Direct epicardial shock wave therapy improves ventricular function and induces angiogenesis in ischemic heart failure

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Objectives: Direct application of low-energy unfocused shock waves induces angiogenesis in ischemic soft tissue. The potential effects of epicardial shock wave therapy applied in direct contact to ischemic myocardium are uncertain.

Methods: For induction of ischemic heart failure in a rodent model, a left anterior descending artery ligation was performed in adult Sprague–Dawley rats. After 4 weeks, reoperation with (treatment group, n = 60) or without (control group, n = 60) epicardial shock wave therapy was performed. Low-energy shock waves were applied in direct contact with the infarcted myocardium (300 impulses at 0.38 mJ/m^2). Additionally, healthy animals (n = 30) with normal myocardium were studied. Angiogenesis, ventricular function upregulation of growth factors, and brain natriuretic peptide levels were analyzed.

Results: Histologic analysis revealed significant angiogenesis 6 weeks (treatment group: 8.2 ± 3.7 vs control group: 2.9 ± 1.9 vessels per field, P = .016) and 14 weeks (treatment group: 7.1 ± 3.1 vs control group: 3.2 ± 1.8 vessels per field, P = .011) after shock wave treatment. In the treatment group ventricular function improved throughout the follow-up period (6 weeks: 37.4% ± 9% [P < .001] and 14 weeks: 39.5% ± 9% [P < .001]). No improvement of ventricular function was observed in the control group (6 weeks: 28.6% ± 5% and 14 weeks: 21.4% ± 5%). Rat brain natriuretic peptide 45 levels were lower in the treatment group compared with those in the control group 6 and 14 weeks after treatment. Vascular endothelial growth factor, Fms-related tyrosine kinase 1, and placental growth factor levels were upregulated after 24 and 48 hours and 7 days in the treatment group. No effects on healthy myocardium were observed.

Conclusion: Direct epicardial low-energy shock wave therapy induces angiogenesis and improves ventricular function in a rodent model of ischemic heart failure.

The increasing incidence of advanced ischemic heart failure and the availability of traditional therapies to selected patients only has driven the need for alternative myocardial regenerative therapies.[1] Based on the consideration that angiogenesis might reverse the pathophysiologic process that leads to ischemic heart failure, a therapy that induces angiogenesis could be developed as an alternative or adjunctive treatment for patients with advanced ischemic heart failure.

Defocused low-energy shock wave therapy (SWT) applied in direct contact to ischemic tissue induces angiogenesis and, consecutively, tissue regeneration in ischemic limbs, skin flaps, and vascular ulcers.[2-6] However, the effects of epicardial SWT applied in direct contact with ischemic myocardium are uncertain.

To address this issue, we tested a system for direct epicardial SWT and initiated an experimental study in a rodent model of ischemia-induced heart failure. We measured the effect of direct epicardial SWT on angiogenesis and followed the animals by means of echocardiographic analysis to study the efficacy of this treatment in the reversal of impaired left ventricular (LV) function. Upregulation of growth factors and development of brain natriuretic peptide (BNP) levels were analyzed. Furthermore, we studied the effects of direct epicardial SWT on healthy myocardium.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the Medical University of Vienna approved this study (GZ: 66.009/0176-BrGT/2005, GZ: 66.009/0217-BrGT/2006). Operations and animal care were provided in accordance with the ''Guide for the care and use of laboratory animals'' (National Institutes of Health, volume 25, no. 28, revised 1996).

Experimental Animals

A total of 210, random, 8- to 10-week-old Sprague–Dawley rats (240–280 g) were obtained from the ''Institut für Labortierkunde und Genetik'' (Himberg, Austria) and inbred in a pathogen-free facility under strict veterinary supervision and maintained in controlled conditions with 12-hour light/dark cycles. The animals received a commercial rat diet and water ad libitum.

Abbreviations and Acronyms

BNP   = brain natriuretic peptide
Flt-1 = Fms-related tyrosine kinase 1
LAD   = left anterior descending artery
LV   = left ventricular
PCR   = polymerase chain reaction
PlGF = placental growth factor
SWT   = shock wave therapy
VEGF = vascular endothelial growth factor
vWF   = von Willebrand factor

Induction of myocardial infarction was performed in 180 rats according to a standardized model by means of left anterior descending artery (LAD) ligation.[7] Animals were anesthetized with ketamine (100 mg/kg), xylazine (Rompum; 10 mg/kg), and isoflurane (2%) and ventilated after orotracheal intubation. LAD ligation was performed through a left minithoracotomy, the pericardium was opened, and the proximal LAD was ligated with Prolene 6-0 sutures to induce a sizable LV infarct. A total of 122 rats survived the LAD ligation. Thirty rats underwent thoracotomy without LAD ligation under the same conditions.

