of patients with peripheral artery disease and intermittent claudication. Circ J 76 : 1486-1493, 2012

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Ischemic Heart Disease

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, PhD; 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/mm^2, 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

Recent progress in emergency care and patient management has improved the prognosis of patients with acute myocardial infarction (AMI).[1–4] However, left ventricular (LV) remodeling after AMI still remains one of the unsolved problems.[5,6] Thus, it is crucial to develop new therapeutic strategies to suppress LV remodeling after AMI. We have developed a non-invasive angiogenic therapy with extracorporeal low-energy shock waves (SW), and have demonstrated its efficacy and safety in a porcine model of chronic myocardial ischemia[7] and patients with angina pectoris.[8,9] Furthermore, we have demonstrated that SW therapy ameliorates LV remodeling after AMI in pigs in vivo.[10,11] However, it remains to be examined whether SW therapy also exerts anti-inflammatory effects on AMI in addition to its angiogenic effects.

Low-energy SW therapy suppresses the production of several cytokines, chemokines, and matrix metalloproteinases in a murine skin graft model,[12] and inhibits tumor necrosis factor (TNF)-α expression induced by lipopolysaccharides in a rat glioma cell line in vitro.[13] In addition, low-energy SW therapy exerts anti-inflammatory effects on orthopedic diseases, such as tendinitis, epicondylitis, plantar fasciitis and several inflammatory tendon diseases.[14] Infiltration of inflammatory cells (eg, macrophages) is critically important in wound healing after AMI, while excessive inflammatory responses deteriorates LV remodeling in the chronic phase.[15–17] In the present study, we thus examined whether SW therapy exerts beneficial anti-inflammatory effects in a rat model of AMI.

Methods

The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes

Received February 23, 2014; revised manuscript received August 27, 2014; accepted August 31, 2014; released online October 2, 2014
Time for primary review: 11 days
Department of Cardiovascular Medicine (Y.A., K.I., K.H., T.S., T.O., Y.K., R.K., K.N., K.S., S.M., H.S.), Department of Medical Microbiology, Mycology and Immunology (Y.A., K.K.), Tohoku University Graduate School of Medicine, Sendai, Japan
The Guest Editor for this article was Ken-ichi Hirata, MD.
Mailing address: Kenta Ito, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan. E-mail: ito-kenta@cardio.med.tohoku.ac.jp
ISSN-1346-9843 doi:10.1253/circj.CJ-14-0230

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 anesthetic inhalation overdose with isoflurane.

Extracorporeal SW Therapy

Based on our previous studies,[7–11,18] we performed low-energy SW therapy (0.1 mJ/mm^2, 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 inhalation 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.

Real-Time Polymerase Chain Reaction (PCR)

We measured the mRNA expression of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β1 in the LV. The heart samples were homogenized and used for total RNA extraction with a RNeasy Plus Mini Kit (QIAGEN, Venlo, Netherlands). cDNA was synthesized by using PrimeScript® RT Master Mix (Takara, Shiga, Japan). The primer sequences were as follows: eNOS (Forward) 5'-CTGTGTGACCCTCACCGATACAA-3' and (Reverse) 5'-AGCACAGCCACGTTAATTTCCA-3'; VEGF (Forward) 5'-GCACGTTGGCTCACTTCCAG-3' and (Reverse) 5'-TGGTCGGAACCAGAATCTTTATCTC-3'; TGF-β1 (Forward) 5'-CATTGCTGTCCCGTGCAGA-3' and (Reverse) 5'-AGGTAACGCCAGGAATTGTTGCTA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward) 5'-GGCACAGTCAAGGCTGAGAATG-3' and (Reverse) 5'-ATGGTGGTGAAGACGCCAGTA-3'. After reverse transcription, real-time PCR was performed with SYBR® Premix Ex Taq™ II (Takara) and a CFX96™ Real-Time system C1000™ Thermal Cycler (Bio-rad, Hercules, CA, USA). The PCR conditions were 40 cycles of 2 s at 98°C and 5 s at 55°C. The mRNA expression levels were compared between the control and SW groups. Results are reported as the quotients of the copy number of the gene of interest, relative to that of GAPDH, as a housekeeping gene.

