Optimal Timing of Extracorporeal Shock Wave Treatment to Protect Ischemic Tissue

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Abstract: Enhancement of flap survival through extracorporeal shock wave treatment (ESWT) is a promising new technique; however, no attempt has been made to define the optimal time point and frequency of ESWT to optimize treatment with ESWT for ischemic indications. Twenty-eight male Wistar rats were randomized into 4 groups and an oversized, random-pattern flap was raised and reattached in place in each animal. ESWT was applied 7 days before (group E7) or immediately after the surgical intervention (group E0). The third group was treated with ESWT 7 days before and additionally immediately after the operation (group E7/0). The fourth group served as a control group and did not receive any ESWT (group C). Seven days after flap harvest the results of flap survival, perfusion, microvessel density, and vascular endothelial growth factor concentrations were assessed. Flap survival was significantly increased in all ESWT groups as compared with the control group. The groups (E7 and E0) that received ESWT pre- or postoperatively showed a significant increase in flap perfusion and microvessel density. Combined pre- and postoperative ESWT application (group E0/E7) did not demonstrate a cumulative effect in any evaluation. In this study, we were be able to prove the effectiveness of ESWT in the protection of ischemic tissue flaps. This study suggests that single postoperative application is the most efficacious protocol for clinical applications of ESWT in the treatment of ischemic tissue.

Key Words: extracorporeal shock wave treatment, ischemia, optimization

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Extracorporeal shock wave treatment (ESWT) is known for its lithotripsy applications in the field of urology. In recent years, different groups described both its mechanical effects in lithotripsy as well as its feasibility to contribute to facilitated wound healing in animal models.[1–6] Experimental investigations on skin flap models in animals showed promising results regarding skin flap survival and proangiogenic growth factor release such as vascular endothelial growth factor (VEGF).[7–11] ESWT improved skin flap survival by reducing ischemia and subsequent necrosis.

Although the dose-dependent effect of ESWT has recently been evaluated using a murine skin flap model,[12] there are fewer publications investigating the optimum number of treatments and the optimal time point of treatment. The purpose of this study was to determine the optimal time point and frequency of ESWT for ischemic compromised tissue.

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MATERIALS AND METHODS

Experimental Design

A total of 28 male Wistar rats weighing 290 to 350 g each were used in this study. In all experiments, anesthesia was induced and maintained by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ketamin 100 mg/mL Gräub, aniMedica GmbH, Senden-Bösensell, Germany) and 5 mg/kg xylazine (Xylazin 2%, Riemser Arzneimittel, Greifswald, Germany). After marking the borders of the skin flap, all animals were randomly divided into 1 of the 4 groups (n = 7 in each group).

All experiments were performed in accordance with the guidelines of the German Animal Welfare Act, and the experimental protocol was approved by a review committee of the state of Baden-Wurttemberg, Germany. All animals were housed under conventional conditions.

Surgical Technique

The surgical procedure was performed by single surgeon (H.K.) on all animals and was identical for all groups. Before any other treatment, all animals were anesthetized and the abdominal area was shaved with an electric razor and then prepared with Octenisept (Schülke and Mayr, Norderstedt, Germany). A cranially based cutaneous flap (8 × 2 cm) was outlined with the medial margin running from the xiphoid 8 cm caudally. This flap is designed in such an extended dimension that it produces a consistent necrosis of the distal half of the flap.[13–15] The flap was immediately returned and sutured back using interrupted 4-0 nonabsorbable sutures and placed onto a silicone sheet (Mepiform, Mölnlycke Health Care, Erkrath-Unterfeldhaus, Germany) to prevent nutrition by the wound bed.

Extracorporeal Shock Wave Treatment

ESWT were applied with 500 impulses at 0.15 mJ/mm^2 (recently revised to 0.23 mJ/mm^2 by the manufacturer) (dermaPACE, Sanuwave Inc, Alpharetta, GA) as a single treatment. Ultrasound transmission gel (Sonogel, Bad Camberg, Germany) was used as a contact medium between the ESW apparatus and skin. The ESWT applicator of 4-cm diameter was positioned perpendicular to the distal half of the flap, because this area represents the ischemic portion of the flap that predictably undergoes necrosis. The applicator was randomly moved to cover full size of the flap. The dosage of ESWT was based on previous studies and recommendations by the manufacturer.[9,12,16,17] ESWT was applied only once 7 days before (group E7) or immediately after the surgical intervention (group E0). A third group was treated 7 days before and again immediately after the operation (group E7/0). Group C served as a control group and did not receive any ESWT.

