Extracorporeal Shock Wave Therapy (ESWT) Minimizes Ischemic Tissue Necrosis Irrespective of Application Time and Promotes Tissue Revascularization by Stimulating Angiogenesis

Rainer Mittermayr, MD,† Joachim Hartinger,* Vlado Antonic, MS,‡§ Alexandra Meinl, PhD,*¶ Sabine Pfeifer,* Alexander Stojadinovic, MD,‡§# Wolfgang Schaden, MD,† and Heinz Redl, PhD**

Objective: To assess the time-dependent treatment effects of extracorporeal shock wave therapy (ESWT) in a standard rodent ischemic epigastric flap model.

Background: ESWT has been shown to accelerate tissue repair in acute and chronic wounds and improve graft survival, but the mechanism remains incompletely understood.

Methods: Shock waves at 0.1 mJ/mm^2 and 5 impulses/s (total 300 impulses) were applied to the epigastric flap ischemic region at various times pre-, immediately and 24 hours postischemic insult. Flap survival; vascular perfusion; vessel number; von Willebrand factor and smooth muscle actin protein expression as well as in vivo vascular endothelial growth factor receptor 2 expression were evaluated at 1, 3, and 7 days postoperatively in ESWT-treated and untreated controls.

Results: Flap perfusion, microvessel number, and survival (through reduced flap contraction and necrosis) were significantly enhanced in the treated groups compared with controls, irrespective of timing of shock wave treatment (preischemia vs. postischemia). Vascular endothelial growth factor receptor 2 expression was dynamically upregulated in response to ESWT.

Conclusion: Shock wave preconditioning and treatment postischemic insult improves skin flap survival through neovascularization and early upregulation of angiogenesis-related growth factors.

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From the *Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria; †AUVA Trauma Center Meidling, Vienna, Austria; ‡Bernhard Gottlieb University School of Dentistry, Vienna, Austria; §Combat Wound Initiative Program, Walter Reed Army Medical Center, Washington, DC; ¶Uniformed Services University of Health Sciences, Bethesda, MD; and #Wound Biology and Translational Research Division, Armed Forces Institute of Pathology, Washington, DC.

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Reprints: Rainer Mittermayr, MD, Donaueschingenstrasse 13, A1200 Vienna, Austria. E-mail: Rainer.Mittermayr@trauma.lbg.ac.at.

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Ischemic necrosis of skin flaps remains a vexing clinical problem. Numerous therapeutic approaches have been undertaken to improve the viability of compromised flaps. These include the postoperative administration of topical agents such as fibrin, soluble mediators and growth factors (eg, recombinant human vascular endothelial growth factor),[1,2] and antiischemic pharmacotherapeutics (nitroglycerine, trolamine salicylate, and nifedipine).[3] Systemically administered agents with some protective effect in ischemic tissue have been tested to include hyperbaric oxygen,[4] corticosteroids,[5] thrombolytics,[6] free radical scavengers,[7] and vasoactive agents (potassium channel agonists like nicorandil, prostacyclin, and other prostanoid modifiers).[8,9] However, no single pharmacological intervention has proven consistently effective. Novel approaches to therapeutic neovascularization are being evaluated currently including adenovirus-mediated transforming growth factor-β, vascular endothelial growth factor (VEGF), and angiopoietin-1 gene therapy;[10–14] fibroblast growth factor-2 delivery by encapsulated genetically engineered myoblasts;[15] and, transplantation of endothelial progenitor cells from human cord blood transfected with the VEGF gene.[16] The cost, complexity and associated risk of these targeted interventions limit widespread utilization aimed at confronting the formidable challenge in wound care and reconstructive surgery, that of ischemic necrosis in distal regions of composite flaps.

Noninvasive, minimal risk and readily applicable treatment modalities have been sought which can accelerate tissue repair and provide treatment response comparable to pharmacologic and targeted approaches, but with more favorable risk-benefit ratio. Various modalities have been shown to stimulate critical healing responses through cellular biochemical signaling, and ultimately accelerated tissue repair, and are gaining acceptance as important adjuncts in complex wound healing. One such modality, extracorporeal shock wave therapy (ESWT), has been utilized in clinical medicine for the past 4 decades for a broad range of indications, and recent data support its use in complex soft tissue wounds in general, ischemic tissue, in particular.[17–20] Although the precise mechanisms of ESWT in ischemic soft tissue are only beginning to be elucidated, early findings support the hypothesis that the application of physical energy or a mechanical stimulus in the form of shock waves can influence cellular events to produce a favorable biological effect promoting repair of compromised tissue.

It seems that shock waves influence a complex spectrum of cellular and biomolecular functions. In particular, recent studies show the overexpression of various growth factors in response to shock waves including proangiogenic factors such as vascular endothelial growth factor[17–20] and nitric oxide synthase.[17,21,22] Importantly, the early proinflammatory immune response to trauma has been shown recently to be consistently suppressed in response to shock wave administration.[21–24] ESWT has been also shown to improve survival of ischemic skin flaps through enhanced tissue revascularization and repair; however, in all of these studies shock waves were administered after the ischemic insult.[17,20,22,25] In fact, the time-dependent effects

(preconditioning vs. postischemic delivery) of shock waves remain indeterminate. The current study was undertaken to assess the efficacy of low energy unfocused shock waves applied at various time points in a standard rodent ischemic epigastric flap model, and to illustrate the positive angiogenic and vasculogenic effects of this biomechanical therapeutic modality.

MATERIAL AND METHODS

Rodent Epigastric Flap Model

Forty adult male Sprague–Dawley rats weighing 350 to 450 g were used in this animal protocol, which was conducted in accordance with the policies, procedures, and responsibilities for the care and use of laboratory animals within the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria. The animals were caged individually in stainless steel cages in open housing conditions at a mean room temperature of 18 ± 2°C with 50 ± 10% humidity on a 12-hour light/12-hour dark cycle with acidified water ad libitum and free dietary access.

Epigastric Skin Flap Surgical Procedure

We used a standardized rodent epigastric flap model for the study of ischemic tissue necrosis[26,27] in which the adipocutaneous skin flap dimension is adopted to the individual animal size. Therefore, anatomic landmarks were used to define the epigastric flap border (approximately 8 × 8 cm). The anatomical epigastric region is physiologically and constantly supplied by the left and right inferior epigastric neurovascular bundles. Ligation of one of these vascular pedicles reproducibly creates ischemic necrosis ipsilateral to the ligature. This model consistently creates full thickness necrosis of approximately 25% of the entire flap, if left untreated. Importantly, flap adherence to the underlying muscular fascia, its recipient site, can also be studied objectively and contributes substantially to flap outcome (quilting sutures = control group in this study).