Western Blot Analysis

We measured protein levels of phosphorylated eNOS (phospho-eNOS), total-eNOS and VEGF in the LV. Samples from the LV were used and the extracted samples were subjected to SDS-PAGE/immunoblot analysis by using the specific antibody for phospho-eNOS at Ser1177 (No.9571; Cell Signaling Technology, Danvers, MA, USA), total-eNOS (No.610298; Becton Dickinson, Franklin Lakes, NJ, USA) and VEGF (sc-507; Santa Cruz). The regions containing proteins were visualized by an electrochemiluminescence Western blotting luminal reagent (RPN2132; GE Healthcare Bioscience, Waukesha, WI, USA). The extents of eNOS phosphorylation and VEGF expression were normalized by that of total eNOS and α-tubulin, respectively.

Cytokine Analysis

We also measured tissue cytokine levels in the border zone of the LV, using a Bio-Plex Pro Rat cytokine custom plate and a Bio-Plex 200 system (Bio-Rad). Data were analyzed using the Bio-Plex Manager 4.1.1 software. Samples were processed and analyzed according to the manufacturer's instructions for the Bio-Plex 200 system (Bio-Rad).

Statistical Analysis

Continuous results are expressed as mean±SEM. 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 ng/ml, P=0.81, n=10; day 6, 0.19±0.26 vs. 0.08±

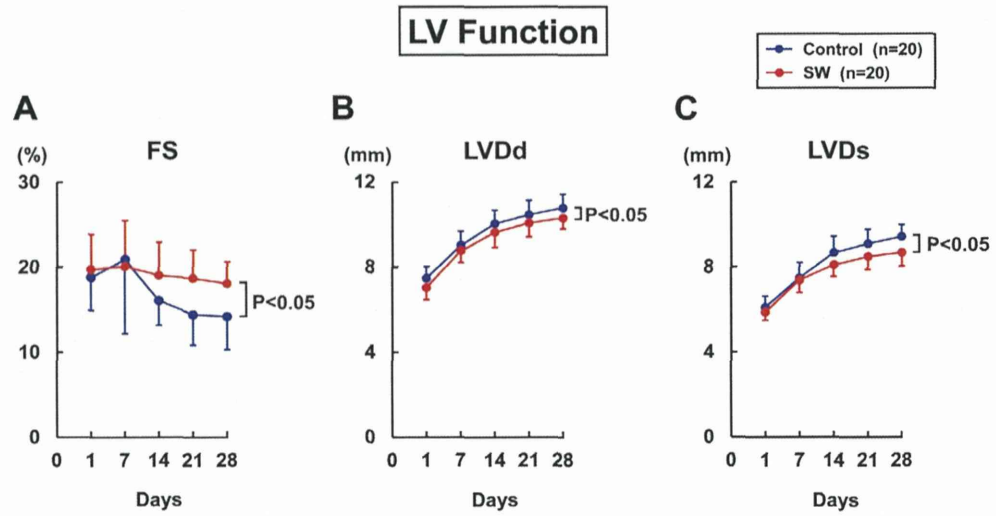

Figure 1. Shock-wave (SW) therapy ameliorates left ventricular (LV) function after acute myocardial infarction. LV function was serially evaluated by echocardiography. (**A**) LV fractional shortening (FS). (**B**) LV end-diastolic dimension (LVDd). (**C**) LV end-systolic dimension (LVDs). Results are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

