Extracorporeal shock wave treatment modulates skin fibroblast recruitment and leukocyte infiltration for enhancing extended skin-flap survival

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ABSTRACT

Extracorporeal shock wave (ESW) treatment has a positive effect of rescuing ischemic skin flaps. This study assessed whether ESW treatment rescues the compromised flap tissue by suppressing the apoptosis of ischemic tissue and recruiting tissue remodeling. We used a random-pattern extended dorsal–skin-flap (10 × 3 cm) rodent model. Thirty-six male Sprague–Dawley rats were divided into three groups. Group I, the control group, received no treatment. Group II received one session of ESW treatment (500 impulses at 0.15 mJ/mm²) immediately after surgery. Group III received two sessions of ESW treatment, immediately and the day after the surgery. Results indicated that the necrotic area in the flaps in group II was significantly smaller than that of the flaps in group I (p < 0.01). Transferase dUTP-nick end labeling (TUNEL) analysis revealed a significant decrease in the number of apoptotic cells in group II. Hydrogen peroxide (H₂O₂) expression in circulation blood was significantly decreased in group II on the day after ESW treatment. Immunohistochemical staining indicated that compared with no treatment, ESW treatment could substantially increase proliferating cell nuclear antigen (PCNA), endothelial nitric oxide synthase, and prolyl 4-hydroxylase (rPH) expression, reduce CD45 expression, and suppress 8-hydroxyguanosine (8-OG) expression in the ischemic zone of the flap tissue. In conclusion, ESW treatment administered at an optimal dosage exerts a positive effect of rescuing ischemic extended skin flaps. The mechanisms of action of ESWs involve modulation of oxygen radicals, attenuation of leukocyte infiltration, decrease in tissue apoptosis, and recruitment of skin fibroblasts, which results in increased flap tissue survival.

Random-pattern skin flaps are still widely used in reconstructive plastic surgery. However, ischemic necrosis of the distal skin flap remains a serious problem, with a high morbidity in reconstructive surgical procedures. The pathogenesis of skin-flap ischemic necrosis remains unclear. The consensus is that cellular activation of proinflammatory mediators, insufficient vascularity, and thrombosis are the principal factors in the pathogenesis of flap ischemic necrosis. Clinical treatment of skin-flap ischemic necrosis remains controversial. Numerous approaches, such as hyperbaric oxygen, ischemic preconditioning, pharmacological agents, and growth factor delivery to ischemic tissues, have been applied to reduce ischemic necrosis in cases of impending skin-flap failure. However, no effective clinical treatment exists for rescuing ischemic tissue necrosis.

Shock waves are high-energy acoustic waves generated with high-voltage explosion and vaporization under water. Recently, extracorporeal shock wave (ESW) treatment has been applied and adapted to different clinical fields. The results of animal experiments and clinical studies have shown that ESWs could induce bony union, cell differentiation, and neovascularization. However, these studies examined ESW treatment for only certain musculoskeletal disorders. The cascade of biological effects associated with ESWs directly correlates with enhanced blood supply and tissue regeneration. Recent studies performed using a rat model have documented that ESWs enhance the survival of the distal portion of the extended island skin flap. However, the biomechanisms of ESWs involved in rescuing ischemic skin flaps remain unclear. In our previous study, ESW treatment was investigated using a random-pattern extended dorsal-skin-flap rodent model. We demonstrated that ESW treatment at an optimal dosage exerts a positive effect of rescuing ischemic skin flaps by increasing tissue perfusion and vascular endothelial growth factor (VEGF) expression and inducing neovascularization. In this study, we attempted to determine whether ESW treatment rescues the compromised flap tissue by suppressing the apoptosis of ischemic tissue cells and leukocyte infiltration and recruiting fibroblasts for tissue regeneration.
MATERIALS AND METHODS

All animals were treated humanely according to the guidelines in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (USA). All animals were housed under conventional conditions. The division of laboratory animal resources at Chang Gung memorial hospital (CGMH), Kaohsiung medical center, provided veterinary care to the rodents. All experiments were approved by the Institutional animal care and use committee (IACUC) at CGMH.