The epigastric skin flap surgical procedure was identical for all rats in this study. The surgical procedure was performed using aseptic technique. Each animal was box-induced using Isoflurane (2 Vol%) and maintained under general anesthesia using Ketamine (60 mg/kg, i.p.) and Xylazine (16 mg/kg, i.p.). Fluid replacement was performed by subcutaneous injection of Ringer´s solution (1 mL/hour). Following induction of general anesthesia, the abdomen of each animal was shaved and depilated (with the exception of the 24-hour Pre-Op study group, see below). Animals were placed in the supine position on a surgical heating pad and rectal temperature was measured continuously and maintained constant at approximately 37°C throughout the surgical procedure. The borders of the epigastric skin flap were then marked using anatomical landmarks (ie, cranially the xiphoid, caudally the pubic region and bilaterally the distinct transition from the thin ventral skin to the coarse dorsal skin). The epigastric flap was additionally divided into 3 distinct vertical zones: Vital Zone (origin of supplying neurovascular bundle), Transition Zone (transition from adequate to insufficient vascular supply), and Ischemic Zone (area of dissected neurovascular bundle). The entire abdominal wall was then scanned using Laser Doppler Imaging (LDI, Moor LDI™, Moor Instruments Ltd., Devon, UK), which depicted the preoperative 2-dimensional perfusion of the flap.

An extended epigastric adipocutaneous flap was raised superficial to the investing abdominal muscular fascia from cranial to caudal, until the entire flap was lifted and isolated, while still pedicled on the inferior epigastric neurovascular bundles, at which time the entire flap was confirmed to be well perfused, viable. To render the epigastric flap ischemic, either the left or the right inferior epigastric neurovascular bundle (according to the study randomization protocol) was doubly ligated with absorbable 5–0 sutures. The interligation bundle was excised to prevent re-anastomoses during the follow-up study period. The flap was then sutured back to its native anatomic orientation using an interrupted technique with 4–0 nonabsorbable sutures.

EXTRACORPOREAL SHOCK WAVE TREATMENT

In this study, we used shock waves of electro-hydraulic origin (DermaGold 180 Tissue Regeneration Technologies, LLC, Woodstock, USA, manufactured by MTS Europe GmbH, Konstanz, Germany). In brief, the shock wave is generated by a high voltage discharge in water generating a plasma bubble expanding at supersonic speed within the water. The expansion velocity of the bubble is decelerated by the surrounding water and, at sound velocity, a shock wave front detaches from the bubble surface traveling through the water. The new soft-focused applicators use a parabolic reflector capable of generating an almost parallel shock wave front with a diameter the size of the reflector to apply the shock waves to a larger area, eg, for wound therapy. Hence, the parabolic reflector permits a large treatment area to be stimulated by the acoustical field. The energy flux density of the soft-focused shock waves is lower than those of the focused shock waves. Our preliminary experimental studies (results not shown) aimed at determining the optimal dose of ESWT in terms of number of impulses, energy flux and impulse frequency for the treatment of soft tissue identified the following shock wave parameters: 300 impulses, energy flux density–0.1 mJ/mm^2, frequency–5 impulses/s. In the rodent epigastric flap study we used a total 300 impulses, and in the transgenic mouse model 30 impulses at the same frequency–5 impulses/s. Control animals were treated identically, however, no shock wave impulses were administered.

Postoperative Care and Follow-up

After completion of the surgical procedure and shock wave treatment, animals received analgesic therapy by subcutaneous administration of 0.01 mg/kg BW Buprenorphin. Thereafter, animals were observed until recovery from anesthesia, and were then single caged. To prevent autocannabalization, teeth were shortened as suggested by Komorowska-Timek et al.[28] Postoperative analgesia was maintained until Day 3 by subcutaneous Carprofen at 4 mg/kg BW. Twenty-four hours, 3 and 7 days postoperatively, all rats were re-anesthetized by Isoflurane (2 Vol%). In a supine position on the surgical heating pad, digital images of the epigastric flaps were taken (for computerized digital management, planimetry) and scanned by the LDI system. On Day 7 animals were euthanized by an overdose of Pentobarbital and flaps were macroscopically evaluated. Full-thickness adipocutaneous biopsies were obtained for histological and immunohistological analysis.

Group Allocation and Treatment

The animals were randomly assigned to a control group and 3 experimental shock wave groups defined by timing of ESWT:

(1) ESWT preconditioning (24-hour Pre-Op; n = 10): After induction of general anesthesia with inhalational Isoflurane 2 Vol%, the abdomen of each animal in this group was shaved and depilated. The epigastric flap borders were marked accordingly as described above. Thereafter, shock waves were applied (0.1 mJ/mm^2, 5 impulses/s, 300 impulses total) to the latter Ischemic Zone (area of dissected neurovascular bundle) through an ultrasound transmission gel applied to the treated area of the planned flap 24 hours before the epigastric skin flap surgical procedure described above;

(2) ESWT immediately postepigastric flap surgery (Post-Op, n = 10): Immediately after suturing the flap back to its native anatomic orientation, shock waves were applied to the ischemic third of the flap,

the Ischemic Zone (area of dissected neurovascular bundle) through an ultrasound transmission gel applied to the treated area;

(3) ESWT 24 hours postepigastric flap surgery (24 hour Post-Op, n = 10): After the epigastric flap procedure the animals were returned to their cages for recovery. Twenty-four hours after the flap surgery shock waves were applied to the ischemic third of the flap, the Ischemic Zone (area of dissected neurovascular bundle), as described above, which was already clearly demarcated (stained blue).

(4) Control Group, Quilting sutures (Control, n = 10): Control animals received the same epigastric flap surgery without any shock wave treatment. To improve tissue adhesion to the recipient site, flaps were additionally affixed in the midportion of the flap with 2 quilting sutures, which secured the flap to the underlying investing muscular fascia.

Digital Image Planimetry

The entire surface area of the flap to include the necrotic flap area was traced onto a transparent acrylic foil, which was then photographed. Digital images of the flap were taken and transferred to a computer and analyzed quantitatively using computerized digital management (CDM) LUCIA morphometric software (version 4.1, Media Cybernetics LP, Silver Spring, MD).

