Low-Energy Shockwave Treatment Promotes Endothelial Progenitor Cell Homing to the Stenotic Pig Kidney

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Abstract
Endothelial progenitor cells (EPCs) patrol the circulation and contribute to endothelial cell regeneration. Atherosclerotic renal artery stenosis (ARAS) induces microvascular loss in the stenotic kidney (STK). Low-energy shockwave therapy (SW) can induce angiogenesis and restore the STK microcirculation, but the underlying mechanism remains unclear. We tested the hypothesis that SW increases EPC homing to the swine STK, associated with capillary regeneration. Normal pigs and pigs after 3 wk of renal artery stenosis were treated with six sessions of low-energy SW (biweekly for three consecutive weeks) or left untreated. Four weeks after completion of treatment, we assessed EPC (CD34+ /KDR+) numbers and levels of the homing-factor stromal cell-derived factor (SDF)-1 in the inferior vena cava and the STK vein and artery, as well as urinary levels of vascular endothelial growth factor (VEGF) and integrin-1β. Subsequently, we assessed STK morphology, capillary count, and expression of the proangiogenic growth factors angiopoietin-I, VEGF, and endothelial nitric oxide synthase ex vivo. A 3-wk low-energy SW regimen improved STK structure, capillary count, and function in ARAS+SW, and EPC numbers and gradients across the STK decreased. Plasma SDF-I and renal expression of angiogenic factors were increased in ARAS+SW, and urinary levels of VEGF and integrin-1β tended to rise during the SW regimen. In conclusion, SW improves ischemic kidney capillary density, which is associated with, and may be at least in part mediated by, promoting EPCs mobilization and homing to the stenotic kidney.

Keywords
low-energy shockwave, endothelial progenitor cells, capillary, renal artery stenosis

Introduction
Renal artery stenosis (RAS) is characterized by a heterogeneous group of pathophysiologic entities and decreased kidney function, of which atherosclerotic RAS (ARAS) is becoming increasingly common1. Recent advances in understanding the pathological features of ARAS implicated in the mechanisms underlying renal damage endothelial cell injury and loss, which result in a fall in renal blood flow (RBF) and development of tissue hypoxia2. Therefore, promoting angiogenesis and restoring capillary density have emerged as important therapeutic targets in ARAS. Microvascular endothelial cells, with complex functions of synthesis, secretion, metabolism, and immunity, are particularly central components of angiogenesis.

Endothelial progenitor cells (EPCs) are circulating, bone marrow–derived cells that are functionally and phenotypically distinct from mature endothelial cells. EPCs can differentiate into vascular endothelial cells to restore dysfunctional endothelium and protect against vascular disease3. Increasing evidence demonstrates that endogenous
circulating EPCs home to sites of neovascularization and differentiate into endothelial cells in situ, or secrete soluble mediators that promote vasculogenesis. Indeed, recent studies have identified protective properties involving transdifferentiation and paracrine effects of EPCs, and demonstrated that exogenously delivered EPCs can participate in restoring the stenotic kidney (STK) microvasculature and stabilize renal function, although it may not be fully normalized4,5. The mobilization from bone marrow and subsequent homing of EPCs are regulated by a variety of mediators, such as stromal cell-derived factor (SDF)-1, stem cell factor (SCF), and matrix metalloproteinase (MMP)-1, which are released by injured tissues to attract the cells and ensure their retention.

Low-energy shockwave therapy (SW) is a noninvasive intervention that has been shown to promote cellular proliferation and metabolism and restore tissue perfusion6. It involves delivery of focused low-energy acoustic waves, generating mechanical forces that affect cellular membranes7. SW power is translated into biological signals via mechanotransduction and activation of angiogenesis, which improves blood flow and blunts inflammation and oxidative stress. Low-energy SW has been shown to be beneficial in several clinical conditions, including bone fracture and tendinopathy8, skin ischemia and skin grafts9, and chronic soft tissue wounds10. We have also shown that cardiac SW reverses cardiac dysfunction and remodeling elicited by ischemia, and ameliorates angina pectoris11-13. Furthermore, in a previous study we found that SW therapy improved the microcirculation and function of the pig STK14. In a mouse model, SW elicits EPC homing to the ischemic hindlimb15. However, whether SW recruits EPCs to the kidney in a large preclinical animal model remains unknown. Therefore, we tested the hypothesis that SW-induced preservation of the STK peritubular capillaries in a unilateral ARAS swine model would be associated with augmented EPC homing.