Four weeks after LAD ligation, the surviving 120 animals with large, echocardiographically proved LV infarcts were randomized to one of 2 groups: reoperation with (treatment group, n = 60) and reoperation without (control group, n = 60) SWT. For reoperation, animals were anesthetized with ketamine (100 mg/kg), xylazine (10 mg/kg), and isoflurane (2%) and ventilated after orotracheal intubation. Rethoracotomy was performed, and the heart was dissected free from the thoracic wall. Homemade air-filled plastic bags were positioned around the heart to expose the LV anterior wall and to avoid shock wave–induced lung injury.[8] A commercially available ultrasound gel serving as contact medium was applied to the LV anterior wall of the heart (Aquasonic; Parker Laboratories, Inc, Fairfield, NJ; Figure 1, A). In the treatment group a total of 300 shock wave impulses were applied to the LV anterior wall by using a CardioGold (CRT/MTS-Europe GmbH, Konstanz, Germany) SWT system and a specially designed applicator (CRT/MTS-Europe GmbH; Figure 1, B). An identical procedure without application of SWT was performed in the control group.

To investigate the effects of SWT on healthy myocardium, a third group was studied (SWT only, n = 30): thoracotomy without LAD ligation and, 4 weeks later, rethoracotomy with SWT on healthy noninfarcted myocardium.

Animals were killed 6 and 14 weeks after treatment, hearts were harvested and processed for immunohistochemistry, and serum was processed for enzyme-linked immunosorbent assays. For real-time polymerase chain reaction (PCR) and Western blotting of proangiogenic cytokines, a subpopulation of animals was killed 24 and 48 hours and 7 days after reoperation.

Shock Wave Therapy

The CardioGold (CRT/MTS-Europe GmbH) SWT system and the used handheld applicator (CRT/MTS-Europe GmbH) were developed for the direct epicardial application of SWT (Figure 1, A). In contrast to SWT systems for nephrolithiasis or percutaneous cardiac SWT that produced focused shock waves, the CardioGold uses a parabolic reflector that produces unfocused, nearly parallel shock waves that allow treatment of a target area with a diameter of 0.5 to 0.7 mm and a penetration depth of 1 to 1.5 cm. The used energy flux density (0.38 mJ/mm²) and the cumulative treatment dose are based on our experience with the treatment of acute and chronic wounds, as well as diabetic and vascular ulcers, compared with that used for percutaneous cardiac SWT and represent about 10% of that used for lithotripsy.[6]

Immunohistochemistry and Quantitative Histology

Tissue was processed for paraffin embedding, and serial sections were stained with hematoxylin and eosin, Goldner, and a polyclonal anti-von Willebrand factor (vWF) antibody (Abcam, Cambridge, United Kingdom). Briefly, deparaffinized sections were preincubated with 3% H_2O_2 and horse serum (Jackson ImmunoResearch, West Grove, Pa) to block endogenous peroxidase and antigen activity before incubation with the primary antibody (vWF, 1:200 dilution). Biotinylated anti-mouse/rabbit IgG (H+L) secondary antibody (Vector Laboratories, Inc, Burlingame, Calif) was used to detect positive staining, followed by streptavidin–biotin peroxidase complex (LSAB®2 streptavidin–horseradish peroxidase; DakoCytomation, Glostrup, Denmark). For quantitative histology, digitalized images were made with a Nikon inverted microscope Eclipse TE2000-U (Nikon Instruments Europe B.V., Badhoevedorp, The Netherlands) at 40× magnification. Quantification was performed with Lucia G image analysis software. Six fields of the myocardial wall in the region of the LAD were evaluated in each heart. Angiogenesis and microvascular density were evaluated according to established procedures.[9] The number of vessels was counted, and vessel lumen areas, vessel wall thickness, and vessel width were measured. Vessels were divided into 3 sizes (small, 0–9 μm; medium, 10–19 μm; large, >20 μm). Additionally, the number of vWF⁺ cells was obtained. A single observer in an observer-blinded fashion performed all analyses.

Echocardiographic Analysis

Before LAD ligation, before SWT or sham operation, and before termination (6 and 14 weeks after SWT), echocardiographic analysis was performed (Philips iE33; Philips Medical Systems, Bothell, Wash; Transducer: S12-1). Rats were anesthetized with isoflurane. Standardized views of the heart were obtained at the papillary muscle level. Ejection fraction and LV diameters and volumes were obtained. Examinations were digitalized and evaluated by an independent experienced investigator.

