Shock Wave Therapy Reduces Necrotic Flap Zones and Induces VEGF Expression in Animal Epigastric Skin Flap Model

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ABSTRACT

The effect of extracorporeal shock wave (ESW) therapy on skin flap survival and growth factor expression was investigated in a rat model using epigastric skin flap. Treatment and control groups each contained 20 animals. ESW effectively enhanced epigastric skin flap survival by significant reduction of areas of necrotic zones. At day 7 after the operation, necrotic zones of 4.2% were found in the ESW-treated group compared with 18.3% in the control group ($p < 0.01$). Concomitantly, in tissue samples adjacent to the necrosis areas, increased vascular endothelial growth factor expression was observed in the ESW-treated animals (median 84.5%, range 57.4 to 94.5%) compared with the control group (median 46.7%, range 29.1 to 93.1%; $p < 0.1$). However, for expression of basic fibroblast growth factor, no difference was found between the two groups. The authors conclude that the success of the shock wave treatment may partly be due to modulation of growth factor expression.

KEYWORDS: Extracorporeal shock wave, skin flap, growth factors

Reduction of tissue necrosis in the distal part of surgical flaps remains a key factor for the success of many procedures in plastic and reconstructive surgery. Various treatments have been demonstrated to improve blood supply and tissue perfusion in compromised tissues, leading to enhanced flap viability. Recent studies show promising results of therapeutic angiogenesis using exogenous growth factors such as vascular endothelial growth factor (VEGF)[1,2] and transforming growth factor-β,[3] respectively. Moreover, extracorporeal shock wave (ESW) therapy has recently been demonstrated to improve skin flap survival by preventing ischemia and subsequent necrosis.[4] Shock wave treatment has also been reported to stimulate expression of growth factors including VEGF at the tendon-bone junction.[5]

However, despite the promising results in wound healing using exogenous growth factors and the evidence that ESW is an effective method in improving skin flap survival, little is known about the presence of naturally occurring growth factors in the wound environment of defects covered by well-vascularized flaps.

In this study, we therefore investigated the potential of ESW therapy to reduce necrosis in skin flaps and to modulate in situ expression of the angiogenic cytokines basic fibroblast growth factor (FGF-2) and VEGF in the tissue adjacent to the necrosis area.

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MATERIALS AND METHODS

The Epigastric Skin Flap Model

The epigastric skin flap model used in this study has been previously described with a modification in flap design.[6] Based solely on the right inferior epigastric vessel, the contralateral distal corner of the flap represents the random portion that predictably undergoes necrosis, amounting to ~30% of the total flap area. The flap is designed in such a way that the lateral branch of the right epigastric artery is excluded and the flap is supplied by the medial arterial branch alone.

Twenty male Sprague-Dawley rats weighing 300 to 500 g were used in this study and were randomly divided into two groups (ESW group and control group, respectively) of 10 animals each. The animals were maintained according to the National Research Council guidelines. Anesthesia was performed by intraperitoneal injection of 50 mg/kg ketamine (Ketanest 100 mg/mL; Fort Dodge Laboratories, Fort Dodge, IA) and 1.3 g/kg body weight Xylazine (Rampun 20 mg/mL; Bayer Corp., Shawnee Mission, KS) with periodic supplementation as needed.

GROUP I: ESW GROUP

Immediately after the surgical intervention (see below for details), the anesthetized animals were placed in a supine position. The ultrasound transmission gel (Pharmaceutical Innovations Inc., Newark, NJ) was used as contact medium between the ESW apparatus and the skin. ESW treatment with 500 impulses at 0.11 mJ/mm^2 (Evotron Sanuwave Inc., Marietta, GA, USA) was given to the left upper corner of the flap This area represents the random portion of the flap, which according to literature predictably undergoes necrosis.

GROUP II: CONTROL GROUP

In the control group, the flap was raised but no shock wave treatment was performed.

SURGICAL TECHNIQUE

Surgical procedures were performed by three plastic surgeons. At the time of surgery, the surgeons were not informed to which experimental group the animals belonged. The rats were anesthetized and the epigastric flap measuring 8×8 cm was outlined on the abdominal skin, the relevant area shaved with an electric razor and then prepped with Betadine and alcohol. The flap was elevated after incising the distal and lateral borders by sharp dissection.[7] Then the inferior epigastric vessels were located bilaterally. The right inferior epigastric artery and vein were left intact, whereas the left inferior epigastric vessels were ligated and divided. Finally, the proximal border of the flap was incised to create a skin island flap pedicled on the right inferior epigastric vessels. Then, the flap was sutured back to its native configuration by using interrupted 4–0 nonabsorbable sutures.

Evaluation

Follow-up evaluation was performed at day 7 postoperatively. The animals were anesthetized, and after standardized digital pictures of the flaps were taken and transferred to the computer, they were sacrificed with an overdose of intraperitoneal pentobarbital (100 mg/kg). The following flap zones were defined for surface area measurement: necrotic zone and total flap area (defined by surgical borders). Surface area of these defined zones was measured by using Image Pro Plus Software (version 4.1, Media Cybernetics LP, Silver Spring, MD) by investigators blinded to the different groups. The results are expressed as percentage relative to surface area of the total flap.