Flap Survival

The entire flap surface area dimension was defined by the flap borders and the flap shrinkage defined as changes of flap dimension over the follow-up period (expressed as percent from presurgical area which was set at 100%). Flap area which seemed black with hair loss in conjunction with induration and loss of skin elasticity was defined as necrotic. Necrotic flap area was expressed as percentage of the entire flap dimension. Flap survival was assessed at Day 1, Day 3, and Day 7 after surgery.

Flap Perfusion Measurements

The LDI (Moor Instruments Ltd.) system was used to evaluate flap perfusion. A low intensity (2 mW) laser light beam (wavelength 632.8 nm) scanned the surface of the epigastric flap skin that generated a 2-dimensional image of flap perfusion. When laser scanning was performed, moving blood cells shifted the frequency of the laser light according to the Doppler principle. Changes in the frequency were displayed as a color-coded image that represented blood flow with dark pixels indicating low flow and bright pixels indicating high flow. All laser scanning measurements were performed without skin contact at a standardized working distance of 20 cm. The LDI scan modus was set at 10 ms/pixel, and the resolution at 256 × 256 pixels. Flap perfusion was documented as colored images and expressed in perfusion units. Perfusion was calculated for each of the 3 vertical zones: Vital Zone, Transition Zone, and Ischemic Zone. As outlined above, the abdomen was scanned before the epigastric flap surgery (baseline). After flap harvesting and rendering the flap ischemic by ligation one of the inferior epigastric neurovascular bundles, a second LDI scan was performed 1 hour after pedicle ligation. Further LDI scans were done at Day 1, Day 3, and Day 7 after surgery.

Macroscopic Evaluation

Flaps were evaluated clinically. Specifically, assessment was made for presence of seroma (in millilitres), flap adherence to the wound bed, and edema formation using a grading scale from 0 (no edema) to 3 (maximum). Flaps were incised proximally and seroma between wound bed and flap if present was aspirated and measured. Thereafter, flaps were prepared from proximally to distally and attention was drawn on the adherence of the flap to its recipient site. In the case of complete laminar flap attachment to the recipient bed the flap adherence was rated 3. If areas were observed without wound bed attachment then flap adherence was rated 2. Presence of small anchoring tissue bridges only was rated 1, and in the case of complete lack of attachment it was rated 0. In addition, the flap tissue was compared with adjacent normal tissue and evaluated in terms of viability and edema formation.

Immunohistochemistry (IHC)

Full-thickness sections from epigastric skin flaps (border from vital to necrotic area) were stored in 10% neutral buffered formalin for 24 to 48 hours followed by dehydration using ascending concentrations of alcohol in preparation for histological and immunohistochemical analyses. The tissue was then embedded in paraffin and 6 μm sections were cut on a rotary microtome, deparaffinized in xylene, rehydrated in graded alcohols, and stained with hematoxylin and eosin (H&E) for standard histology. Immunohistochemical staining of vascular endothelial cells was performed using an antibody against von Willebrand Factor (vWF) to visualize vascular density (ie, total amount of vessels within the flap tissue section). To determine mature and functional vessels, we additionally used an antibody against smooth muscle actin (SMA).

In brief, slides of 6 μm tissue sections were warmed to 60°C for 30 minutes and then deparaffinized with xylene and rehydrated in descending alcohol series. Samples for vWF antibody were pretreated with proteinase K (DAKO, Glostrup, Denmark) for 10 minutes. Then all samples were treated with peroxide (1.5% H_2O_2 in TBS) for 30 minutes at room temperature to deactivate endogenous peroxidase activity. After rinsing with TRIS-buffered saline for 10 minutes, sections were incubated with 2.5% horse serum (Vector Laboratories, Burlinghame, CA). Thereafter, tissue sections were incubated with the primary vWF antibody (polyclonal rabbit anti-human, DAKO, Glostrup, Denmark) or primary SMA antibody (monoclonal mouse anti-human, Clone 1A4, Sigma Aldrich, St. Louis) over night at 4°C and then washed with TRIS-buffered saline. ImmPRESS anti-rabbit or anti-mouse micropolymers (Vector laboratories, Burlinghame, CA) were then added and incubated for 30 minutes at room temperature. After washing, staining (red pigmentation) was performed by VECTOR NovaRed Substrate Kit (Vector laboratories, Burlinghame, CA) for 6 minutes. The slides were then counterstained with hematoxylin, dehydrated and mounted permanently with Roti-Histokit II (Carl Roth, Karlsruhe, Germany).

Evaluation was done in a blinded manner. Two independent observers assessed microvessel density in the flap sections by enumerating the number of vWF^+ and SMA^+ vessels in consecutive 8–16 high-power fields (at 200× magnification) across 3 areas of each flap section (subepithelial, intradermal, and intramuscular) in the transition zone of viable to necrotic flap area.

vWF^+ vessels and SMA^+ vessels were counted within these 3 areas and mean values calculated for each study group. The ratio of SMA^+/vWF^+ was calculated to demonstrate the relationship between mature functional vessels (SMA^+) and all endothelial structures (vWF^+).

Transgenic Mouse Model

Given the somewhat remote effect in the rodent epigastric flap study (higher perfusion in the transition and in the vital zone, although only the ischemic zone was treated with ESWT, Fig. 3) we conducted a second study in transgenic uninjured mice to evaluate if shock wave treatment influence the main angiogenic receptor (ie, VEGF-R2), and to determine if shock wave-related VEGF-R2 upregulation when present is apparent only on the treated hind limb or is also seen remotely on the not directly treated contra-lateral hind limb in the same animal.

Transgenic mice (VEGF-R2-luc mice [FVB/N-Tg(VEGF-R2-luc)Xen], n = 5/group) were used to study in vivo response of uninjured tissue to ESWT on a local (treated hind limb) and systemic (contra-lateral hind limb) level. These mice carry a transgene, which contains a 4.5-kb murine VEGF-R2 promoter fragment that drives the expression of a firefly luciferase reporter protein[19,29] allowing in vivo visualization of VEGF-R2 expression and indirectly angiogenic response to shock wave treatment.