Random-pattern skin-flap model

A modified McFarlane skin-flap model was used in this study. The procedures followed in the study have been described previously. Rat dorsum was shaved, and a caudal 10 × 3 cm random-pattern extended dorsal flap was obtained. The palpable joint was excised as the anatomical landmarks for defining the flap base. Under sterile conditions, incisions were made and the entire flap was undermined below the level of the dorsal fascia. To harvest the flap, it was elevated without perforating the cutaneous vessels, which supply the base. The skin flap was sutured back to its native position using 4-0 silk sutures. Following the surgery, the rats were returned to their cages in the animal holding room after they recovered from anesthesia.

Experimental design

Thirty-six male Sprague–Dawley rats weighing 250–300 g were divided equally into three groups (n = 12 in each group). They were anesthetized with an intraperitoneal injection of 6% chloro-hydrate (5 mL/kg; Riedel-de Haén®). To reduce saliva secretion, atropine (0.1 mg/kg) was administered intramuscularly peri- and postoperatively.

In all the three groups, skin flaps were elevated and sutured in their native position, as described above. Group I (control group) flaps did not receive ESW treatment postoperatively. Group II flaps were treated with one session of ESW treatment (Ossatron®; HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) at a dose of 500 impulses at 14 kV (equivalent to 0.15 mJ/mm² energy flux density) immediately after the surgery. Group III flaps received two sessions of ESW treatment at the above-mentioned dose: one immediately after flap suturing and the other on the following day. The time required to administer 500 impulses of ESW is 10 minutes. The dosages and timing of ESW treatment were those used in our previous studies. Ultrasound transmission gel (Pharmaceutical Innovations Inc., Newark, NJ) was applied as the contact medium between the ESW apparatus and the skin. ESWs were applied to five areas from the mid-part of the dorsal flap to the distal corner. These areas represented the various ischemic flap portions that typically undergo necrosis.

All rats were observed daily and follow-up examinations were performed on postoperative day 7. Although the edge effect of the flap wound margin might be a factor affecting the flap survival, however, the edge effect was overlooked because the same procedure was used for all the animals. The necrotic area in the distal portion of the skin flap was well demarcated, and it could be easily identified by gross observation on day 7 postoperatively. Oxygen radicals in circulating blood were detected by flow cytometry on day 1 (immediately after ESW therapy) and day 2 postoperatively (the day after ESW) and on the same days in group I. The expressions of anti-8-hydroxyguanosine (8-OG; Serotec®, Oxford, UK) and endothelial nitric oxide synthase (eNOS; BD Pharmingen®, SanDiego, CA) were examined. Cellular proliferation was assessed by examining the expression of proliferating cell nuclear antigen (PCNA: Upstate Biotechnology®, Lake Placid, NY); leukocyte infiltration, by detecting the expression of CD45 (BD Pharmingen®), and tissue remodeling and collagen processing was examined by the expression of procollagen 4-hydroxylase (rPH; Chemicon®, Temecula, CA), a marker within fibroblasts that are actively producing procollagen. The expressions of CD45, 8-OG, eNOS, rPH, and PCNA were examined by immunohistochemical (IHC) staining on day 7 postoperatively. The terminal deoxynucleotidyl transferase digoxigenin-labeled uridine triphosphate (dUTP)-nick end labeling (TUNEL; Roche®, Mannheim, Germany) assay was performed to detect apoptotic cells. The surviving flap area was also determined on day 7 postoperatively. The animals were sacrificed by an intraperitoneal injection of 100 mg/kg ketamine on postoperative day 7.