Real-Time PCR Analysis

Total RNA was isolated from rat heart tissue (anterior wall) by using a TRIzol extraction (Invitrogen, Lofer, Austria), according to the manufacturer's protocol. cDNA was synthesized with M-MuLV Reverse Transcriptase (Fermentas, Burlington, Ontario, Canada) and 2 μg of total RNA primed with oligo dT-primer. After reverse transcription of RNA into cDNA, real-time PCR was used to monitor gene expression with the FastStart DNA Master SYBR Green kit and a LightCycler instrument (Roche Applied Science, Vienna, Austria), according to the standard procedure. The primer sequences (sense/antisense) were vascular endothelial growth factor (VEGF): 5′-TCCTGCAGCATAGCAGATGT-3′/5′-GCGAGTCTGTGTTTTTGCAG-3′; placental growth factor (PlGF): 5′-ACTGTGTGGCGCTAAAGACA-3′/5′-TTCCTCAGTCTGTGGGGTTT-3′; and Fms-related tyrosine kinase 1 (Flt-1): 5′-GGAGGCGAGGATTACAGTGA-3′/5′-GGAGGCGAGGATTACAGTGA-3′. LCDA Version 3.5.28 was used for PCR data analysis (Roche Applied Science). The specificity of the amplification product was determined by performing a melting curve analysis. Standard curves for expression of each gene were generated by means of serial dilution of known quantities of the respective cDNA gene template. Relative quantification of the signals was done by normalizing the signals of the different genes with the β_2-microglobulin signal. Measurements were done in triplicate.

Western Blotting

Rat heart tissues were lysed in solubilization buffer (10 mmol/L Tris–HCl, 50 mmol/L NaCl, 1% Triton X-100, 30 mmol/L sodium pyrophosphate, 100 μmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× Complete ethylenediamine tetraacetic acid–free Protease Inhibitor Cocktail (Roche Applied Science). Insoluble material was removed by means of centrifugation (15,000 rpm for 15 minutes at 4°C). Tissue lysates (50 μg per lane) were separated by means of sodium dodecylsulfate–polyacrylamide gel electrophoresis before electrophoretic transfer onto 0.2-μm
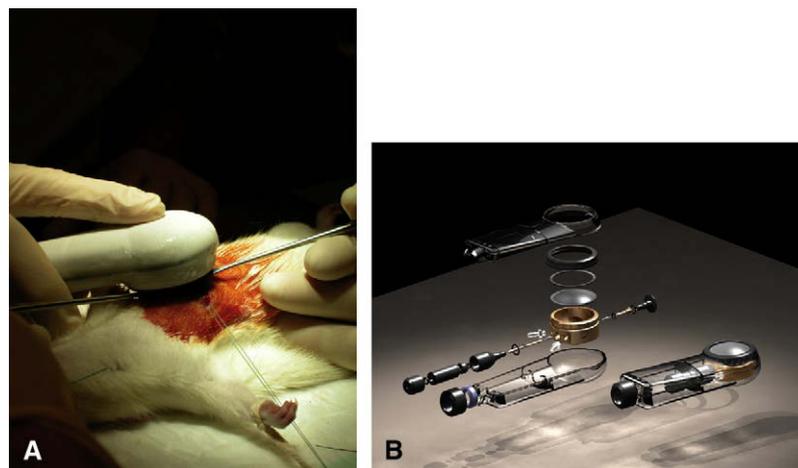
FIGURE 1. A, Direct epicardial shock wave therapy. B, Technical drawing of the applicator for direct epicardial shock wave therapy.

supported nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif). The blots were probed with polyclonal antibodies against VEGF and PlGF (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) before incubation with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare, Buckinghamshire, United Kingdom; Millipore, Billerica, Mass). Proteins were detected on the membrane by using SuperSignal West Pico Chemoluminnescent Substrate (Pierce, Rockford, Ill), and specific protein bands were quantified by normalizing the signals of the different proteins to the Ponceau S stain of the membrane with ImageQuant Version 5.0 software (Molecular Dynamics, Sunnyvale, Calif).

Enzyme-Linked Immunosorbent Assay for Rat BNP-45

Enzyme-linked immunosorbent assay for rat BNP-45 (AssayMax; AssayPro, St Charles, Mo) was performed according to the manufacturer's protocol. Fifty microliters of standard or a serum sample were added to each well. The wells were incubated for 2 hours at room temperature and then aspirated and washed 5 times. Biotinylated rat BNP-45 antibody (200 μL) was added to each well and incubated for 2 hours at room temperature, followed by 5 washes. Then 50 μL of streptavidin–peroxidase conjugate was added and incubated for 30 minutes at room temperature, followed by 5 washes. Afterward, 50 μL of chromogen substrate solution was added to each well and incubated for 7 minutes at room temperature. The reaction was stopped by adding 50 μL of stop solution. Then the optical density was detected immediately by using a microplate reader (Anthos, Salzburg, Austria) at 450 nm.