Preparation and Shock Wave Application

General anesthesia for each mouse was box-induced using isoflurane (2 Vol%) and maintained with Ketamine (60 mg/kg, IP) and xylazine (7.5 mg/kg, IP). Thereafter, both hindlimbs of each individual were shaved with an electric clipper followed by depilation. Mice were injected with luciferin (150 mg/kg, IP) and imaged with an in vivo imaging system (IVSI; VivoVision® IVIS®, Xenogen, CA) to acquire a background image signal. Animals were then assigned to control group or to treatment group following the randomization protocol. Control group animals did not receive any treatment. Shock waves (energy flux density – 0.1 mJ/mm^2, frequency – 5 impulses/s, 30 impulses) were applied on one of the hind limbs of animals assigned to treatment group. One hour posttreatment and on Day 1, Day 3, and Day 7 follow-up measurements with the IVIS in vivo imaging system were performed. The bioluminescence signal from the in vivo luciferase activity was measured in emitted photon counts per second and quantified using Living Image Software (Xenogen). Pretreatment bioluminescence activity was set to 100% and the subsequent measurements were referenced to this baseline.

Statistics

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using a standard software package (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA). Normal distribution of data was tested by Bartlett's test for equal variances. One-way analysis of variance was used to compare mean values of specific variables between study groups. Post hoc testing was performed using the Tukey multiple comparison test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Flap Survival

Ligation of the unilateral neurovascular inferior epigastric bundle resulted in acute and permanent flap ischemia, which when left untreated, progressed to full-thickness necrosis in all control animals. The results are expressed as percentage of necrotic area relative to the total flap surface area (tissue loss rate = necrotic area/total surface area × 100%). By Day 3 after the inferior epigastric pedicle ligation the necrotic area occupied 19% ± 2 of the total epigastric flap area in the Control group, and by Day 7, it had progressed to 24% ± 2 tissue loss as determined by digital image planimetry.

The tissue loss rate was reduced by ESWT (Fig. 1), irrespective of time of shock wave delivery (24-hour Pre-Op, Post-Op, 24-hour Post-Op). The tissue loss rate by Day 3 in the ESWT-treated groups according to time of delivery, 24-hour Pre-Op, Post-Op, and 24-hour Post-Op was 13% ± 2, 12% ± 2 ($P < 0.05$ vs. Control), and 13% ± 1, respectively. One week after inferior epigastric pedicle ligation the tissue loss rate was significantly reduced for all ESWT-treated groups relative to Controls ($P < 0.001$): 24-hour Pre-OP: 15% ± 2, Post-Op: 12% ± 2, 24-hour Post-Op: 11% ± 1 vs. Control: 24% ± 2). There was no significant difference in extent of necrosis among the 3 ESWT-treated groups at either the third or seventh postoperative day. Unlike the untreated Controls where significant increase in tissue loss occurred during the Day 3 to Day 7 time interval, the necrotic flap area in ESWT-treated groups remained stable.

Epigastric flap contraction was evident in all animals after surgery. However, the extent of contraction was significantly lower in shock wave treated animals. Flap surface area measured 65% ± 3 in Control animals, and 80% ± 5 in shock wave treated groups ($P < 0.05$ vs. Control) on postoperative day 7 (Fig. 2).

Macroscopic Evaluation

Adherence of a flap to its recipient site is important to neovascularisation of ischemic tissue. Flap tissue edema formation and/or seroma formation under the flap may significantly disrupt this process. Seroma formation was a common finding in this model, occurring in approximately a third of the animals in each study group. Although flap adherence in the Ischemic Zone was generally better in the shock

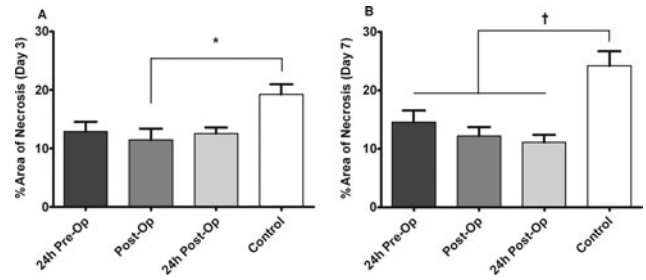

FIGURE 1. Necrosis in percentage of entire flap surface area on Day 3 and Day 7 after surgery (ligation of unilateral epigastric pedicle) assessed by digital image planimetry analysis. Significant reduction of extent of flap tissue necrosis was seen in all shock wave treated groups in comparison to the Control group irrespective of application time at Day 7 (B, †$P < 0.001$). Although a marked decrease in extent of flap necrosis is seen in all shock wave treated groups at Day 3 (A), only application of low energy unfocused shock waves immediately Post-Op resulted in statistically significant reduction in extent of flap necrosis this early in the postoperative follow-up period relative to Controls (*$P < 0.05$). Within the different shock wave treatment groups no significant difference was found. Data are presented as means ± SEM.

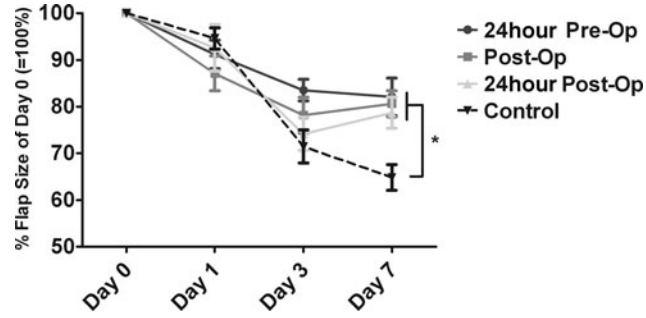

Figure 2. Overall flap contraction over a 7-day postoperative follow-up interval expressed as percentage of initial total flap surface area (=100%). Significantly reduced flap contraction is demonstrated for the groups treated with shock waves in comparison to the Control group on Day 7 postsurgery (*$P < 0.05$). Data are presented as mean ± SEM.

wave treated groups, no significant difference in adherence was found relative to Controls. Overall flap adherence was similar across groups in the Vital and Transition Zones of the flap.

We have also documented the flap tissue edema formation in all animal groups. Flap tissue edema was similar in the Ischemic Zone in all study groups, while edema formation in the Vital Zone of the flap was significantly more pronounced ($P < 0.05$) in the Control group in comparison to the shock wave treated groups (Table 1). The shock wave treated group receiving treatment 24 hours after inferior epigastric pedicle ligation (24-hour Post-Op) had significantly lower Transition Zone edema ($P < 0.05$) compared with all other study groups.