The histology and expression of VEGF and FGF-2 were also examined at day 7 postoperatively from tissue samples adjacent to the necrosis area. The skin samples were fixed in a 10% formalin solution and routinely processed for paraffin embedding.

For immunohistochemistry, a monoclonal antibody for VEGF (sc-7269; dilution 1:50) and a polyclonal antibody for FGF-2 (sc-79; dilution 1:20), both obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), were used. The sections (1 to 2 μm thick) were deparaffinized in xylene and hydrated through a graded series of ethanol, rinsed for 5 minutes in distilled water, and treated with pronase (Sigma-Aldrich, Vienna, Austria) at 37°C to retrieve antigens. Subsequently, the slides were incubated with 1.5% H_2O_2 in methanol. To block unspecific binding, the sections were additionally treated with a blocking solution (Boehringer Blocking reagent, Roche Diagnostics GmbH, Vienna, Austria) for 45 minutes. Sections were then incubated with the primary antibodies for 1 hour. After washing with Tris-HCl buffer, pH 7.6 (Merck, Darmstadt, Germany), positive reactions for FGF-2 were visualized by using the streptavidin-biotin peroxidase technique with diaminobenzidine as chromogen. Positive reactions for VEGF was visualized by using the alkaline phosphatase anti-alkaline phosphatase method (Dako, Glostrup, Denmark) and Fast red (Serotec, Düsseldorf, Germany) as a substrate. Placenta was used for positive controls, whereas nonrelevant immunoglobulin G instead of the specific antibodies was applied for negative controls.

VEGF and FGF-2 expression was assessed as ratio of positive staining cells to total number of cells in five randomly selected high-power fields on each slide.

FGF-2 was detected in stromal fibroblasts as well as in inflammatory cells but not in endothelial cells. VEGF was positive in the epidermis and in stromal fibroblasts but not in endothelial cells.

Statistical Analysis

The Kruskall-Wallis test was used to test the equality of median percent necrotic area between the ESW group

and the control group. Two-tailed Wilcoxon rank sum test was used on all pairs of interest. No correction was made for multiple testing. Results were expressed as mean ± standard deviation and considered significant when $p < 0.05$.

RESULTS

Flap Survival

At day 7 postoperatively, none of the flaps in the control group and the ESW group showed any signs of infection, seroma, or hematoma formation. The regions of survival and necrosis were clearly demarcated in every flap. The areas of necrotic zones were significantly ($p < 0.01$) smaller in the ESW group (median 4.2%, range 1.9 to 17.3%) compared with the control group (median 18.3%, range 8.8 to 31.1%).

Growth Factor Expression

In both groups, abdominal skin tissue samples adjacent to the necrosis area underwent immunohistochemical evaluation for the presence of cells staining positive for VEGF and FGF-2, respectively. At day 7 postoperatively, samples from animals receiving ESW treatment showed more VEGF-positive cells (median 84.5%, range 57.4 to 94.5%) than comparable sections from untreated control samples (median 46.7%, range 29.1 to 93.1%; $p < 0.1$). The comparison of VEGF-positive staining cells from tissue samples with different treatments is shown in Fig. 1.

In contrast to VEGF, at postoperative day 7 immunohistochemical analysis for FGF-2-positive staining cells did not reveal relevant differences between similar tissue samples from ESW-treated animals (median 90.5%, range 67.1 to 96.0%) and untreated animals (median 89.0%, range 74.6 to 94.0%; Fig. 2).

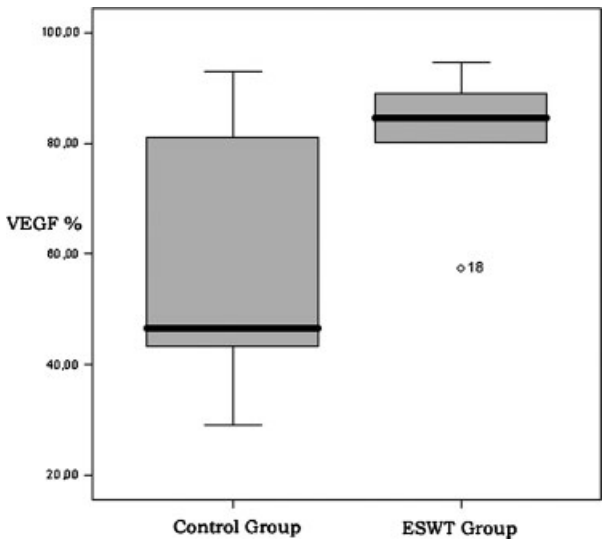

Figure 1 Comparison of percentage of VEGF-positive cells from tissue samples with different treatments. ESW treatment versus no treatment: $p < 0.1$. At day 7 postoperatively, specimens were subjected to immunohistochemical staining using an antibody against VEGF.