Statistical Analysis

Statistical analysis was performed with SPSS 14.0 software (SPSS, Inc, Chicago, Ill). Categorical variables are expressed as frequency distributions and percentages; continuous variables are expressed as means $\pm$ standard deviation. Categorical variables were compared by means of χ^2 or Fisher's exact tests, as appropriate. Continuous variables were either compared with analysis of variance (Bonferroni) after testing for normality of distribution or the Mann–Whitney test.

RESULTS
Animal Outcome

A total of 122 rats were alive 4 weeks after LAD ligation. Of those, 2 had only minor LV infarcts and were excluded from further analysis. The remaining 120 rats with large, echocardiographically proved LV infarcts were randomized to the treatment and control groups in a 1:1 ratio. On reoperation, 4 animals in the treatment group and 5 animals in the control group died immediately after induction of anesthesia. No deaths were observed during or after SWT. No deaths occurred in the SWT-only group.

Quantitative Histology and Immunohistochemistry

Quantitative histology of the LV anterior wall revealed more and larger vessels, indicating a higher microvascular density in the treatment group compared with the control group 6 and 14 weeks after treatment (Figure 2). We observed more vWF$^+$ endothelial cells in the treatment group compared with the control group 6 and 14 weeks after treatment (Figure 3, A). Fourteen weeks after treatment, we found more vital cells in the area of myocardial infarction in the treatment group compared with the control group (Figure 3, B). Quantitative histology of the (noninfarcted) LV posterior wall revealed no difference in microvascular density and endothelial cells, as well as vital cell count, 6 and 14 weeks after treatment. In SWT-only animals we observed no structural changes of the myocardium in the treated area. Furthermore, we could not detect any histologic signs of myocardial damage, including cellular infiltrates, extravasates, edema, cell membrane, or cell nuclei damage and hypertrophy.

Ventricular Function

Baseline LV ejection function was comparable between the treatment and control groups (treatment group, 59.0% $\pm$ 4%; control group, 55.7% $\pm$ 9%; $P = .581$). Four weeks after LAD ligation (before reoperation with or without treatment), LV function was impaired in the treatment (21.3% $\pm$ 10%) and control (21.6% $\pm$ 7%) groups compared with
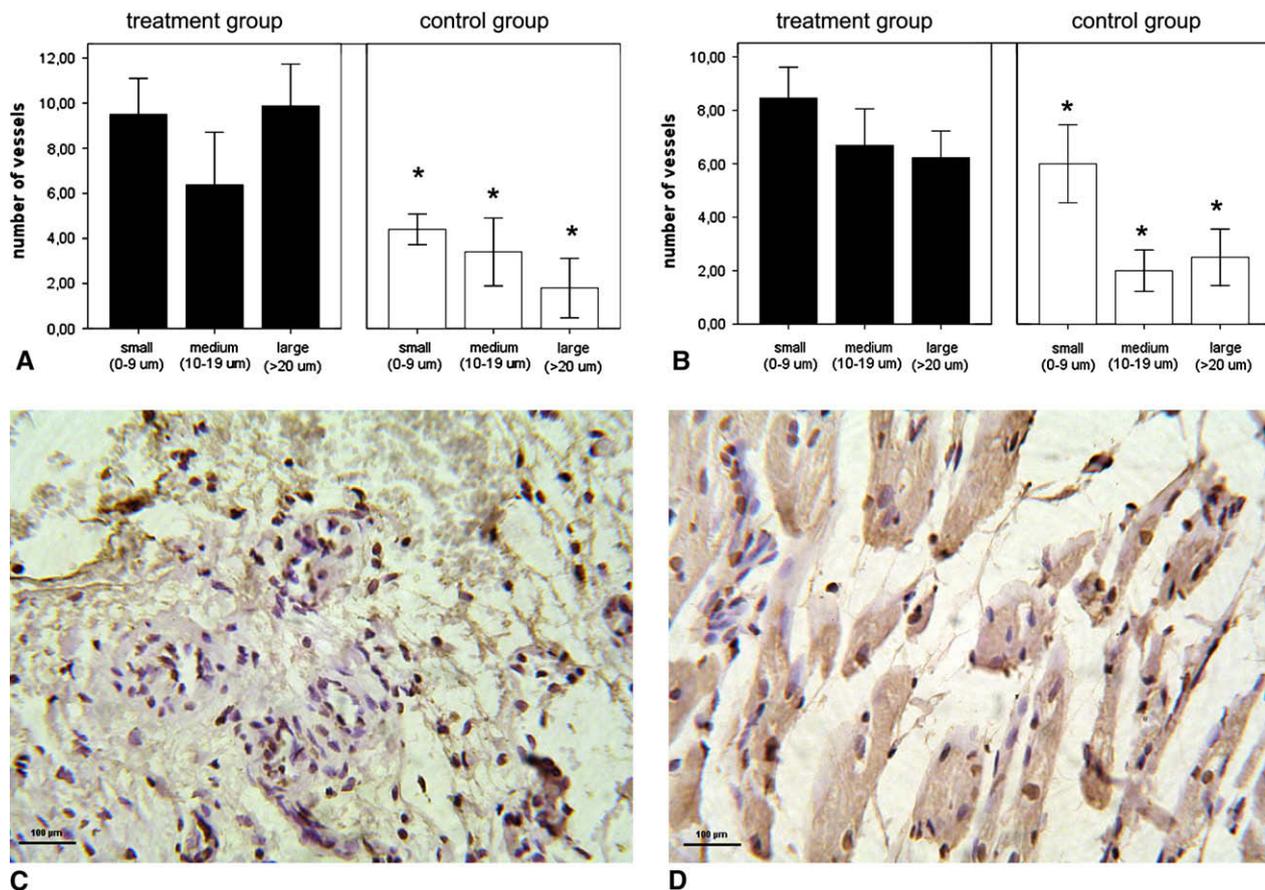
FIGURE 2. A, Microvascular density and vessel size expressed as the number of vessels per field (± standard deviation) in the treatment and control groups 6 weeks after treatment. *P < .05, treatment versus control groups. B, Microvascular density and vessel size expressed as the number of vessels per field (± standard deviation) in the treatment and control groups 14 weeks after treatment. *P < .05, treatment versus control groups. C, Factor VIII staining of the anterior wall treatment group. D, Factor VIII staining of the anterior wall control group.