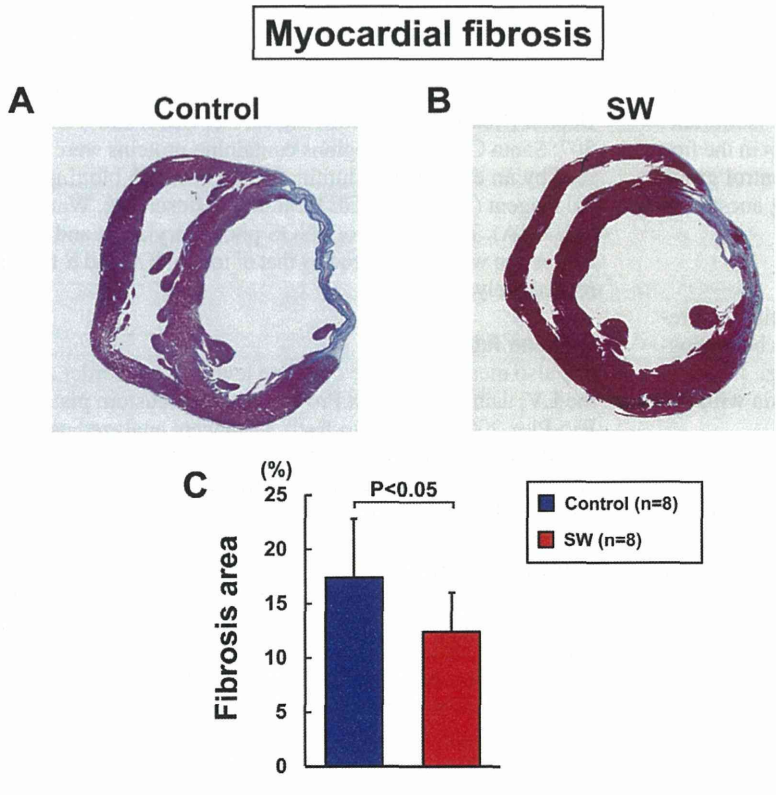

Figure 2. Shock-wave (SW) therapy ameliorates left ventricular (LV) fibrosis after acute myocardial infarction. (**A**,**B**) Masson-trichrome staining in the control (**A**) and the SW group (**B**). Fibrotic area was stained in blue. (**C**) LV fibrosis on day 28. Fibrotic area was quantified as a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