All animals were observed daily and on the seventh day after flap harvesting, full-thickness necrosis was fully developed in each rat. Animals were reanesthetized and follow-up evaluations were performed.

Skin Flap Survival

With a clear demarcation in the distal part of the flap, standardized digital pictures of the abdominal wall were taken and transferred to the computer. The mean area of flap necrosis, defined by surgical borders (expressed as a percentage of the total flap area), was calculated for each animal using ImageJ-Software (NIH, United States). The results were expressed as a percentage relative to surface area of the total flap.

Indocyanine Green (ICG) Laser Fluoroscopy

ICG fluorescence was induced and recorded using a laser-fluorescence imaging device (IC-View, Pulsion Medical Systems AG, Munich, Germany), comprising a near-infrared laser light source (0.16 W, wavelength λ 780 nm) and a near-infrared–filtered digital camcorder. All animals received an injection (indocyanine) of 0.3 mg/kg bodyweight into the penis vein. Subsequently, fluorescence was recorded with a digital camera producing gray scale images that were transferred to an image analyzing system (ImageJ, NIH, United States). The distal half of the flap was evaluated as the region of interest. The perfusion index was recorded in relation to surrounding skin with normal blood flow as an increase of gray value over time.

Microvessel Density

Microvessel density (MVD) was determined by using the endothelial cell marker CD31. Skin specimens were divided into proximal and distal halves respective to the flap design. A third of the surviving proximal half was shock frozen in liquid nitrogen and stored at −80°C afterward. For immunohistochemical investigations, 5-μm thick sections were cut from the snap-frozen tissue and mounted on coated slides provided by DAKO (DAKO REAL, catalogue number S2024, Germany). Immunohistochemistry was performed semi-automatically using VENTANA Benchmark XT, Strasbourg, France. For CD31 detection the mouse anti-rat CD31 antibody by Serotec (catalogue MCA 1334GA) was used in a dilution of 1:50. No antigen retrieval was necessary prior to primary antibody exposure for 32 minutes at 37°C. Primary antibody binding sites were visualized using the ultra View Universal DAB Detection Kit (VENTANA, Roche, Mannheim, Germany). Nuclei were automatically counterstained implementing hematoxylin and Bluing Reagent (VENTANA, Roche, Mannheim, Germany). Following immunohistochemistry, slides were shortly rinsed in lukewarm soap water to remove oil residues from the staining process. Then, slides were shortly incubated in an ascending alcohol series. Prior to cover slipping, slides were incubated twice in acetone and xylol. Finally, slides were cover-slipped (Eukitt, Kindler GmbH, Freiburg, Germany). From each specimen, 3 representative digital images of CD31 stained skin flap sections were captured at 200× magnification using a photomicroscope (BX-51. Olympus, Tokyo, Japan). Morphometric studies were facilitated by analysis software (cellF, Olympus, Tokyo, Japan). Two independent observers assessed MVD by enumerating the number of CD31-positive vessels in consecutive 3 high-power fields across each central tissue section. Digital analysis software, ImageJ, NIH, was used for standardized analysis of pixel density to enumerate the CD31-positive areas within each skin flap section.

Vascular Endothelial Growth Factor

In 4 animals of each group, skin specimens were divided into proximal and distal halves respective to the oversized flap design with the distal half being critically perfused. From each portion of flap (proximal and distal half), 3 skin were samples immediately snap-frozen in liquid nitrogen after harvesting and then stored at −80°C. Total protein content of VEGF in homogenized skin samples was measured by enzyme-linked immunosorbent assay using a commercially available kit (rat Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Concentration was expressed as picograms of VEGF per milligram of total protein in the sample. Row data below lowest values of the internal standard curve were defined as 0 mg VEGF.

Statistical Analysis

All values were expressed as the mean standard error of the mean. Statistical analysis was performed with one-way ANOVA followed by post hoc Fisher's least significant difference. Statistical significance was set at $P < 0.05$. Results are presented in box plots with the box representing the interquartile range and the line within the box being the median. Vertical lines represent the adjacent values.