baseline values (both $P < .0001$). In the treatment group LV function improved 6 ($37.4\% \pm 9\%$, $P < .001$) and 14 ($39.5\% \pm 9\%$, $P < .001$) weeks after SWT. LV function remained impaired in the control group 6 ($28.6\% \pm 5\%$) and 14 ($21.4\% \pm 5\%$) weeks after treatment (Figure 4). In SWT-only animals with noninfarcted myocardium, we observed normal LV function throughout the entire study period.

Expression of VEGF, Flt-1, and PlGF

We observed an upregulation of mRNA levels of VEGF, Flt-1, and PlGF 24 and 48 hours and 7 days after treatment in the treatment group compared with the control group (Figure 5, A-C). Furthermore, we observed an upregulation of VEGF protein level 7 days after treatment in the treatment group compared with the control group (Figure 5, D). We observed no significant commensurate upregulation of PlGF 24 and 48 hours and 7 days after treatment. Six and 14 weeks after treatment, we observed no difference in mRNA and protein levels of VEGF, Flt-1, and PlGF in the treatment group compared with the control group (data not shown).

Serum Levels of Rat BNP-45

We measured lower serum levels of rat BNP-45 6 and 14 weeks after treatment in the treatment group compared with the control group (Figure 6). The lowest (normal) serum levels of rat BNP-45 were observed in SWT-only animals throughout the study period.

DISCUSSION

We demonstrate that direct epicardial SWT induces angiogenesis and reverses ischemic heart failure, as shown by improved LV function and lower serum levels of BNP in a rodent model of ischemic heart failure.