Flap Perfusion Measurement

To evaluate the influence of shock wave application on ischemic tissue perfusion we used the LDI system depicting a 2-dimensional color-coded image of superficial epigastric flap perfusion. Perfusion was calculated for 3 distinct vertical zones: Vital Zone (origin of the feeding vessels), Transition Zone (border between normal and ischemic tissue), and Ischemic Zone. LDI scan of the abdomen before epigastric flap surgery (baseline) identified a significant increase ($P < 0.001$) in flap perfusion in response to shock wave preconditioning (24-hour Pre-Op) in all 3 anatomic zones (V, T, I) compared with the 3 other groups (Control, Post-Op, 24-hour Post-Op; Fig. 3). As anticipated, induction of ischemia (inferior epigastric pedicle ligation) resulted in compromised tissue perfusion in all study groups, and was most pronounced in the Ischemic Zone. Flap perfusion in both the Vital and Transition Zones remained significantly higher ($P < 0.05$) immediately after surgery in the shock wave pretreated group (24-h Pre-Op) compared with all other study groups.

As early as 1 day into the follow-up period, both the shock wave pretreated (24-hour Pre-Op) animals and the group treated with ESWT immediately postoperatively (Post-Op) showed statistically significant increase in flap perfusion compared to Control and 24-hour Post-Op group in both the Vital and Transition Zones, and had recovered to preoperative baseline or greater perfusion unit levels seen in the Pre-Op shock wave group (Fig. 3). Flaps in both the Controls, and the 24-h Post-Op group, where ESWT was applied 24 hours after inferior epigastric pedicle ligation, had a clearly demarcated Ischemic Zone.

By Day 3 flaps treated with ESWT 24-hour Post-Op showed a statistically significant increase in perfusion compared to Controls in all 3 flap zones ($P < 0.01$), and perfusion units were comparable ($P > 0.05$) to the other 2 ESWT-treated groups. A continued trend in progressively improved tissue perfusion was evident in both the Pre-Op and immediate Post-Op shock waves on Day 3.

In fact, all flaps treated with ESWT, irrespective of time of administration of shock waves demonstrated statistically significant higher perfusion on Day 3 and on Day 7 after surgery compared with the Control group across all anatomic zones (Fig. 3). Although perfusion in the Vital and Transition Zones decreased slightly in the interval between Day 3 and Day 7, it further increased in the Ischemic Zone in the ESWT-treated animals during that time period. The flap perfusion in the control group remained low for the duration of the 1-week follow-up period.

vWF and SMA Protein Expression (IHC)

Quantitative immunohistochemical analysis of shock wave effects on microvessel revascularization of the ischemic epigastric flap was conducted by blinded observers assessing vWF-stained tissue sections, and counting the number of vWF-positive vessels per high-power field (×200 magnification) across 3 areas of each flap section (subepithelial, intradermal, and intramuscular) in the Transition Zone of viable to necrotic flap area. Eight to 16 consecutive high-power fields across each graft section were enumerated (Fig. 4A). An increased number of vWF-positive blood vessels were identified within the various layers of the epigastric flaps of ESWT-treated animals in comparison to Control flaps. This was most pronounced within the intradermal layer of the flaps receiving ESWT immediately after operation, as a significantly greater number of vWF^+ stained endothelial structures were found within the intradermal layer of the flaps in the Post-Op group compared to Controls ($P < 0.01$) and ESWT pretreatment (24-hour Pre-Op) groups ($P < 0.05$).

The shock wave effects on mature and functional vessels within the ischemic flaps were evaluated. To demonstrate the relationship between mature functional vessels (SMA^+) and all positively staining endothelial structures, the ratio of SMA^+/vWF^+ was calculated. Quantitative analysis identified a significantly greater number of mature vessels within the subepithelial layer of ESWT-treated flaps relative to Controls (Fig. 4B). This finding held true irrespective of timing of shock wave application and underscored the induction of mature and functional angiogenesis within the subepithelial layer of the flap through the delivery of low energy unfocused shock waves. In addition, the 24-hour Pre-Op ESWT group demonstrated a significantly greater ratio of SMA^+/vWF^+ within the intramuscular flap layer (paniculus carnosus muscle) compared with Controls. Within the dermal layer of the flap (intradermal) both the Pre-Op and

TABLE 1. Evaluation of Seroma Formation, Flap Adherence, and Edema Formation on Day 7 After Surgery

	Seroma		Flap Adherence			Edema			
	Occurrence	mL	Vital Zone	Transition Zone	Necrotic Zone	Vital Zone	Transition Zone	Necrotic Zone	
24-hour Pre-Op	3 out of 10	1.3	0.4	1.1	1.8	1.1	1.2	2.0	Mean
		0.4	0.2	0.3	0.4	0.2	0.2	0.2	SEM
Post-Op	4 out of 10	4.8	1.0	1.4	2.0	1.0	1.7	2.0	Mean
		2.9	0.4	0.3	0.4	0.2	0.2	0.2	SEM
24-hour Post-Op	3 out of 10	2.2	0.4	0.7	1.2	0.2	0.3‡	1.0	Mean
		1.4	0.2	0.2	0.4	0.1	0.1	0.4	SEM
Control	3 out of 10	2.0	0.3	1.0	0.6	2.2*	2.0	2.0	Mean
		0.4	0.2	0.5	0.2	0.5	0.3	0.5	SEM
			0 – no		3 – full	0 – no		3 – severe	

Incidence and volume (mL) of seroma was recorded. Flap adherence and edema formation was rated according a scale from 0 (none) to 3 (maximum, severe). Data are presented as mean ± SEM.

*$P < 0.05$ vs. shock wave treated groups;

‡$P < 0.05$ vs. control, 24-hour Pre-Op and Post-Op group.