Materials and Methods

Animals Experiment and SW Treatments

All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee. Twenty-four domestic female pigs (50 to 60 kg) were observed for 16 wk. Pigs were randomly divided into four groups that were treated or untreated by SW: NC (normal control), NC+SW, ARAS, and ARAS+SW (n = 6 each).

RAS was induced under fluoroscopic guidance by placing a local irritant coil in the right main renal artery after a 6-wk high-fat diet containing 2% cholesterol (Harlan Teklad, Madison, WI, USA). Normal pigs were fed with standard chow (Purina Animal Nutrition, Shoreview, MN, USA). All animals continued their assigned diets and had free access to water for the remaining observation period.

Three weeks after induction of ARAS or sham, a SW regimen was initiated (Omnispec Vetspec Model, MediSpec® LTD, Germantown, MD, USA; spark voltage 10 to 24 kV; energy density 0.09 mJ/mm²; frequency 120 pulse/min). As we previously described14, anesthetized pigs were laid prone, the skin of the back shaved, and ultrasound gel applied to their right flank. An ultrasound probe was placed at the lateral aspect of the STK along its long axis, and the SW applicator positioned perpendicularly above the kidney to distribute energy through the kidney along its short axis. Evenly distributed 1-cm zones across the entire kidney were treated with 200 rapid shots each. Treatments were applied twice a week for three consecutive weeks (a total of six sessions). In a subset of ARAS pigs undergoing SW, urine samples were collected at the first and last weeks of the 3-wk SW regimen to assess urinary levels of the proangiogenic factors vascular endothelial growth factor (VEGF) and integrin-β1.

The pigs were then anesthetized again with Telazol (Zoetis, Inc, Kalamazoo, MI, USA) and Xylazine (Xylamed, VetOne, Bimeda, MTC Animal Health, Cambridge, ON Canada), intubated, and cannulated 4 wk after SW treatment. Blood samples were collected from the inferior vena cava (IVC) and the STK vein and artery through a catheter placed under fluoroscopic guidance, and mean arterial pressure (MAP) measured using an arterial catheter. Glomerular filtration rate (GFR), RBF, and renal regional perfusion (ml/min/g tissue) were measured using Multidetector CT (Somatic Sensation-128, Siemens Medical Solution, Forchheim, Germany). RBF was quantified before and after systemic infusion of acetylcholine (5 μg/kg/min, Sigma), an endothelium-dependent vasodilator that dilates primarily renal microvessels16,17. Images were acquired following a central venous injection of Omnipaque (iohexol, 350 mgI/ml, distributed by GE Healthcare, Marlborough, MA, USA), and renal functional parameters calculated from time–density curves, as previously described18. Urine and blood samples were collected for measurement of biochemical parameters. The pigs were euthanized (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) 3 d after in vivo studies, and kidneys collected for histological and molecular assays.

EPC Quantification

Mononuclear cells were isolated from fresh IVC and renal vein and artery blood (10 ml) by the density-gradient method. EPCs were obtained from mononuclear cells and quantified using FlowSight™ (Amnis, Seattle, WA, USA) imaging flow cytometry by acquiring at least 10,000 positive events, and expressed as percentage. EPCs were characterized using Amnis® Image Data Exploration and Analysis Software (IDEAS) after immunostaining with monoclonal antibodies against the common EPCs markers CD34 (ab81289, Abcam, Cambridge, UK) and kinase-insert domain receptor (KDR, sc-504, Santa-Cruz Biotechnology, Dallas, TX, USA) (Fig. 1A). The flow-gating strategy included a first positive gate for single cells, followed by positive gate for CD34 and KDR (Fig. 1). Thresholds for
positivity were determined from histograms generated by single-color-stained positive and negative control beads.

EPCs were then counted in blood samples obtained from the renal artery, renal vein, and IVC, and gradients across the kidneys determined by subtracting the numbers of renal arterial from venous EPC counts.

**Renal Histology**

Kidney sections were stained with periodic acid-Schiff (PAS) and Masson’s trichrome, and then examined by light microscopy (Zeiss, Jena, Germany) in a blinded manner. Twenty randomly selected nonoverlapping regions from each kidney were analyzed. The PAS-stained paraffin sections were analyzed using a blinded scoring method using a grid. For each square, the presence of tubule injury, including intratubular proteinaceous casts or tubular atrophy, was documented. The final score is presented as the percentage of positive squares.