Based on the broad clinical experience with percutaneous lithotripsy, first studies on SWT for myocardial regeneration followed the principle of distant percutaneous application.[10-14] These studies generated knowledge on the regenerative potential of SWT of ischemic myocardium, as demonstrated by induction of angiogenesis, reduction of infarct size, and improvement of ventricular function in pig models.[11,13] First experience in a very
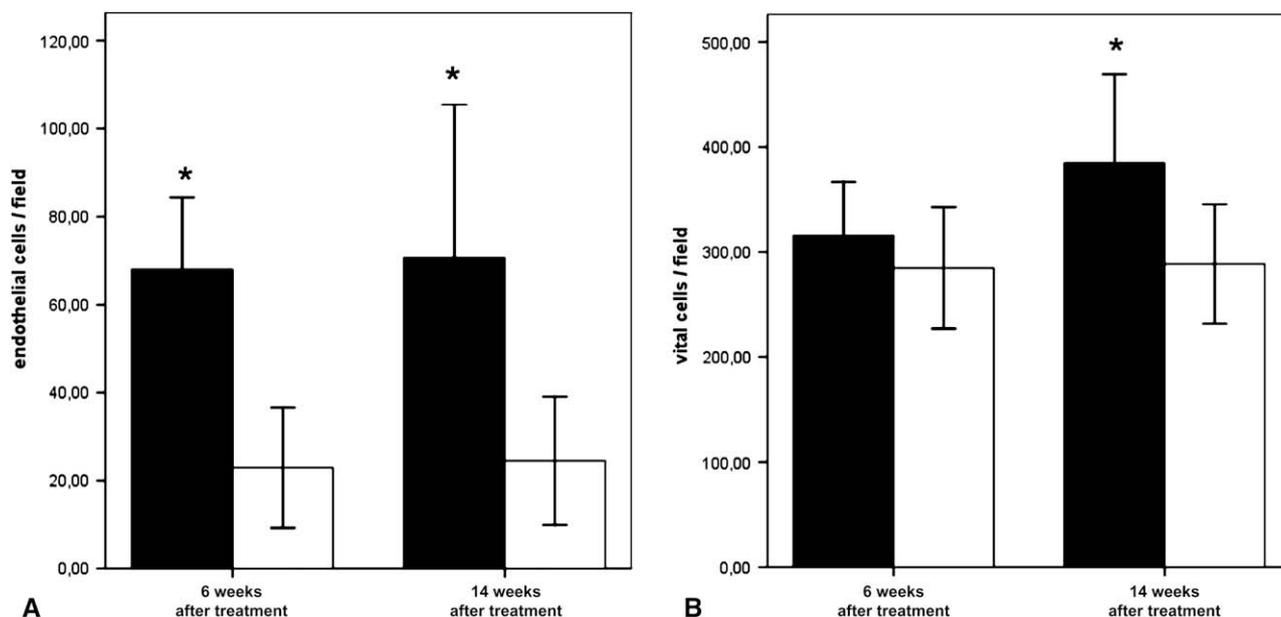
FIGURE 3. A, Number of endothelial (Factor VIII–positive) cells per field (± standard deviation) 6 and 14 weeks after treatment in the treatment and control groups. Black bar Treatment group; *white bar*, control group. *$P < .05$, treatment versus control groups. B, Number of vital cells per field (± standard deviation) in the infarct scar 6 and 14 weeks after treatment in the treatment and control groups. *Black bar*, myocardial infarction with shock wave therapy; *white bar*, myocardial infarction without shock wave therapy. *$P < .05$, treatment versus control groups.

limited clinical series of patients with diffuse coronary artery disease not amendable to percutaneous or surgical revascularization indicates that percutaneous cardiac SWT induces relief of symptoms of angina pectoris.[12,14] Results with regard to improvement of exercise capacity and regional myocardial perfusion remain inconsistent.[12,14] Despite these preliminary encouraging results, percutaneous SWT has not achieved broad clinical acceptance to date, most probably because of the fact that only microspots of ischemic myocardium can be treated with percutaneous SWT and treatment of whole infarct scars or large ischemia in single sessions is impossible.[12,14] This fact necessitates repeated treatment sessions to treat a substantial amount of the whole ischemic area, which renders this technique time-consuming, expensive, and complex.[11-14] A more defocused application allowing treatment of larger ischemic areas is limited by the dependence on appropriate acoustic windows because shock waves can severely injure lung tissue if applied over it.[15] Considering the impressive angiogenic potential of defocused low-energy shock waves applied in direct contact with ischemic soft tissues, we therefore tested a therapy system for low-energy unfocused direct epicardial SWT.[2-6] The obvious benefit of direct epicardial SWT is that the open approach allows precise identification of treatment areas and enables treatment of whole infarct scars and large areas of diffusely diseased myocardium in a single session with a justifiable expenditure of time in combination with conventional bypass surgery.

Direct epicardial SWT effectively induced significant angiogenesis throughout the entire follow-up period, as demonstrated by means of histologic analysis. We detected increased numbers of small, medium, and large vessels, as well as vWF+ endothelial cells, in treated areas of ischemic myocardium. This intense angiogenic effect relates to the observed early upregulation of VEGF, Flt-1, and PlGF. VEGF and Flt-1 are well-defined promoters of angiogenesis[16-18] and have been shown to induce angiogenesis and improve regional blood flow in preclinical and first clinical studies when injected into areas of chronic limb ischemia and ischemic myocardium.[19-21] Upregulation of VEGF
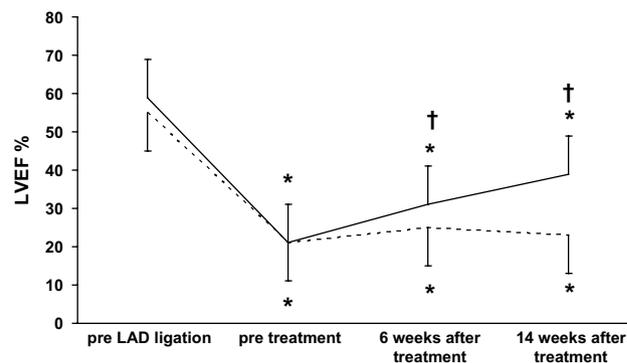