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-

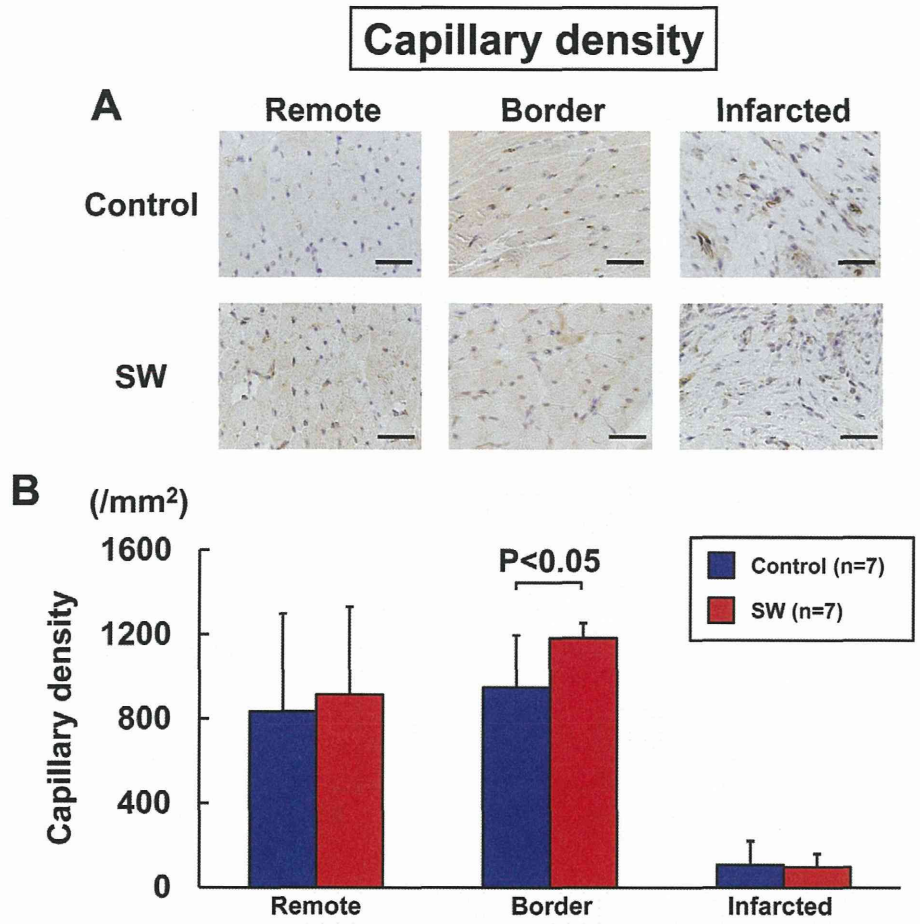

Figure 3. Shock-wave (SW) therapy enhances capillary density after acute myocardial infarction. The number of CD31-positive cells on day 28 was counted at ×400 magnification. Scale bars represent 50 μm. Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.

mension (LVDs) were progressively increased by day 28 in the control group, which was significantly ameliorated in the SW group (**Figures 1B,C**). In the sham-operated animals, SW therapy did not affect FS, LVDd or LVDs (**Figure S1**). Thus, SW therapy significantly ameliorated LV remodeling after AMI in rats in vivo, as we previously noted in pigs in vivo.[10,11] However, there was no significant difference in mortality between the SW and the control groups.

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/mm^2, P<0.05; **Figure 3**).

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 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