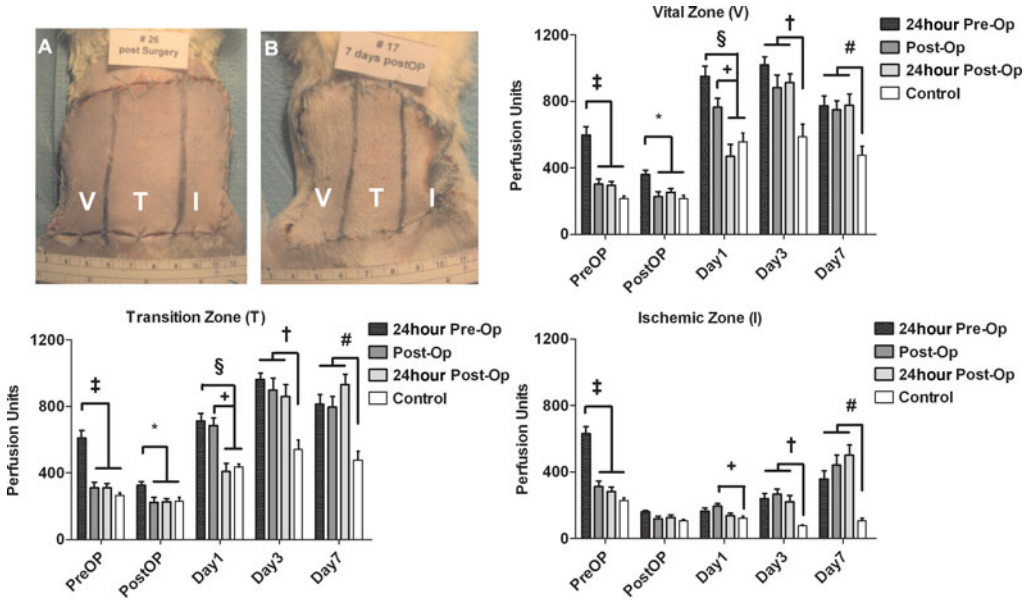

Figure 3. Superficial flap perfusion assessed by the Laser Doppler Imaging system in 3 defined vertical flap Zones: (V, Vital; T, Transition; I: Ischemic) expressed as perfusion units. Representative image of the flaps on day of surgery and Day 7 after surgery (A: Immediate Post-Surgery; B: Day 7 Post-Surgery). Shock wave preconditioning 24 hours before surgery (24-hour Pre-Op) resulted in a significantly increased flap perfusion before ischemic insult (‡$P < 0.001$ vs. all other study groups) in all epigastric flap areas (Graphs V, T and I). Induction of ischemia through unilateral epigastric pedicle ligation caused a remarkable drop in flap perfusion in all groups, although perfusion in the 24-hour Pre-Op shock wave treated group remained significantly higher (*$P < 0.05$ vs. all other study groups) during the early Post-Op period in both the Vital and Transition Zones of the ischemic epigastric flap. Shock wave treatment immediately after surgery (Post-Op group) resulted in a significant increase within the first 24 hours (Day 1) in all flap Zones (+$P < 0.05$) while perfusion in the 24-hour Pre-Op group also increased and stayed statistically significant vs. Control and 24-hour Post-Op group in the Vital and Transition Zone (§$P < 0.001$). Similarly, treating the Ischemic Zone 24 hours post-operatively (24-hour Post-Op group) resulted in a significantly increased perfusion on Day 3 in all flap Zones. In fact, on Day 3 all shock wave treated groups had similar flap perfusion, all differing significantly from the Control group (†$P < 0.05$). Although the perfusion in both the Vital and Transition Zones tended to decrease slightly in most animals by Day 7, the Ischemic Zone was characterized by a further increase in perfusion for all shock wave treated groups. All shock wave treated flaps showed a significantly higher perfusion index on Day 7 in comparison to the Control group (#$P < 0.05$). Data are presented as mean ± SEM.

immediate Post-Op ESWT groups had significantly higher proportions of mature functional (SMA^+) vessels within the ischemic flaps (Fig. 4B). Representative images of anti-SMA IHC stained sections in Figure 4C revealed more mature functional vessels within the various layers of the epigastric flaps of ESWT-treated animals in comparison to Control flaps.

Transgenic VEGF-R2/luc Mouse Model

The IVIS in vivo imaging system was used to quantify *in vivo* hind limb luciferase activity in transgenic VEGF-R2-luc mice 1-hour posttreatment and on Day 1, Day 3, and Day 7 of the follow-up. Shock wave treatment of hind limbs in the uninjured transgenic VEGF-R2/luc mice resulted in a steadily rising bioluminescence signal over the 7-day follow-up period, consistent with sustained overexpression of VEGF-R2 1-week after shock wave treatment (Fig. 5). In Control group hind limbs bioluminescent signals remained at basal level during the entire follow-up period. Although a marked (2-fold change on Day 7) increase in bioluminescent luciferase activity the in ESWT group was apparent in both the shock wave treated hind limb and the untreated contra-lateral hind limb. This observation failed to reach a statistically significant difference relative to the Control group.

To summarize, VEGF-R2 levels in the treated uninjured group were higher in both the shock wave treated limb and the untreated contra-lateral hind limb of the shock wave treated animal in comparison to controls, although not statistically significant. In contrast, the control group demonstrated baseline VEGF-R2 levels in both untreated hind limbs.

DISCUSSION

The current study suggests that a single treatment of 300 impulses of defocused shock waves at an energy flux density of 0.1 mJ/mm^2 applied 24 hours pre-, immediately or 24 hours postoperatively significantly improves ischemic epigastric flap perfusion, microvessel number, and survival. This shock wave preconditioning and postischemic tissue-sparing effect is related to early upregulation of angiogenesis-related growth factor receptor, VEGF-R2.

Although previous animal studies have shown that shock waves reduce the extent of necrosis in the distal aspects of ischemic flaps, most of these studies involved ESWT application after the ischemic insult.[17,20,22,25,30] This study evaluated the time-dependent effects of shock waves on ischemic flaps, most importantly assessing tissue-sparing effects of shock wave preconditioning. The findings show that local treatment of tissue subjected to persistent ischemia by low energy shock waves significantly reduces the rate and extent of flap tissue loss. This protective effect was independent of time of shock wave administration, either electively before induction of ischemia,