FIGURE 4. Development of left ventricular function expressed as left ventricular ejection fraction (*LVEF*; percentage ± standard deviation) in the treatment and control groups. *Solid line*, Treatment group; *dotted line*, control group. *$P < .05$ compared with before left anterior descending artery (*LAD*) ligation. †$P < .05$, treatment versus control groups.
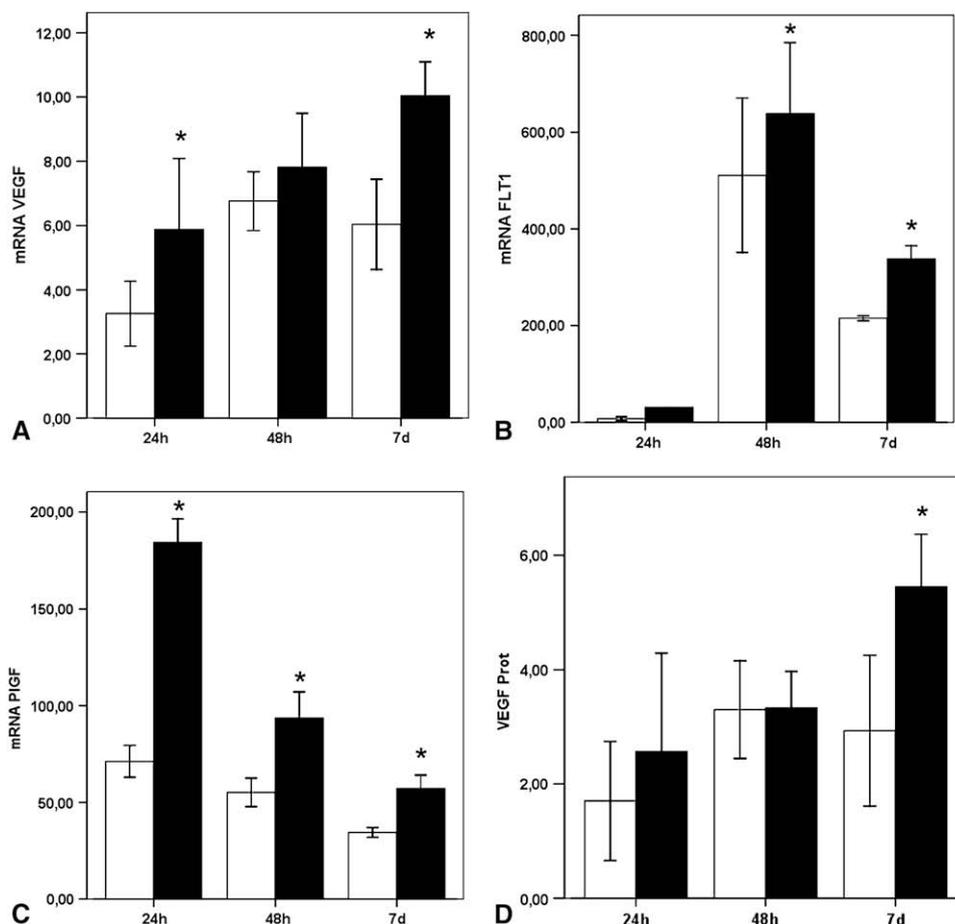
FIGURE 5. A, Expression of vascular endothelial growth factor *(VEGF)* mRNA in the treatment and control groups 24 and 48 hours and 7 days after treatment. *Black bar*, Treatment group; *white bar*, control group. *P < .05, treatment versus control groups. B, Expression of Fms-related tyrosine kinase 1 *(FLT1)* mRNA in the treatment and control groups 24 and 48 hours and 7 days after treatment. *Black bar*, Treatment group; *white bar*, control group. *P < .05, treatment versus control groups. C, Expression of placental growth factor *(PlGF)* mRNA in the treatment and control groups 24 and 48 hours and 7 days after treatment. *Black bar*, Treatment group; *white bar*, control group. *P < .05, treatment versus control groups. D, Expression of vascular endothelial growth factor *(VEGF)* protein in the treatment and control groups 24 and 48 hours and 7 days after treatment. *Black bar*, Treatment group; *white bar*, control group. *P < .05, treatment versus control groups.