Estimation of flap necrosis area

Nonviable and viable skin areas in the flap were assessed on day 7 postoperatively using the template technique described previously. Gross observation identified a clear line demarcating the living tissue from the necrotic tissue. The weight of the entire flap, including the necrotic and viable areas, was measured by tracing the flap onto a transparent graph paper; the traced portion was cut and its weight on the rat was estimated. The weight of the necrotic tissue was calculated as A / A , where A is the weight of the original flap area and A is the weight of the necrotic area on day 7 postoperatively.

Immunohistochemical staining

Punch biopsy samples in the transitional zone at the mid-third of the dorsal flap were obtained after various treatments at 1 week. These transitional zones represented the various ischemic flap portions that typically undergo necrosis. IHC was performed using a horseradish peroxidase-diaminobenzidine (HRP-DAB) system staining kit (R&D Inc., Minneapolis, MN), as described previously. The transitional necrotic zone of the flap tissue was examined. Polyclonal anti-8-OG, anti-eNOS, anti-rPH, anti-CD45, and anti-PCNA antibodies at 1 : 100 dilutions in phosphate-buffered saline (PBS) were used as the primary antibodies for 1 hour. Tissue sections were then incubated with biotinylated goat anti-rabbit antibodies for 30 minutes. Specific binding to primary antibodies was visualized by enzymatic conversion of the chromogenic substrate DAB into a brown precipitate by HRP. The slides were mounted, cleared, and cover-slipped and subsequently examined under a light microscope (Carl Zeiss, Gottingen, Germany).
TUNEL assay
Biopsy samples were obtained after various treatments at 1 week. To identify apoptotic cells by means of direct immunoperoxidase detection of digoxigenin-labeled genomic DNA, an in situ cell death detection kit (Roche®) was used according to the manufacturer’s instructions. Formalin-fixed and paraffin-embedded tissue blocks were sectioned at 6 µm thickness and placed on coated slides. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol series and PBS. The specimens were permeated with 0.1% Triton X-100 in 0.1% sodium citrate. TdT and dUTP were added, and the sections were incubated for 1 hour at 37 °C. The sections were washed with PBS, and the digoxigenin-dUTP incorporated was detected by incubation with anti-digoxigenin Fab fragments conjugated with alkaline phosphate (AP) at 37 °C for 30 minutes. The reaction product was visualized using a 5-bromo-4-chloro-3-indolyolphosphate/nitro blue tetrazolium (BCIP/NBT)-buffered substrate in AP buffer (dilution, 1: 50) at room temperature for 10 minutes. The TdT enzyme was replaced with distilled water in the negative control. Finally, each specimen was mounted under a glass coverslip and analyzed for the number of apoptotic cells under a light microscope.

Histomorphometrical examination
Punch biopsy samples were obtained (six rats’ samples/per group) after various treatments at 1 week. For immunostaining quantification, sections were analyzed using a Zeiss Axioskop 2 plus microscope (Carl Zeiss). Four random images from each selected area were then taken under ×400 magnifications. All images of each specimen were captured using a cool CCD camera (SNAP-Pro c.f. Digital kit; Media Cybernetics, Silver Spring, MD). Images were analyzed using IMAGE-PRO® Plus image-analysis software (Media Cybernetics) as described previously.15,16,24 The number of positive immunolabeled cells and total cells in each area were counted, and the percentages of positive-labeled cells were calculated. One pathologist blinded to the treatment regimen performed measurements on all sections under ×400 magnifications.

Detection of oxygen radicals (O2 and hydrogen peroxide (H2O2)) by flow cytometry
A 50 µL whole-blood sample was lysed using an ammonium chloride potassium (ACK) buffer and washed until it was almost clear. The sample was then centrifuged at 1,450 × g for 5 minutes. To all the samples, 50 µM of 2’,7’-dichlorofluorescein diacetate (DCFH; Eastman Kodak, Rochester, NY) was added to detect H2O2, and 10 µM of histofluorescence (HE) was added to detect O2 at 37 °C in the dark for 15 minutes. Subsequently, the samples were subjected to flow cytometric analysis for the detection of oxygen radicals. The results were analyzed using a software program LYSIS II (Becton–Dickinson, Palo Alto, CA).