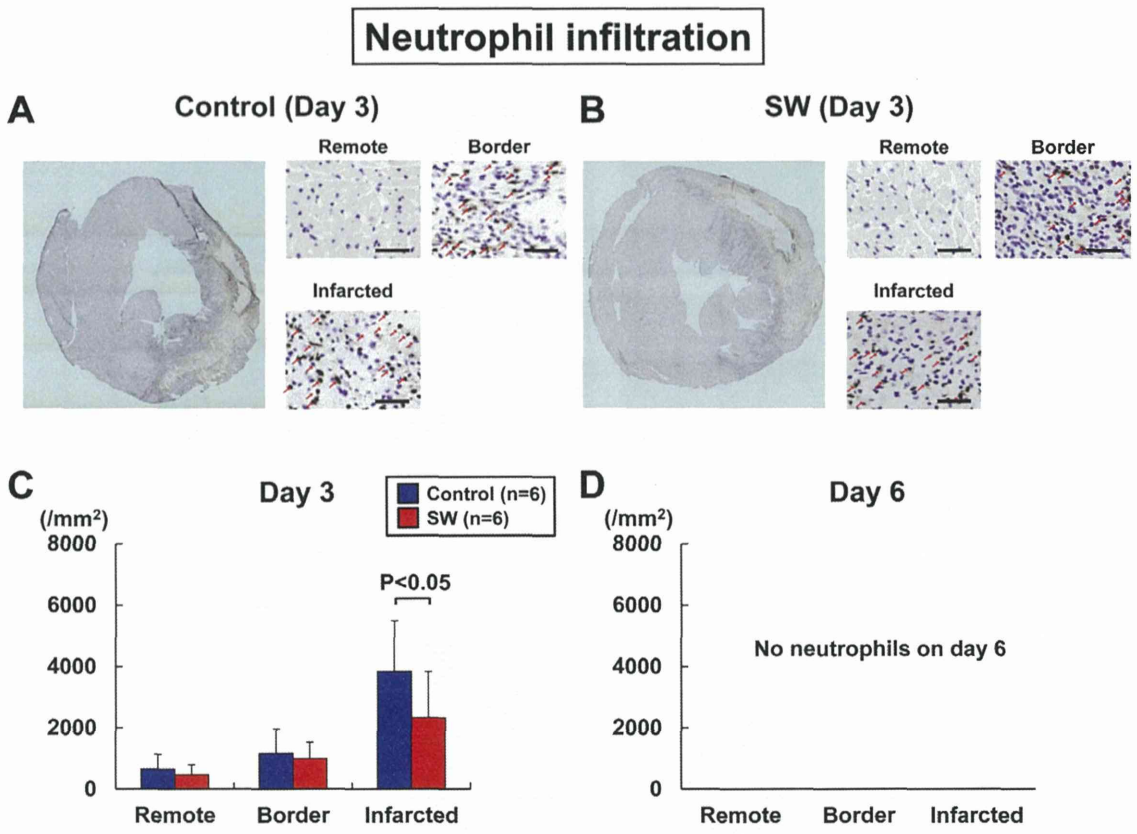

Figure 4. Shock-wave (SW) therapy suppresses neutrophil infiltration after acute myocardial infarction. (**A**,**B**) Immunohistochemical staining of neutrophils in the control group (**A**) and the SW group (**B**) on day 3. Scale bars represent 50 μm. (**C**,**D**) The number of infiltrated neutrophils in the control and the SW group on day 3 (**C**) and day 6 (**D**). The number of neutrophils was counted at ×400 magnification. No neutrophils were detected in either remote, border or the infarcted area on day 6. Results are expressed as mean±SEM. P<0.05 for the control vs. the SW group.

was similarly elevated in both groups (**Figures 6D–F**). Western blot analysis showed that the ratio of phospho-eNOS to total-eNOS, a marker of eNOS activity, was higher in the SW group than in the control group at day 3 (**Figures 6G–I**), while the protein levels of VEGF at day 6 was higher in the control group (**Figures 6J–L**).

Expression of TGF-β1

Messenger RNA expression of TGF-β1 in the remote area was significantly lower in the SW group than in the control group at day 3. The expression of TGF-β1 was higher in the border area while that in the infarcted area was lower in the SW group than in the control group at day 6 (**Figures 7A–C**). The number of TGF-β1-positive cells was significantly lower in the border and infarcted area in the SW group than in the control group at day 3 (**Figure 7D**). However, the number of TGF-β1-positive cells was lower in the control group in the remote and infarcted area at day 6 (**Figure 7E**). These results suggest that the infiltration of TGF-β1-positive cells was suppressed and delayed by SW therapy.

Myocardial Cytokine Levels

Myocardial levels of inflammation-related cytokines were measured in the border area where inflammatory cells infiltrated. 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