RESULTS

Skin Flap Survival (Planimetric Measurement)

Mean skin flap survival was determined using digital photographs of each animal 7 days after flap harvest. The group that received ESWT immediately after surgery (E0) showed the highest mean skin flap survival compared with the control group (68.2% ± 12.7% vs. 42.7% ± 10%, $P < 0.001$). The group that received ESWT as preconditioning (group E7) showed a mean flap survival of 57.7% ± 14.5%. This was also significantly higher than the control group ($P = 0.015$). ESWT applied as preconditioning and then again after flap harvesting (group E7/0) also resulted in a significantly higher mean flap survival of 62.6% ± 9.3% when compared with the control group ($P = 0.002$). There was no statistically significant difference in skin flap survival among ESWT groups (Fig. 1).

ICG Laser Fluoroscopy

Skin flap perfusion was measured 7 days after graft harvesting. Because of experimental limitations it was not possible to acquire data from every animal (Table 1). Values from the ESWT groups were compared with that of the control group. Group E0 that received ESWT immediately postoperative showed a mean perfused area of 65.1% ± 10.8%, which is significantly more than that of the control group (group C: 50.5% ± 9.8%, $P = 0.037$). The group that was treated 7 days preoperatively (group E7) showed a mean perfused area of 62.4% ± 8%. In group E7/0 that received 2 ESWT treatments pre- and postoperatively, a value of 59.6% ± 11.5% could be measured. Most likely due to low case numbers, statistical significance could not be proven for groups E7 and E7/0 when compared with the control group ($P = 0.065$, 0.159, respectively). ESWT groups did not differ significantly among each other (Fig. 2).

Microvessel Density

Quantitative MVD was determined using immunohistochemical incubated tissue. This revealed a significantly higher value in group E7 than in the control group (23.0 ± 14.4 vs. 11.0 ± 1.3, $P = 0.001$). Also, group E0 that received ESWT immediately postoperatively showed a significantly higher count of CD31-positive vessels per high power field (17.3 ± 3.4, $P = 0.012$). Group E7/0 did not show a statistically significant higher MVD (14.1 ± 2.7, $P = 0.181$). ESWT groups did not differ significantly among each other (Fig. 3).

Vascular Endothelial Growth Factor

Enzyme-linked immunosorbent assay results were expressed as picograms of VEGF per milligram of total protein in the sample.

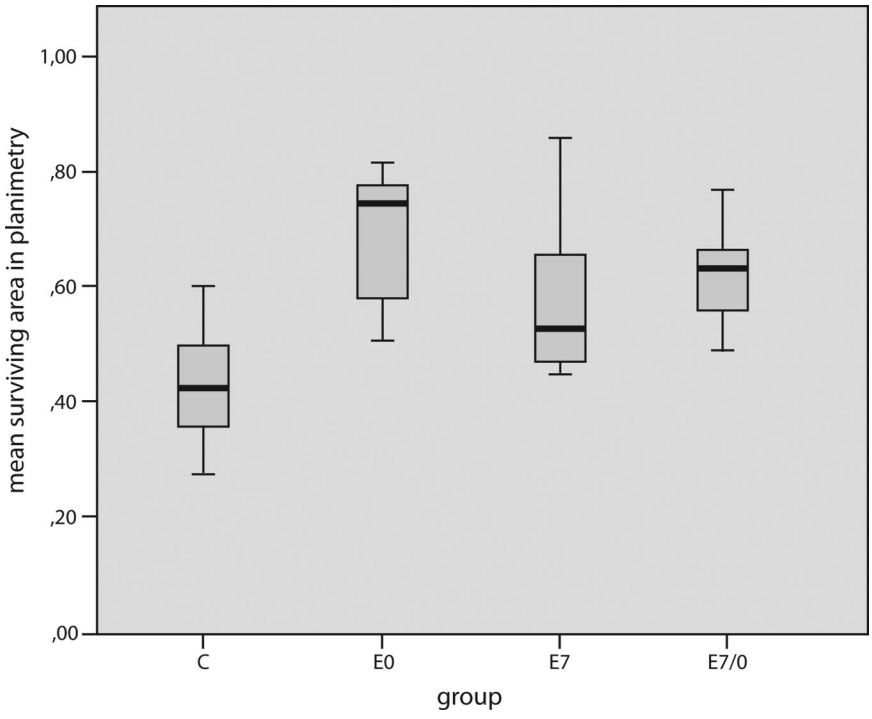

FIGURE 1. Box plot shows the mean surviving area at day 7 postoperatively.