Data management and statistical analysis
Experimental results are presented as means ± SE. One-way analysis of variance (ANOVA) was used to determine the differences in the various expressions among the three groups in a normal distribution. Post hoc comparisons were analyzed by the Tukey method. A value of p < 0.05 was considered statistically significant.

RESULTS
Optimal ESW treatment rescued ischemic skin flap tissue
The distal necrotic area in the random-pattern extended skin flaps in group II (flaps treated with one session of ESW treatment) was significantly smaller than that in group I (control group) (13 ± 2.6% vs. 42 ± 5.7%; p = 0.003). On the other hand, the necrotic area in group III (flaps treated with two sessions of ESW treatment) was insignificantly smaller than that in group I (29 ± 6.2% vs. 42 ± 5.7%; p = 0.09). These results indicate that ESW treatment at an optimal dosage has a positive effect of promoting flap tissue survival.

ESW suppressed leukocyte infiltration in the ischemic zone of flap tissue
The transitional ischemic portion of the flap tissue was subjected to a histological examination. Hematoxylin and eosin (H&E) staining indicated that a single ESW session markedly reduced leukocyte infiltration as compared with no ESW treatment. Leukocyte infiltration was assessed by observing the CD45 expression in the ischemic zone of the flap tissue by HRP-DAB IHC staining. The results indicated that compared with no ESW treatment, both single and two ESW sessions markedly reduced CD45 expression in the region extending from the dermis to the subcutaneous muscular layers in the ischemic zone of the flap (Figure 1). On the other hand, group II flaps showed a significantly lower CD45 expression level than the group III flaps.

ESW decreased apoptosis in the ischemic zone of flap tissue
The presence of apoptotic cells in the ischemic zone of the flap tissue after ESW treatment was investigated by the TUNEL assay. The results revealed apoptotic cells with a relatively greater DNA damage and fragmentation in group I flaps. Compared with group I flaps, groups II and III flaps exhibited a marked reduction in the number of apoptotic cells from the dermis to the subcutaneous muscular layers of the ischemic zone. This result indicates that ESW, when applied at an optimal dosage, could decrease cell apoptosis in the ischemic skin-flap tissue (Figure 2).

ESW recruited skin fibroblasts and tissue remodeling
Cellular proliferation was analyzed by observing the PCNA expression in the ischemic zone of the flap tissue. PCNA expression was higher, especially in the basal layers of the dermis and subcutaneous layers in groups II and III than in group I (Figure 3A). However, PCNA expression was significantly increased in group II compared with that in group III. In contrast, IHC staining revealed that the rPH expression in the ischemic zone of the flap tissue was...
significantly higher on day 7 in group II than in group I (Figure 3B). Meanwhile, the rPH expression in the ischemic zone of the flap tissue was significantly higher in group II than in group III. These results indicate that ESW treatment at an optimal dosage significantly promotes fibroblast proliferation and tissue remodeling in the ischemic skin flap.

ESW down-regulated oxygen radical burst and promoted eNOS expression

Oxygen radical expressions in the circulating leukocytes were detected by flow cytometry. No significant differences existed in the expressions of H₂O₂-DCFH or O₂-HE in the circulating leukocytes between the control and the ESW-treated groups at day 1 postoperatively (after ESW therapy immediately). The O₂-HE expression revealed a mild decrease but no significant differences between the control and the ESW-treated groups at day 2 postoperatively (the day after ESW therapy). However, compared with group I, groups II and III exhibited an apparent decrease in the H₂O₂-DCFH expression the day after ESW treatment (Figure 4). Further, we also examined the expression of 8-OG, a byproduct of ischemia, in the transitional ischemic zone of the flap tissue by IHC. The 8-OG expression in the ischemic zone was significantly lower on day 7 postoperatively in group II than in group I (Figure 5A). Compared with group I, group III exhibited a minimal decrease in 8-OG expression. In contrast, IHC revealed that the eNOS expression in the ischemic zone of the flap tissue, especially in the basal layers of the dermis and subcutaneous layers, was higher in groups II and III than in group I (Figure 5B). These results indicate that ESW treatment at an optimal dosage promotes ischemic flap survival by suppressing oxygen radical burst and promoting eNOS expression.