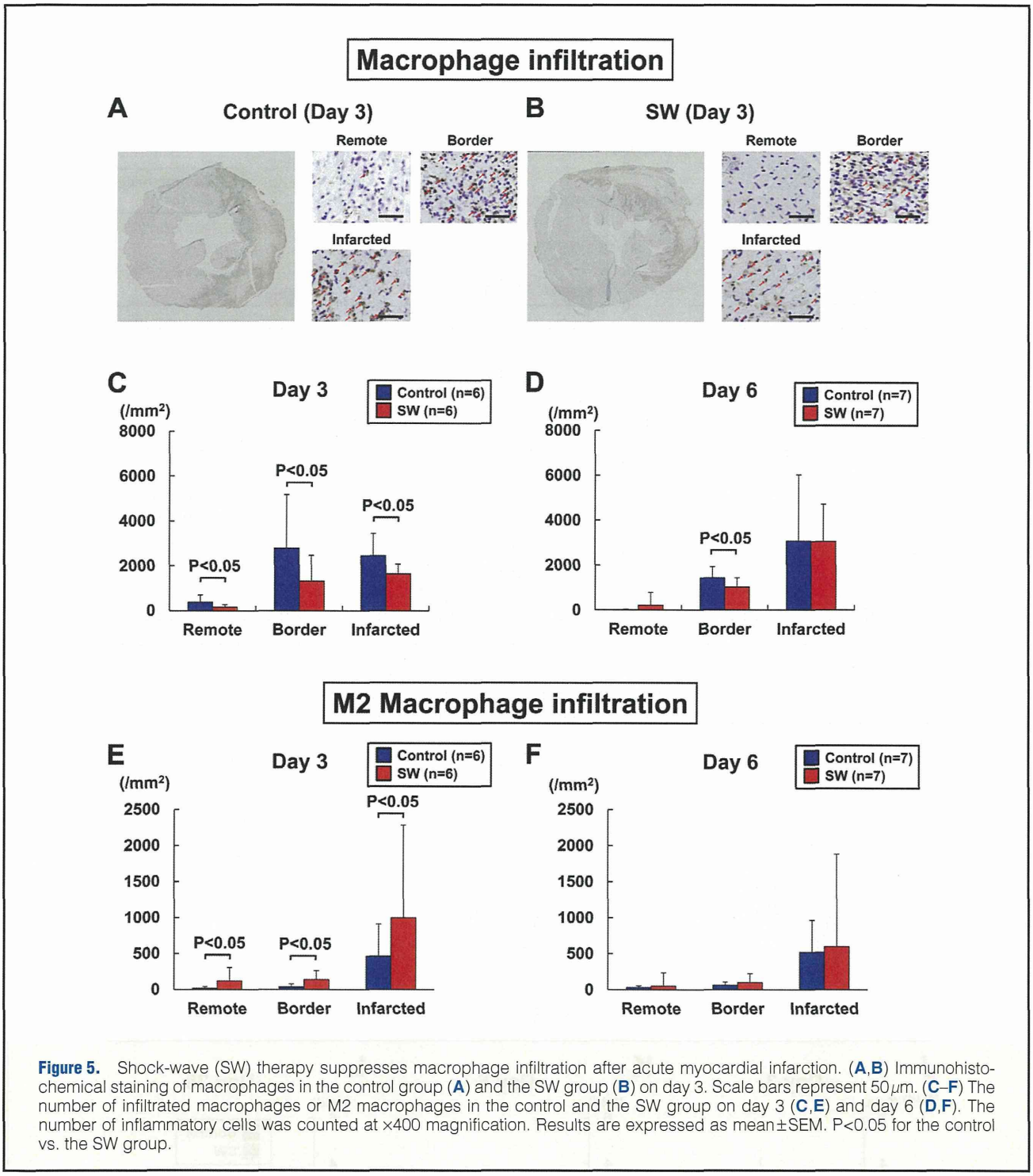

Figure 5. Shock-wave (SW) therapy suppresses macrophage infiltration after acute myocardial infarction. (**A**,**B**) Immunohistochemical staining of macrophages in the control group (**A**) and the SW group (**B**) on day 3. Scale bars represent 50 μm. (**C**–**F**) The number of infiltrated macrophages 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.

in vivo.

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.[10,11] 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.[15–17]

Suppression of Post-MI Inflammatory Responses by SW Therapy

Inflammatory cells play important roles in myocardial tissue repair after AMI. Infiltration of inflammatory cells (eg, macrophages) is essential in wound healing, while excessive in-