TABLE 1. Mean Results and Case Numbers

Group	Planimetry MSA	Fluoroscopy MPA	CD31 MNV/HPF	VEGF MVEGF/P
C	42.7% ± 10.0%	50.5% ± 09.8%	11.0 ± 1.3	0.0445 ± 0.0735
E0	68.2% ± 12.7%	65.1% ± 10.8%	17.3 ± 3.4	0.0846 ± 0.1748
E7	57.7% ± 14.5%	62.4% ± 08.0%	23.0 ± 14.4	0.1400 ± 0.1442
E7/0	62.6% ± 9.3%	59.6% ± 11.5%	14.1 ± 2.7	0.0412 ± 0.0639

MSA indicates mean surviving area; VEGF, vascular endothelial growth factor; MPA, mean perfused area; MNV/HPF, mean number of vessels per HPF (magnification 400×); MVEGF/P, mean pg VEGF per milligram protein.

Results did not differ significantly between the 4 groups because of very low levels of VEGF in the samples (E0: 0.0846 ± 0.1748, E7: 0.1400 ± 0.1442, E7/0: 0.0412 ± 0.0639, C: 0.0445 ± 0.0735).

Data are summarized in Table 1 and Figure 4.

DISCUSSION

Necrosis of pedicled skin flaps remains a major operative problem that is frequently encountered clinically. To address this issue, various approaches using unspecific local or systemic physical stressors, pharmacological agents, and growth factors have been establish to further refine and simplify alternative methods to increase skin-flap viability. However, most of these techniques require an invasive and expensive administration of the beneficial substance and the results achieved with these approaches in experimental studies may not necessarily be reproduced in the clinical setting.[13,18–25] Among these, ESWT is a promising new procedure. Recently, an increasing number of studies has highlighted ESWT's feasibility in experimental flap surgery.[8,9,11,16] The protocols used to administer ESWT may play a substantial role in its clinical efficacy. Kamelger et al recently reported the appropriate number of ESWT impulses to increase flap survival in a comprised epigastric skin flap in rats.[12] However, no attempt has been made to define the optimal time point and frequency of ESWT in skin flaps, so far. This study determined that administration of ESWT at different time points resulted in significantly different flap survival rates when compared with the control group. ESWT immediately after the surgical procedure significantly improved the survival of the skin flap with an increase of MVD. These findings are in agreement with the studies of Meirer et al who demonstrated enhancement of epigastric skin flap survival after one session of ESWT (500 impulses at 0.22 mJ/mm^2).[9,16] Subsequent studies confirmed these findings and showed that blood perfusion, expression of nitric oxide and VEGF, vasodilatation of pre-existing vessels at early postoperative stage, and neovascularization at late stage were all significantly promoted by one session of ESWT.

In most reports, one session of ESWT was administered immediately after the surgical procedure, but Kuo et al reported the effect of multiple postoperative sessions of ESWT.[8] A modified skin random flap model in a rodent was used, and animals were administered 500 impulses of 0.15 mJ/mm^2 (recently revised to 0.25 mJ/mm^2 by the manufacturer) immediately after the surgery and the day after surgery. There was a smaller, but statistically insignificant reduction in the necrotic area in flaps treated with ESWT once a day for 2 days compared with that in the control group. Therefore, no additional experimental group with multiple postoperative ESWT was included in our study.

In a previous pilot study, we were able to show that preoperative ESWT may enhance survival of an axial pattern flap in a rodent model.[17] However, our pilot study had minor limitations in its experimental design. During this current study, it was shown that ESWT 7 days before flap elevation significantly improved the survival of the ischemic flap with a noticeable increase in vessel density when compared with the control group. Interestingly, the combination of pre- and postoperative ESWT resulted in a significantly higher mean flap survival when compared with the control group, but there was not a statistically higher MVD compared with control. Therefore, we hypothesize that combination of pre- and postoperative ESWT induced too much energy density, and pressure