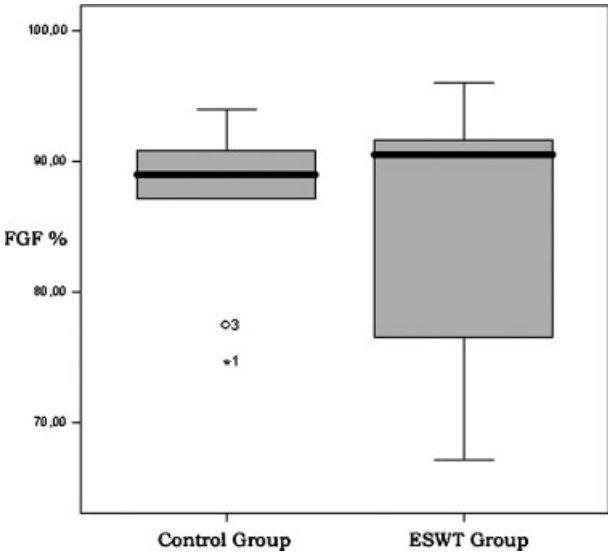

Figure 2 Comparison of percentage of FGF-2-positive cells from tissue samples with different treatments. ESW treatment versus no treatment: $p > 0.1$. At day 7 postoperatively, specimens were subjected to immunohistochemical staining using an antibody against FGF-2.

DISCUSSION

Skin flap loss due to insufficient blood supply and tissue perfusion remains a critical issue in plastic and reconstructive surgery. To overcome this problem, various treatments have been introduced and shown to improve skin flap survival. The role of neovascularization as a pivotal factor in ensuring flap viability has led to the therapeutic use of angiogenic polypeptide growth factors. Several growth factors have been explored in several animal models and reported to improve skin flap survival.[3,8–11] In most studies of flap survival, these growth factors were administered by a single bolus injection. Recently, adenovirus- and plasmid-mediated VEGF gene therapy was also demonstrated to be effective in prevention of ischemia and subsequent skin flap necrosis, resulting in enhanced skin flap survival.[1,2,4,12,13] Although VEGF and other growth factors such as transforming growth factor β 1 (TGF-β1), endothelial cell growth factor, FGF-2, and platelet-derived growth factor exert pleiotrophic effects, angiogenesis and the formation of new blood vessels depend on the combined activity of these and probably also other growth factors.

ESW therapy has demonstrated speedy and promising results in orthopedics and traumatology. Although it was suggested that ESW treatment stimulates the early endogenous expression of growth factors, the exact mechanism of shock wave therapy remains

unknown. For orthopedics, Wang reported a significant rise of growth factors such as VEGF as well as endothelial nitric oxide synthase and proliferating cell nuclear antigen after ESW treatment.[14] Moreover, the same group demonstrated that ESW treatment promotes the healing of fractures and injuries by stimulated expression of the growth factors mentioned above[5] as well as TGF-β1.[15]

With regard to plastic surgical perspectives, ESW therapy has recently been demonstrated to improve skin flap survival.[16] Moreover, ESW treatment resulted in even better skin flap survival than adenovirus-mediated VEGF gene therapy.[4] However, compared with ESW treatments in orthopedics, the ESW treatments for reconstruction of soft tissue defects were done at significant lower doses to avoid complications such as microtrauma and hematoma. With application of 500 impulses at 0.11 mJ/mm^2, we did not encounter any of the aforementioned complications.

The results from the current study confirm the findings from earlier reports[4,16] with regard to improved flap survival due to ESW therapy. The respective mean skin survival areas at day 7 postoperatively were 95.8% in the ESW-treated group and 81.7% in the control group. The difference of surviving area between the two groups was statistically significant ($p < 0.01$).

With regard to the effect of ESW therapy on the expression of endogenous growth factors, tissue samples from animals receiving ESW treatment showed more VEGF-positive cells (median 84.5%, range 57.4 to 94.5%) than comparable sections from untreated control samples (median 46.7%, range 29.1 to 93.1%; $p < 0.1$) at postoperative day 7. In contrast to VEGF, no relevant difference for FGF-2-positive staining cells was revealed at the same time point. In both the ESW-treated and the control groups, FGF-2 was detected in around 90% of the cells. This may reflect a qualitative or quantitative difference between expression of VEGF and FGF-2 but also could be due to a difference in the kinetics of the expression of these growth factors. For wound angiogenesis, FGF-2 was suggested to act as initial angiogenic stimulus followed by a subsequent and more prolonged angiogenic stimulus mediated by VEGF.[17]

In conclusion, the results from the current study demonstrate that low-dose ESW treatment can significantly improve skin flap survival. The concomitant rise in endogenous VEGF expression but apparently unchanged FGF-2 expression suggests that the mechanism of ESW at least partly acts via induction of specific growth factors. Although continued research will be necessary to look into the linkage of ESW therapy to the kinetics of endogenous growth factor expression, this developing technique seems to have the potential to become a feasible and cost-effective method to improve blood supply in ischemic tissue. This may enable plastic surgeons to better treat future patients undergoing reconstructive surgery.

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