DISCUSSION

Random-pattern skin flaps are still widely used in reconstructive surgery. However, necrosis of skin flaps remains a serious complication in reconstructive surgical procedures. Random-pattern skin flaps are still widely used in reconstructive surgery. However, necrosis of skin flaps remains a serious complication in reconstructive surgical procedures. The distal part of a random-pattern flap is more prone to ischemia and consequent necrosis. Although distal–skin-flap ischemic necrosis is a common complication after skin-flap surgery, the underlying pathogenic mechanism remains unclear. Several approaches have been developed to reduce ischemic necrosis in unsuccessful skin flaps. Although several methods exist for augmenting tissue perfusion in flap ischemia, suppression of leukocyte inflammation and induction of tissue regeneration are considered as the primary factors involved in flap-tissue survival.

Several studies have proposed the beneficial effects of ESW treatment in bone fracture and tendon healing. Recently, Meier et al. demonstrated the rescue effect of ESW treatment on extended epigastric artery skin island flaps in a rodent model. In our previous study, we have investigated the efficacy of ESW treatment in random-pattern dorsal skin flaps in a rodent model. Experimental results indicate that ESW treatment at an optimal dosage rescues the compromised distal flap tissue by increasing tissue perfusion and inducing neovascularization. Nevertheless, the biological mechanism by which ESWs enhance ischemic flap-tissue healing remains to be determined.

The literature has reported that leukocyte inflammation is an important factor predisposing a flap to ischemic necrosis. In the present study, histological analysis of
the ischemic zone of the flap tissue shows that inflammatory cell infiltration was attenuated by the one ESW treatment as compared with no treatment. IHC revealed that leukocyte infiltration, which was assessed by detecting CD45 expression, in the ischemic zone of the flap tissue was markedly reduced by the one-session ESW treatment as compared with no treatment. These data show that inflammatory cell infiltration was attenuated by the immediate postoperative ESW treatment. Shock wave-enhanced flap survival is associated with the suppression of a pro-inflammatory response.

In this study, we investigated whether ESW treatment rescues the compromised skin-flap tissue by suppressing the apoptosis of ischemic tissue cells. The presence of apoptotic cells was analyzed by the TUNEL assay. Experimental data revealed that compared with no treatment, ESW treatment markedly reduced the number of apoptotic cells in the ischemic zone of the flap tissue. This indicated that ESW treatment at an optimal dosage could decrease cell apoptosis in the ischemic zone of the flap, thereby promoting flap-tissue survival.

In contrast, we investigated the role of ESWs in recruiting cellular proliferation and tissue remodeling. We

Figure 2. ESW decreased apoptosis in the transitional ischemic zone of the flap tissue. The presence of apoptotic cells in the ischemic zone of the flap was investigated in all the three groups using the TUNEL assay. Experiment results revealed apoptotic cells with relatively greater DNA damage in the ischemic zone of skin flap tissue in the control group. Compared with no treatment, the application of ESW once or twice markedly reduced apoptotic cell expression in the region extending from the dermis to the subcutaneous muscular layers of the ischemic zone of the flap tissue. The scale bar=5μm; *p < 0.001 vs. controls; #p < 0.001 significant differences in ESW-1 vs. ESW-2 groups. ESW-1, ESW treatment once; ESW-2, ESW treatment twice; TUNEL, transferase dUTP-nick end labeling.