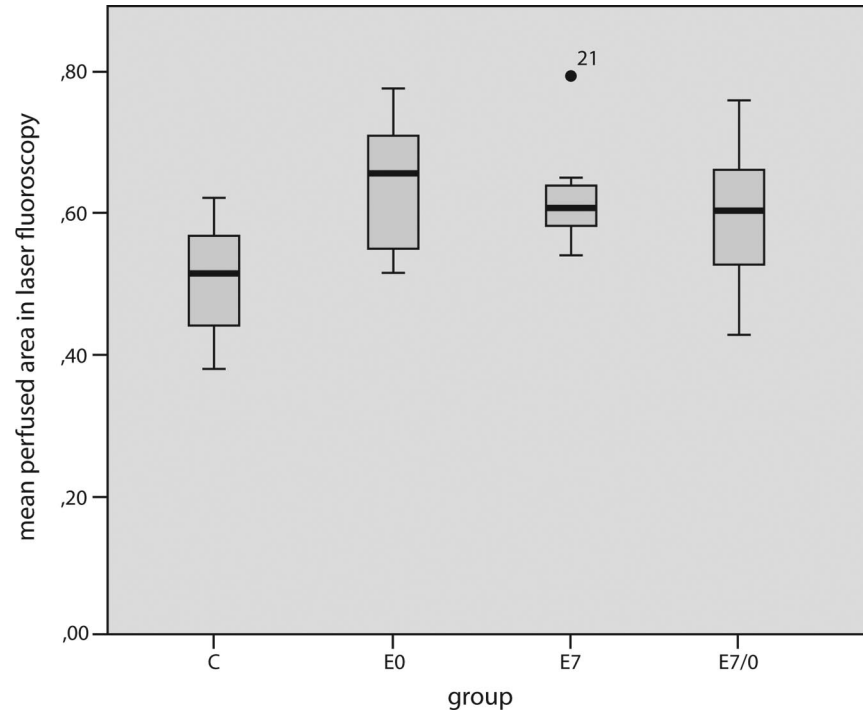

FIGURE 2. Box plot of indocyanine perfusion index measured on day 7 postoperatively.

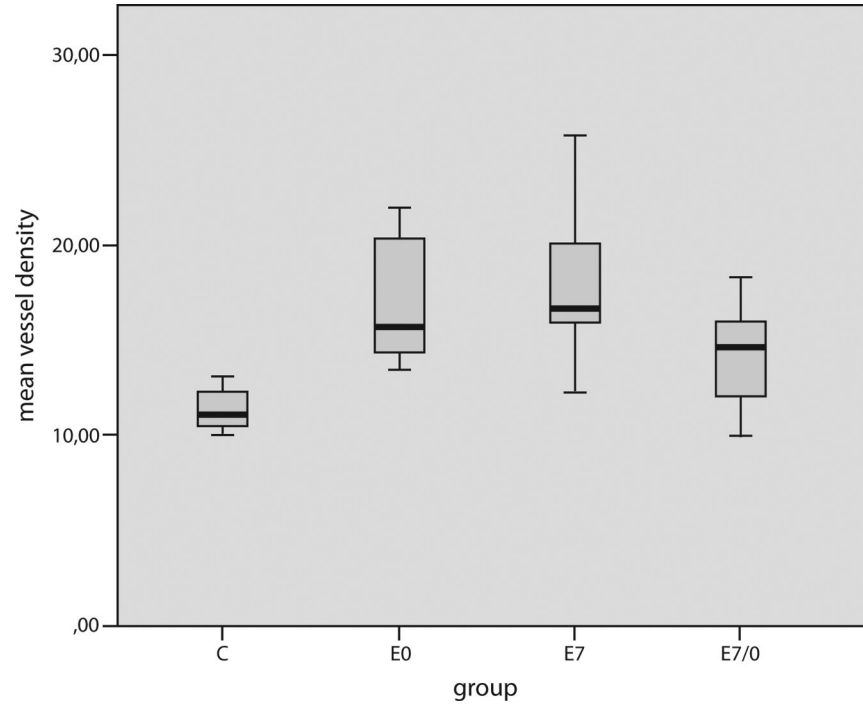

FIGURE 3. Diagram showing the distribution of mean vessel density (vessels per high power field) in all experimental groups.

distribution that resulted in localized flap tissue damage and blockade of blood perfusion and neovascularization.

In contrast to other authors, we were not able to show the strong connection between ESWT and the growth factor VEGF.[9] It is conceivable that the timing of the examination (day 7) and the small amount of tissue might not be adequate to detect upregulation of VEGF. Furthermore, a continuous decrease in VEGF concentration 3 days after flap harvest without significantly higher VEGF concentrations on day 7 has been described.[14] Although our results did not show statistical differences in the VEGF concentrations, we strongly believe that ESWT facilitated vasodilatation, and opening of pre-existing vessels or choke vessels in the flaps. According to Yan et al, we presumed that an increase in blood supply in the first 3 days after ESWT could be mainly attributed to vasodilatation of microcirculation and opening of pre-existing vessels. This early phase