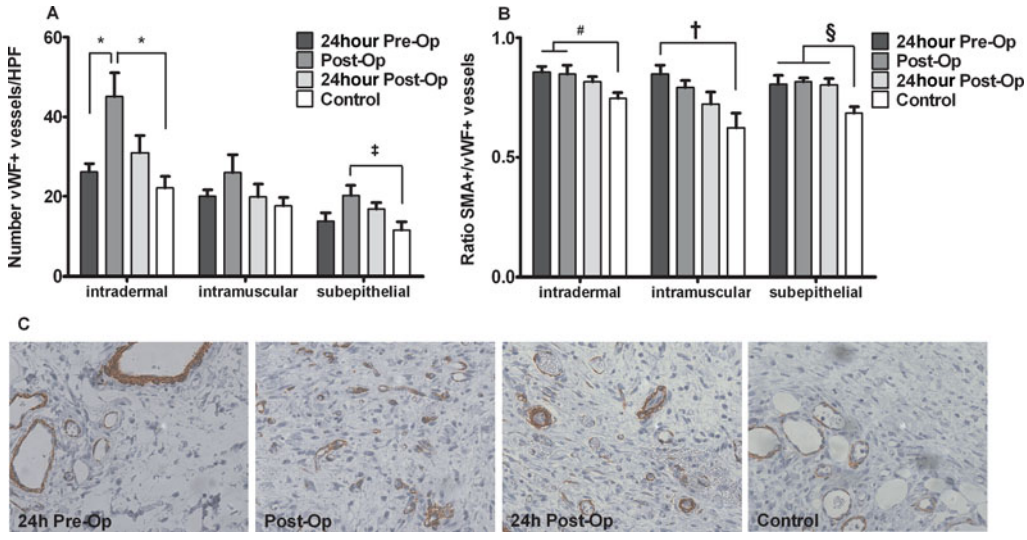

Figure 4. Induction of angiogenesis via ESWT. (A) Number of vWF$^+$ stained endothelial structures. ESWT administered immediately after induction of ischemia (Post-Op) was associated with a significantly higher quantity of amount vWF$^+$ stained endothelial structures in the intradermal layer of the epigastric flap in comparison to Control and 24-hour Pre-Op group ($^*P < 0.05$). Additionally, the Post-Op group had a significantly higher quantity of amount vWF$^+$ stained endothelial structures in the subepithelial flap layer relative to Controls ($‡P < 0.05$). (B) Ratio of mature, functional vessels (SMA$^+$) in relation to total count of endothelial structures (vWF$^+$) revealed a statistically significant higher quantity of mature vessels in the subepithelial layer in all shock wave treated groups when compared with Controls ($§P < 0.05$). In the intramuscular layer (paniculus carnosus) the 24-hour Pre-Op group demonstrated significantly more functional vessels ($†P < 0.05$), whereas in the intradermal layer both the 24-hour Pre-Op group and the immediate Post-Op group showed significantly higher amounts of functional vessels ($\#P < 0.05$). (C) Representative IHC sections of each study group showing SMA$^+$ stained mature and functional vessels. Data are presented as mean ± SEM.

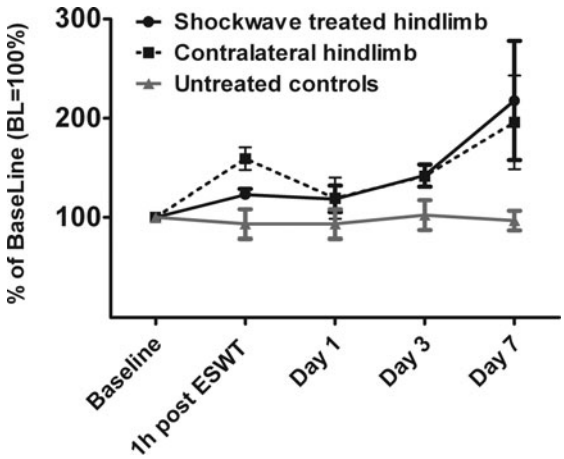

Figure 5. Expression of VEGF-R2 in an uninjured hind limb model in transgenic mice. Emitted photons were normalized to pretreatment baseline values (=100%) and expressed as a percentage of this baseline. Analyzing expression levels of the receptor (principal receptor in angiogenesis) revealed a continuous increase from time of ESWT to Day 7 in the ESW treated hind limb with a 2-fold higher gene expression level of VEGF-R2 in comparison to the Control group, although not statistically significant. Interestingly, the untreated contra-lateral hind limb of shock wave-treated animals showed higher receptor expression compared with Controls. n = 5/group. Data are expressed as mean ± SEM.

immediately after ischemia or when tissue ischemia was clinically apparent, 24-hour Post-Op. A significant reduction in extent of necrosis to ~10% (vs. 24% in Controls) of the total ischemic flap area was achieved in all shock wave treated groups, and uniformly limited (10–14%) flap necrosis was seen in these treated groups at Day 3 and Day 7 postischemia. These results are comparable with other studies using exogenous growth factors (eg, therapeutic angiogenesis) to limit ischemic tissue necrosis.[1,2]

There are a number of clinically relevant attributes of therapeutic low energy shock waves that are distinctly more advantageous than pharmacotherapeutic angiogenesis. Shock wave therapy is a safe, easy-to-use, noninvasive, low-cost modality, devoid of any drug interactions. This represents a significant advantage in terms of patient safety, tolerance, and compliance, as well as healthcare-related costs.[31] The positive effects of ESWT in terms of limiting ischemic tissue necrosis observed here was further shown by others in various ischemic flap animal models;[22,25,30,32] contrary to our study, ESWT was generally applied immediately after induction of ischemia. This study suggests that tissue preconditioning via shock wave application is a potentially feasible, noninvasive, clinically relevant approach to protecting tissue against ischemic necrosis, comparable to invasive, staged, surgical interventions, which carry relatively higher risk. Similarly, a recent study has shown that application of low energy ESWT as early as 7 days before operation significantly improves epigastric flap survival in a rodent model.[33]

Importantly, low energy shock waves in the current study were found to interrupt progression of tissue loss in flaps with clinically apparent ischemic necrosis. To our knowledge, this is the first published data demonstrating this protective, delayed treatment effect of therapeutic shock waves in necrotic soft tissue flaps. We explored potential

mechanisms through which shock waves may enhance ischemic flap viability using laser Doppler quantitative image-based assessment of flap perfusion. A significant increase in tissue perfusion was apparent in all flap Zones even though shock waves were applied only to the ischemic third of the epigastric flap, the Ischemic Zone. This enhanced perfusion effect of ESWT was therefore apparent not only at the local, but also the regional level. Assessment of preconditioning perfusion effects identified a sustained increase in flap perfusion in response to shock wave administration 24 hours before operation. After ligation of the inferior epigastric pedicle, perfusion decreased across all study groups; however, flap Ischemic Zone perfusion progressively increased over the follow-up period, achieving an approximately 3-fold greater flap perfusion index by Day 7 in the shock wave groups relative to Controls. Interestingly, perfusion was not significantly different across all flap Zones amongst all ESWT-groups by Day 7. Expectedly, extent of ischemic necrosis on Day 1 was no different in Control and the 24-hour Post-Op ESWT groups; however, the tissue perfusion promoting effect of delayed ESWT became apparent and statistically significant by Day 3, when a perfusion index no different than that of animals pretreated with ESWT or treated immediately after induction of ischemia was attained. This dynamic increase in local-regional ischemic flap perfusion is consistent with previous shock wave studies.[30,34]