Interstitial fibrosis was quantified in Masson’s trichrome-stained paraffin sections; images were captured in 10 random fields per kidney, and the mean area of positive staining quantified using Image-J software (NIH, Bethesda, MA, USA). For capillary count, tissues were fixed and stained for the endothelial cell marker CD31 (MCA1747, AbD Serotec, Kidlington, UK) and the nuclear stain DAPI. Images were taken by microscopy (Zeiss). Peritubular capillaries identified as CD31-positive cells showing one nucleus were counted in each field using Image-J software (NIH).

**Angiogenic and Homing Factors**

Renal angiogenic activity was assessed by the expression of VEGF, its downstream effector endothelial nitric oxide synthase (eNOS), as well as angiopeititin-1 (Ang-1) that acts in concert with VEGF for microvascular stabilization. EPC homing factors were assessed by renal tissue expression...
of the EPC homing mediator MMP-1\(^{19}\), as well as blood levels of SDF-1 and SCF.

MMP-1 and Ang-1 expression was detected using western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated at 4°C overnight with primary antibodies against MMP-1 (ab137332, Abcam), Ang-1 (sc-8357, Santa-Cruz), and β-actin. After three washes with PBST/5 min, the nitrocellulose membranes were blocked, incubated at 4°C overnight with primary antibodies against MMP-1 (ab137332, Abcam), Ang-1 (sc-8357, Santa-Cruz), and β-actin. After washing, the membranes were visualized with enhanced chemiluminescence. Blots were quantified with densitometry using Image-J software (NIH).

Blood levels of SDF-1 (MBS735811, MyBiosource, San Diego, CA, USA), SCF (MBS2020518, MyBiosource), plasma renin activity (72-REN_CT2, ALPCO Diagnostics, Salem, NH, USA), serum creatinine (KBO2-H1, Arbor Assays, Ann Arbor, MI, USA), and urinary proteins (K024-H1, Arbor Assays) were measured using kits according to the manufacturers’ instructions.

For immunofluorescence, kidneys were fixed and stained for immunoreactivity of VEGF (ab53465, Abcam) and eNOS (ab5589, Abcam). Images were taken by microscopy (Zeiss) and analyzed by Image-J software (NIH).

### Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and results are expressed as mean ± standard deviation for normally distributed variables, and median (range) for non-Gaussian distributed data. Comparisons within groups were performed using the paired Student’s t-test and among groups using analysis of variance and unpaired t-test with Bonferroni correction. A statistical difference was considered significant for \( P \leq 0.05 \).

### Results

#### Systemic Characteristics

As shown in Table 1, body weight showed no significant difference among the four groups. Plasma renin activity and the angiographic degree of stenosis were similarly greater in ARAS and ARAS+SW animals compared to NC (\( P \leq 0.043 \)). ARAS pigs also showed a significant increase in urinary protein, serum creatinine, and MAP compared to NC, which were all improved after SW treatment (\( P \leq 0.037 \) vs. untreated ARAS).

#### Low-Energy SW Promotes EPC Homing

CD34+/KDR+ circulating EPCs were identified by imaging flow cytometry (Fig. 1A, B) and counted (Fig. 1C) in blood samples. Four weeks after protocol completion, circulating EPC numbers were decreased in the IVC of the untreated ARAS group (Fig. 1B, C, \( P \leq 0.048 \) vs. NC), but significantly improved in ARAS+SW (\( P \leq 0.039 \) vs. ARAS). Similarly, EPC numbers were decreased (\( P \leq 0.01 \) vs. NC) in the renal artery of ARAS, but not in ARAS+SW. There was no change in EPC numbers in the STK renal vein.

The EPCs gradient across the kidney (STK vein–artery) was significantly improved in ARAS+SW (\( P \leq 0.048 \) compared to ARAS), suggesting increased retention of EPCs in the STK.

#### Low-Energy SW Increases Expression of Homing and Angiogenesis Factors

SDF-1 levels were lower in the renal artery, renal vein, and IVC of the ARAS group, but were significantly increased and normalized in ARAS pigs treated with SW therapy (Fig. 2). There was no change in the levels of SCF. Renal expression of the angiogenic factor Ang-1 was downregulated in ARAS
and significantly increased after SW treatment to the kidney ($P = 0.037$ vs. NC), and significantly increased after SW treatment to the kidney ($P = 0.041$ vs. ARAS). The expression of the EPCs homing factor MMP-1 did not differ among the groups, although it tended to rise ($P = 0.07$ vs. NC) in the ARAS kidney. Furthermore, urinary levels of VEGF ($P = 0.058$) and integrin-1β ($P = 0.09$) tended to increase in ARAS+SW pigs over the course of the SW regimen (Fig. 2D).