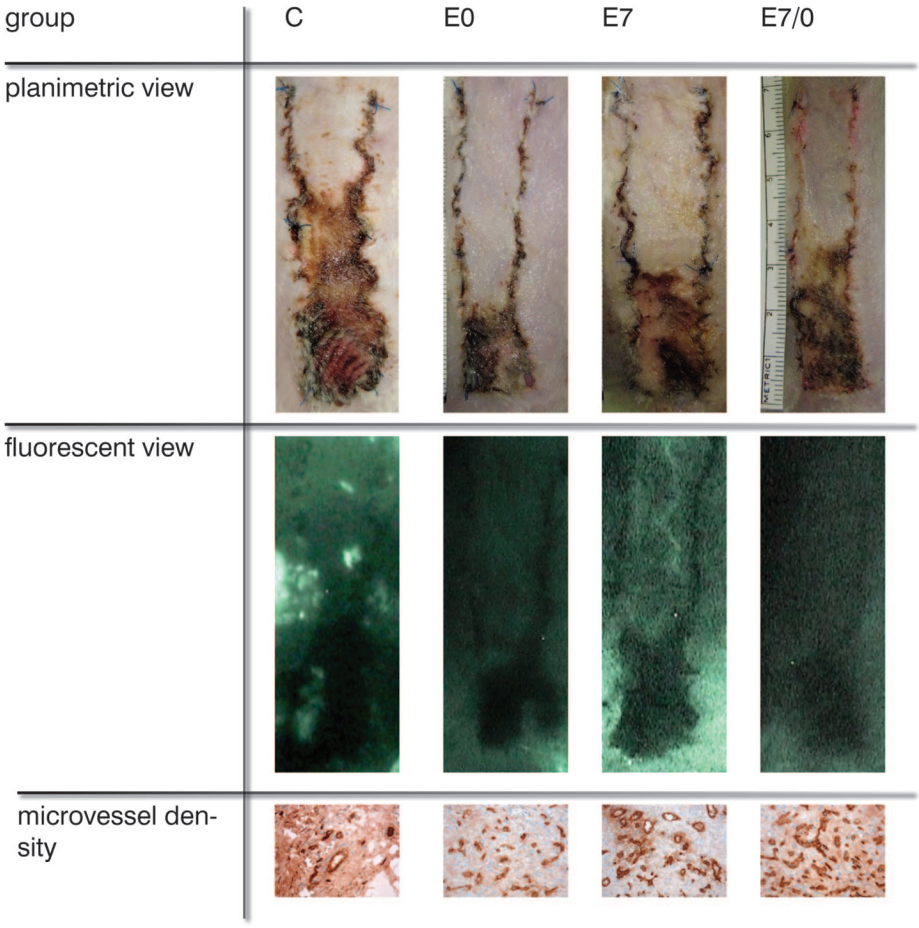

FIGURE 4. Comparison between representative samples of all 4 groups at day 7 postoperatively. Planimetric view in upper row shows survival of the flap tissue. Fluorescent view indicates skin flap perfusion in relation to the surrounding skin with unchanged blood flow. MVD was demonstrated by histologic sections with CD31 immunostaining.

provides the ischemic tissue with the minimum requirement of oxygen and nutrition to stay alive until new vessels are formed.[11]

In agreement with other previous studies, this study showed that ESWT enhances skin flap survival.[7–11,16,17] Pre- or postoperative single dose application of ESWT and the combination of 2 applications of ESWT pre- and postoperatively resulted in a statistically significant increase of flap survival compared with the control group. Further studies are necessary to determine more information regarding the molecular events in ischemic tissue after ESWT and surgical intervention. However, only the single pre- or postoperative application of ESWT showed a significant increase in vessel density and flap perfusion which strongly indicates neovascularization. On the basis of these findings, we would recommend the preoperative ESWT in more complex clinical situations with a patient population whose vascular conditions are frequently insufficient due to diabetes or arterial occlusive disease. The postoperative ESWT may be used in acute clinical situation when intraoperative microvascular perfusion failure occurs with a high risk of loss of nutritional oxygen supply and distal ischemic tissue damage.

In the past, a wide range of different approaches proved to be effective in protecting ischemic tissue. However, most of the proposed techniques were either time consuming or invasive and consequently did not become routine in clinical practice. ESWT offers the benefits of protecting ischemic tissue without surgical delay or additional operative morbidity. In summary, this rodent study indicated that ESWT at an optimal time improves ischemic skin flap survival.

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