Figure 3. ESW treatment up-regulated the PCNA and rPH expressions in the transitional ischemia zone of the flap tissue, as revealed by HRP-DAB IHC staining. Cellular proliferation was assessed by examining the PCNA expression in the ischemic zone of the flap tissue. IHC results indicated that PCNA expression was increased in the flaps treated with one ESW treatment as compared with that in the control flaps. There was a significant difference in PCNA expression between the group that was treated with ESW twice and the control group. The rPH expression in the ischemic zone of the flap tissue was significantly increased on day 7 in group II, which was treated with one ESW treatment as compared with the control group that received no treatment. There was also significant increase in the rPH expression in the ischemic zone between the group treated with ESW treatment twice and the controls. *p < 0.001 vs. controls; #p < 0.001 significant differences in ESW-1 vs. ESW-2 groups. ESW-1, ESW treatment once; ESW-2, ESW treatment twice; HRP-DAB IHC, horseradish peroxidase-diaminobenzidine immunohistochemical; PCNA, proliferating cell nuclear antigen; rPH, prolyl 4-hydroxylase. Scale bar=5μm.
examined the ischemic zone 1 week after the ESW treatments. Experimental data showed markedly elevated PCNA expression, particularly in fibroblasts, in group II. Tissue remodeling represented as rPH expression, an enzyme that modified proline residues in procollagen to allow stable assembly of mature type I collagen, was obviously higher in group II than in group I. These findings indicate that topical ESW application reduced tissue necrosis by increasing cellular proliferation, especially by recruiting fibroblast proliferation and actively producing procollagen, thereby attenuating flap-tissue ischemic injury and increasing tissue repair.

The contribution of free radicals to ischemic tissue damage has been investigated. Oxidative stress has been implicated as an early mediator of tissue damage in postischemic tissue injury in a variety of models. In contrast, oxygen radicals are also known to play an important role in regulating cell proliferation and metabolism. Studies have indicated that ESW treatment modulates oxygen radical production, regulates osteoprogenitor cell growth, and promotes bony module formation in vitro. In this study, reactive oxygen species in the circulating leukocytes were detected by flow cytometry. The results revealed that ESW application for one day caused an apparent decrease in the H2O2–DCFH expression of the circulating leukocytes as compared with no treatment. However, the O2-HE expression was not associated with significant decrease one day after ESW treatment. The O2-HE expression might have remained unchanged in circulating leukocytes because the superoxide anion radical transformed largely via a reaction catalyzed by the enzyme superoxide dismutase to H2O2 substances. In contrast, the results of IHC indicated that the expression level of 8-OG, a byproduct of ischemia, was significantly lower on day 7 in group II than in group I. Further, the eNOS expression level in the ischemic zone of the flap tissue after application of ESWs was significantly greater than that after no treatment. Taken together, these results

**Figure 4.** ESW treatment down-regulated hydrogen peroxide (H2O2) expressions in the circulating leukocytes. H2O2–DCFH expressions in the circulating leukocytes were detected by flow cytometry. Compared with the controls, there was a significant decrease in the H2O2–DCFH expression in the flaps treated with ESW treatment on the day after ESW application. *p < 0.01 vs. controls. ESW-1, ESW treatment once; ESW-2, ESW treatment twice. MFI, mean fluorescence intensity.

**Figure 5.** ESW treatment down-regulated 8-OG expression and promoted eNOS expression in the transitional ischemia zone of the flap tissue, as assessed by HRP-DAB IHC staining. Experimental results indicated that 8-OG expression in the ischemic zone of the flap tissue was significantly decreased on day 7 in group II, which was treated with one session of ESW as compared with that in the control group, which received no treatment. Compared with the control group, group III, which was treated with ESW treatment twice, showed a minimal decrease in 8-OG expression. On the other hand, IHC results revealed that eNOS expression in the ischemic zone of the flap tissue was significantly higher in the group treated with one session or two sessions of ESW than in the controls. *p < 0.001 vs. controls; **p < 0.001 significant differences in ESW-1 vs. ESW-2 groups.
Extracorporeal shock waves and ischemic skin-flap survival

Kuo et al.


Wang CJ, Wang FS, Yang KD, Weng LH, Hsu CC, Huang CS, Yang LC. Shock wave therapy induces