**Low-Energy SW Restores Renal Morphology and Capillary Density**

Determine whether SW regulates STK capillary density, we counted CD31+ peritubular capillaries in the renal tubulo-interstitium. ARAS markedly reduced the number of capillaries ($P = 0.012$ compared with NC), which
significantly improved after SW treatment ($P = 0.027$ vs. ARAS) (Fig. 3A, F), indicating that SW enhanced capillary density. Similarly, immunoreactivity of the proangiogenic factors VEGF and eNOS was downregulated in ARAS, but SW restored their expression (Fig. 3B, C and G, H), although neither was fully normalized.
Tubulointerstitial fibrosis increased in ARAS compared with NC and NC+SW, and significantly decreased in ARAS+SW, as did renal tubular score (Fig. 3D, E and I, J).

**Low-Energy SW Preserves Renal Function in ARAS**

Renal perfusion, RBF, and GFR were unchanged in NC+SW, but were decreased in the ARAS STK ($P = 0.027$ vs. NC). However, these were all restored in ARAS+SW. Renal perfusion response to acetylcholine was attenuated in ARAS ($P = 0.047$ vs. NC), but restored to normal levels in ARAS+SW ($P = 0.027$ vs. ARAS), suggesting that SW improved microvascular endothelial function in the ARAS STK.

**Discussion**

This study demonstrates that increased number of peritubular capillaries in the stenotic ARAS kidney after SW therapy was associated with increased release of homing factors for EPCs, as well as elevated levels of EPCs in the stenotic renal artery and in the systemic circulation. Furthermore, SW therapy significantly improved RBF, microvascular endothelial function, and renal function. These observations suggest that SW improves ischemic kidney capillary density and microvascular function, which is associated with, and may be at least in part mediated by, promoting EPCs mobilization and homing to the STK.

ARAS represents a chronic ischemic renal disease, which is often accompanied by comorbidities like dyslipidemia, coronary artery disease, and peripheral arterial disease, and results in renovascular hypertension and ischemic nephropathy. The pathogenic mechanisms leading to ischemic kidney damage involve vascular endothelial cell injury and loss, renal tubular atrophy, interstitial fibrosis, and renin-angiotensin system activation.

Currently, there are no accepted noninvasive treatments to alleviate tissue damage in ARAS, and even invasive renal artery stenting does not bestow major benefits beyond optimal medical therapy. Therefore, identifying new therapeutic modalities in ARAS is of utmost importance. Low-energy SW is an effective, safe, and noninvasive intervention to ameliorate tissue ischemia. SW involves delivery of sound waves that induce physical cell cavitation and activation of angiogenic signaling to promote cell proliferation, metabolism, and repair. Hayashi and colleagues found an increase in capillary number using SW to induce wound healing. In the ischemic heart, SW upregulated mRNA expression of VEGF and its receptor and decreased tissue damage, and both experimental and clinical studies have shown improvement in myocardial blood flow and cardiac function after SW therapy.

In the kidney, peritubular capillaries transport oxygen and nutrients to renal tubular and interstitial cells, serving a pivotal role in sustaining normal kidney hemodynamics and function. Increasing evidence has demonstrated that renal injury progression is associated with peritubular capillary loss in both experimental animals and patients. Renal ischemia may cause endothelial cell dysfunction, swelling, and obstruction of peritubular capillaries and microvessels, leading to hypoxic injury, which stimulates interstitial fibrosis. Thus, increasing capillary vessels density is a fundamental approach to mitigating renal interstitial fibrosis in ARAS, and angiogenesis serves a crucial role in this process. Microvascular and capillary endothelial cells are the main effectors of angiogenesis.

We have previously shown that SW restored the number of large renal vessels (up to 500 µm in size) in ARAS, improved STK function, and blunted fibrosis. The current study extends our previous studies, and focused on peritubular capillaries, central determinants of renal function and predictors of renal disease progression. We found that their density was reduced in ARAS, but increased in ARAS treated with SW, and that this was linked to recruitment of EPCs. Furthermore, this study shows that SW also improves also function of STK microvessels (RBF response to acetylcholine) in ARAS pigs. Endothelial dysfunction has been implicated in the pathomechanism of chronic kidney disease progression, as well as in development of its cardiovascular ramifications. Therefore, improvement of the renal microcirculation likely contributed to decreasing development of renal injury distal to the stenosis.