Our results suggest that ESWT administered before ischemic insult may favorably alter the early tissue response to ischemia. The observed increase in VEGF-R2 expression observed in the hind limb experiments in uninjured mice provides supporting data in this regard. A study conducted by Zimpfer et al[35] demonstrates induction of angiogenesis and ventricular functional improvement in shock wave treated ischemic myocardium. In that study shock wave stimulated angiogenesis was evident in relatively higher expression of VEGF-2R, similar to our study. Importantly, shock waves did not create any morphological disruption of normal myocardium in the Zimpfer study. Reichenberger et al[33] have also shown that shock wave preconditioning (1 week before surgery) in a rat epigastric skin flap model reduces the extent of ischemic necrosis. Kuo et al[30] showed significant increase in flap perfusion and decrease of necrotic tissue area in groups receiving ESWT immediately after the flap was rendered ischemic. Extracorporeal shock wave therapy treatment applied immediately after full thickness skin isografting in a murine model has an early pro-angiogenic and anti-inflammatory effect promoting tissue revascularization and wound healing by augmenting angiogenesis and dampening inflammation.[19] However, studies are lacking to assess if the same favorable tissue effects of ESWT are observed irrespective of timing of shock wave delivery relative to ischemic insult. Our results indicate that ischemic epigastric flap perfusion in the shock wave treated groups steadily increased 24 hours after epigastric pedicle ligation, significantly over that of untreated flaps, irrespective of timing of ESWT administration (24 hours preoperatively vs. immediately after pedicle ligation), and these higher perfusion levels in the 2 ESWT treated flaps persisted throughout the remainder of the study period (7 days).

We have previously shown that ESWT stimulates angiogenesis and suppresses pro-inflammatory responses in ischemic tissue.[19,23] Angiogenesis and inflammatory responses are not divergent biological processes; in fact the temporal governance of one over the other has a defining effect on the ultimate outcome of tissue repair and regeneration. In the current study we evaluated further shock wave effects on microvessel revascularization of the ischemic flap. A distinctly higher amount of vWF^+ cells (marker for endothelial structures) were found in all ESWT groups relative to Controls, although only vWF staining in the intradermal flap layer of the ESWT Post-Op group achieved statistical significance relative to Controls. However, quantification of newly formed mature functional vessels (SMA^+ stained vessels) in relation to the vWF^+ endothelial structures suggested a potential new mechanism of ESWT. We observed an increased number of blood vessels, especially pronounced in the intradermal zone, less pronounced in deeper flap layers over the 7-day study period in shock wave treated flaps. We hypothesize that the tissue response to shock waves is dependent on the pathology of the treated tissue. For flap survival, superficial vessels are crucial to recovery whereas deeper layers seem to have a relatively more abundant blood supply adequate to sustain viability. Shockwave therapy is not a unidirectional therapeutic means such as application of growth factors, where one might expect equal distribution of induced neovessels within different zones. Shockwaves seem to have multifaceted effects, which remain incompletely understood. Previous authors have hypothesized that shockwaves induce a favorable response in compromised tissue, possibly explaining the observed nonsignificant change in counted vessels in deeper dermal layers due to adequate preexisting perfusion contributing to relative resiliency when rendered ischemic, and the observed significant quantitative change in counted vessels in the more superficial layer having relative paucity of vasculature and reduced resiliency to ischemia, where ESWT-induction of new vessels is evident.

Having demonstrated a local and regional tissue-sparing effect of ESWT in this study, we further sought to evaluate a possible systemic response and gain greater understanding of the angiogenic effect of low energy shock waves. As VEGF receptor 2 is well accepted as the main receptor governing angiogenesis, we conducted experiments on transgenic mice coexpressing the enzyme luciferase with VEGF-R2. Results from this transgenic mouse study evaluating local (treated hind limb) and remote (contra-lateral hind limb) effects of ESWT on uninjured tissue support the protein expression (IHC vWF and SMA) patterns in the ischemic rodent epigastric flaps. Bioluminescence of photons emitted from luciferin turnover via co-expressed luciferase provided an indirect marker of VEGF-R2 gene expression. Two-fold increase in bioluminescent luciferase activity 7 days posttreatment in both shock wave treated and the untreated contralateral hind limbs of mice exposed to ESWT did not reach statistical significance. However, higher VEGF-R2 expression levels were observed on the contra-lateral (not directly treated) hind limb of the ESWT-treated transgenic mice unlike the steady baseline level of bioluminescence luciferase activity in the Control (no ESWT exposure) group. Published experimental data to date, and clinical observations suggest that ESWT may exert a systemic effect. Aicher et al further defined the local-regional and systemic effects and mechanism of action of shock wave preconditioning in nonischemic and chronically ischemic hind limbs of nude rats, demonstrating enhanced recruitment and homing of circulating endothelial progenitor cells through enhanced shock wave treated tissue expression of chemo-attractants, stromal cell-derived factor 1 and VEGF.[36] In our uninjured hind limb shock wave treated and untreated mouse experiments VEGF-R2 levels were quantified from both uninjured groups of animals; 1 group received ESWT on 1 limb and the other group had no ESWT (controls). The VEGF-R2 levels in the shock wave treated uninjured group were higher in both the shock wave treated limb and the untreated contra-lateral hind limb of the shock wave treated animal, although not significantly different relative to controls. In contrast, the control group demonstrated baseline VEGF-R2 levels in both untreated hind limbs. This finding led us to hypothesize a potential systemic effect of ESWT, which remains to be established. Findings in the rat ischemic flap model also suggest an effect extending beyond the local shock wave treated area, as higher perfusion levels were observed not only in the ischemic zone in which shock waves were applied, but also the transition and the vital zone in the shock wave treated rats. We plan to evaluate systemic effects of therapeutic shock waves in future similar studies by quantifying serum proinflammatory cytokines, growth

factors and chemokines in all study groups. These studies evaluating systemic effects (ie, relevant protein biomarkers levels in serum) of ESWT are necessary to better understand the observed phenomenon in this study.

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