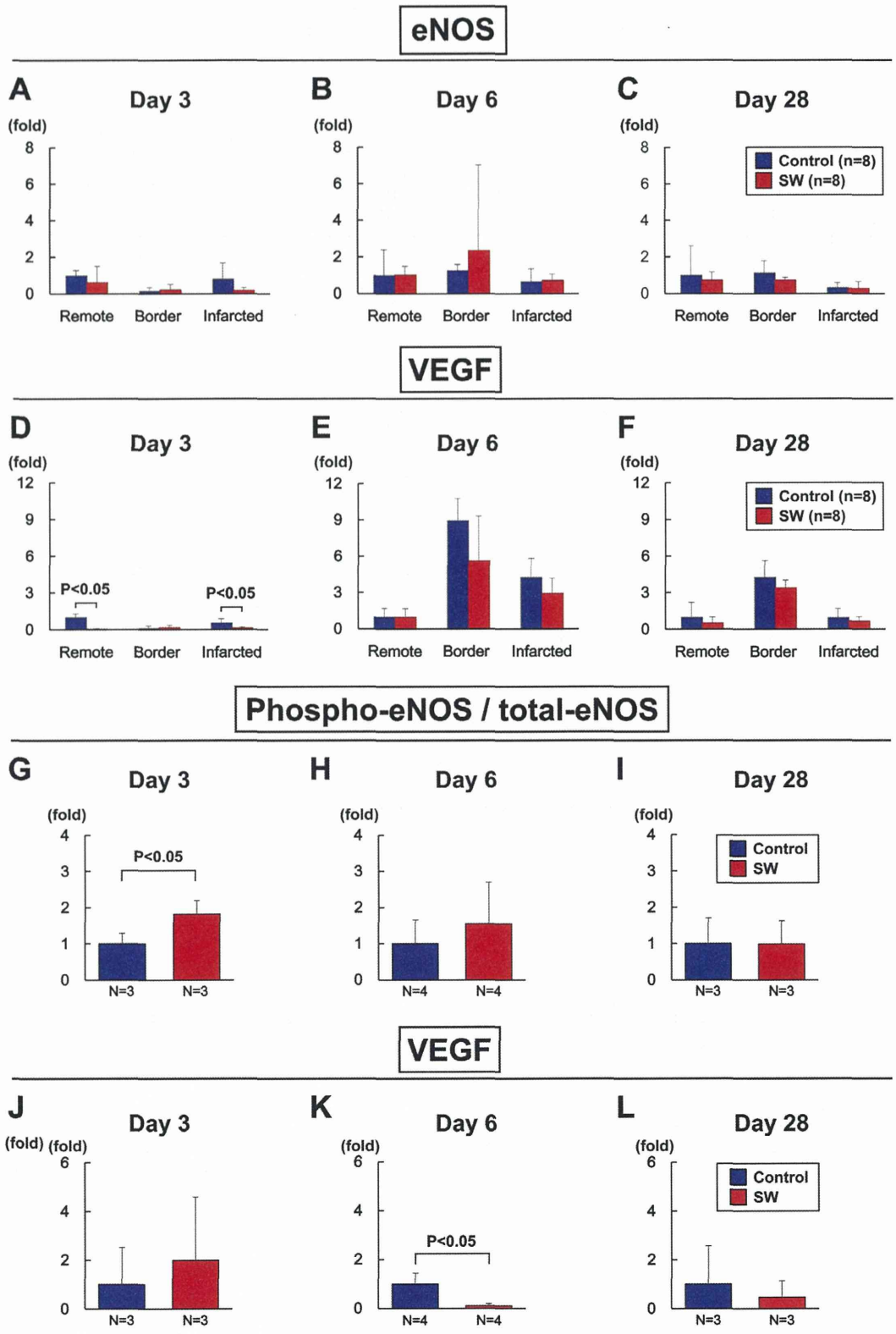

Figure 6. Shock-wave (SW) therapy enhances endothelial nitric oxide synthase (eNOS) activity after acute myocardial infarction. (**A**–**C**) mRNA expression levels of eNOS on day 3 (**A**), day 6 (**B**) and day 28 (**C**). (**D**–**F**) The expression levels of vascular endothelial growth factor (VEGF) on day 3 (**D**), day 6 (**E**) and day 28 (**F**). (**G**–**I**) The ratio of phosphorylated eNOS (phosphor-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.