EPCs are circulating bone marrow–derived progenitor cells that contribute to capillary formation and angiogenesis via migration, adhesion, and replacement of damaged endothelial cells. As a result, EPCs can assist in capillary formation, restore blood supply in ischemic tissues, and alleviate endothelial dysfunction. When circulating EPCs home to sites of vascular injury, they can differentiate into mature endothelial cells to participate in repair, or release proangiogenic mediators.

Pertinently, we observed in the ARAS STK downregulation of Ang-1, VEGF, and eNOS, which are essential for endothelial cell survival, vascular branching, and pericyte recruitment. This was associated with a fall in RBF and GFR, and development of renal interstitial fibrosis. Importantly, these were all improved in ARAS pigs treated with SW, in association with increased levels of EPCs in the systemic and renal circulation, as well as a fall in their cross-kidney gradient, suggesting an increase in both homing and retention of EPCs in the STK. Previous studies had shown that SW mobilized and increased EPCs homing into the ischemic murine hindlimb, and improved its functional recovery. Furthermore, in patients with post-infarction heart failure, pretreatment with SW improved left-ventricular ejection fraction 4 mo after intracoronary administration of bone marrow–derived mononuclear cells. Importantly, our study shows that SW therapy induces recruitment of endogenous EPCs to the STK of a large preclinical animal model, and thereby boosts self-repair.
The link between SW delivery and kidney improvement is strengthened by the increase in urinary levels of VEGF and integrin-1β over the course of the SW regimen. Integrin-1β is involved in mobilization, migration, and survival of endothelial and progenitor cells, and might have thus contributed to EPC recruitment. The mechanisms by which SW favored mobilization of EPC from the bone marrow might have also included release of SDF-1, an inducible chemokine that regulates multiple physiological processes and plays a major role in reendothelialization of injured vessels and revascularization of ischemic tissues. In particular, SDF-1 exerts a crucial role in mobilization of proangiogenic hematopoietic cells from the bone marrow. We observed a fall in SDF-1 levels in ARAS, and an increase in its IVC, STK vein, and STK artery levels after SW treatment, which might have contributed to EPCs mobilization from the bone marrow into the peripheral circulation. EPCs from the circulation are then directed to the kidney, possibly by adhesion molecules and integrins, to participate in renal repair of damaged endothelial cells and promote regeneration of capillaries. Upregulated expression of Ang-1 in EPCs from ARAS also supports their potential for cell homing and vascular proliferation. Contrarily, SCF levels remained unchanged after SW treatment, arguing against its role in EPCs recruitment in our model. In addition, MMP-1 expression tended to rise in the untreated STK, and might contribute to compensatory but unsuccessful renal homing and retention of EPCs in our ARAS model. MMP-1 can not only promote EPCs recruitment, but also induce the growth of endothelial cells and subsequent sprouting angiogenesis.

Limitations

Our studies are limited by using relatively young animals and relatively short period of observation. Tracking the fate of endogenous EPCs in the SW-treated kidney is difficult, because several types of renal parenchymal cells might co-express CD34 and KDR without necessarily being EPCs. We focused on mature, CD34+/KDR+ EPCs, but did not monitor alternative EPC populations, such as less mature CD133+ cells. Urinary levels of VEGF and integrin-1β tended to rise during the SW regimen, but this increase has not reached statistical significance due to the small sample size and variability of available urine samples. The SW regimen and machine settings that can best achieve EPCs recruitment also need to be optimized in future studies.

Conclusion

The present study demonstrates that a proangiogenic effect of SW on renal capillaries in chronic experimental renovascular disease is accompanied by mobilization and recruitment of endogenous EPCs to the kidney. This in turn leads to improved microvascular function, capillary density, and renal function and structure. These studies highlight SW therapy as a promising strategy to restore the kidney microcirculation in renovascular disease. Further studies are needed to gain insight into the molecular mechanisms linking SW and EPCs in the STK.

Ethical Approval

This study was approved by our Institutional Animal Care and Use Committee.

Statement of Human and Animal Rights

This article does not contain any studies with human subjects. All procedures with animal subjects in this study were approved by Mayo Clinic Institutional Animal Care and Use Committee.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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