and Flt-1 has previously been shown to induce angiogenesis in ischemic soft tissues, including myocardium, after SWT.[2,4,11] However, VEGF and Flt-1 alone induce only transient angiogenesis, and the majority of vessels regresses when the angiogenic stimulus fades away.[16,17,22] The sustainability of the angiogenic effect throughout the entire follow-up period might be explained by the concomitant up-regulation of PlGF. PlGF reportedly amplifies the angiogenic activity of VEGF, induces further VEGF release from fibroblasts, and, most importantly, stimulates the maturation of vessels through coverage with smooth muscle cells, which leads to stabilization and durability of new vessels.[23-25] Therefore the observed upregulation of VEGF in combination with PlGF might be responsible for the stable angiogenetic effect observed after a single application of SWT. Although the exact mechanism of mechanotransduction that links the extracellular stimulus of SWT to the

upregulation of proangiogenetic cytokines remains uncertain, there is growing evidence that cavitation phenomena that occur during SWT induce localized stress on cell membranes, with consecutive membrane hyperpolarization and ras activation.[26-28] Moreover, SWT has been shown to induce nonenzymatic nitric oxide synthesis and induction of stress fibers and intracellular gaps.[27,28] Nevertheless, the precise mechanisms for the shock wave–induced biochemical effects remains to be examined.

A single application of direct epicardial SWT resulted in a durable improvement of LV function, as assessed by means of echocardiographic analysis. With regard to improvement of LV function, various synergistic mechanisms have to be discussed. SWT-induced angiogenesis appears to play a primary role for the improved myocardial function. Angiogenesis in chronic ischemic myocardium and border zones of myocardial infarcts has been shown to reverse the
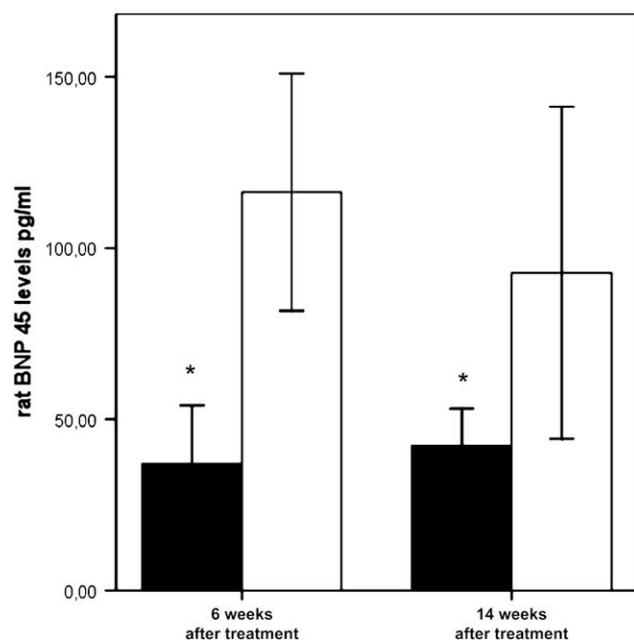
FIGURE 6. Rat brain natriuretic peptide 45 serum levels expressed as picograms per milliliter (± standard deviation) 6 and 14 weeks after treatment in the treatment and control groups. *Black bar*, Treatment group; *white bar*, control group. *$P < .05$, treatment versus control groups.

process of inadequate oxygenation and nutrient supply to ischemic but viable myocardium, thereby promoting the survival of tenuous cardiomyocytes and preventing the process of apoptosis and collagen deposition that leads to ventricular dilatation and ventricular remodeling.[29,30] Furthermore, the observed shock wave–mediated improvement of cell survival in the infarct and the infarct border zone might attenuate the process of scar thinning, which promotes the deleterious process of progressive ventricular dilation and remodeling.[31,32] This buttressing effect to the infarcted ventricular wall with improvement of the infarct's passive mechanical properties might be similar to that reported after cellular transfer.[31,32] The efficacy of SWT on reversal of impaired ventricular function in the animal model is supported by our measures of the serum levels of BNP as a marker for the prognosis of ischemic heart failure.[33] Lower serum levels of BNP throughout the entire follow-up period of 14 weeks indicate a significant and beneficial effect of SWT on heart failure.

The extent of angiogenesis and improvement of ventricular function after direct epicardial SWT in the present study compares with published results on gene therapy and cell transplantation with regard to induction of angiogenesis and improvement of ventricular function.[32,34] Although the initiating stimulus is different, cell therapy and SWT seem to have a common final path. The main benefit of direct epicardial SWT is its reproducibility and ease, which renders this technology convenient for surgical use as an adjunctive to bypass surgery.

In summary, direct epicardial SWT induces angiogenesis and reverses symptoms of ischemic heart failure, as shown by improved LV function and lower serum levels of BNP.

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