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Extracorporeal Shock Wave Enhanced Extended Skin Flap Tissue Survival via Increase of Topical Blood Perfusion and Associated with Suppression of Tissue Pro-inflammation

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Objective. **Distal skin flap ischemic necrosis is a significant challenge in reconstructive surgery. This study assessed whether extracorporeal shock wave (ESW) treatment rescues compromised flap tissue by enhancing tissue perfusion and is associated with suppression of inflammatory response.**

Methods. **This study used the dorsal skin random flap model in a rodent. Thirty-six male Sprague Dawley rats were divided into three groups. Group I, a control group, received no treatment. Group II was administrated 500 impulses of ESW treatment at 0.15 mJ/mm^2 as a single treatment immediately postoperatively. Group III received 500 impulses of ESW at 0.15 mJ/mm^2 applied immediately postoperatively and the day following surgery. Flap blood perfusion was detected by laser Doppler. Flap survival/necrosis area and histological staining of flap ischemia zone was performed on day 7 postoperatively. The tumor necrosis factor alpha, vascular endothelial growth factor, and proliferating cell nuclear antigen expression were evaluated with immunohistochemical staining.**

Results. **Experimental results indicated that the necrotic area of the flaps in Group II was significantly reduced compared with that in the control group (13 ± 2.6%** ***versus*** **42 ± 5.7%, $P < 0.01$). There was small and insignificant reduction in the necrotic area in Group III compared with the controls. Flap tissue blood perfusion was significantly increased postoperatively in Group II. Histological staining indicated that ESW treatment substantially increased vascular endothelial growth factor and proliferating cell nuclear antigen expressions, reduced leukocyte infiltration, and suppression of tumor necrosis factor alpha expression in flap tissue ischemic zones in Group II compared with that in controls.**

Conclusion. **Optimal dosage of ESW treatment has a positive effect in rescuing ischemic zone of flap by increasing tissue perfusion and is associated with suppressing inflammatory response.**

Key Words: **extracorporeal shock waves; ischemic flap tissue survival.**

INTRODUCTION

Distal skin tissue ischemia compromised flap viability is a significant challenge during reconstruction surgery. The pathogenesis of skin flap ischemic necrosis remains unclear. The consensus is that insufficient vascularity, unpredictable vasospasm, thrombosis, and cellular activation of pro-inflammatory mediators are the principal factors in the pathogenesis of flap ischemic necrosis [1–5]. Clinical treatment of flap tissue ischemic necrosis remains controversial. Conventional management typically includes topical dressings changed repeatedly, and healing via a secondary intention or via secondary reconstructive procedure is frequently inevitable. Numerous approaches, such as hyperbaric oxygen, ischemic preconditioning, pharmacological agents, or growth factor delivered to ischemic tissues, have been applied to induce angiogenesis in ischemic flaps [6–13]. However, potential side effects and cost effectiveness are significant factors associated with these treatments.

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Shock waves are high-energy acoustic waves generated under-water with high voltage explosion and vaporization [14]. Extracorporeal shock wave (ESW) therapy is widely accepted as a technique for treating patients with urinary stones. Recently, ESW treatment was applied and adapted to different clinical fields [14]. Results of animal experiments and clinical studies have demonstrated that ESW induced bony union, cell differentiation, and neovascularization [15–20]. However, these studies examined ESW treatment for certain musculo-skeletal disorders. Neovascularization and revascularization in the tendon, bone, and tendon-bone interface are associated with increased expressions of angiogenic growth factors, including vascular endothelial growth factor (VEGF), proliferating cell nuclear antigen (PCNA), bone morphogenetic protein-2, etc. [14, 16–18, 21, 22]. The cascade of biological effects associated with ESW is directly correlated with enhanced blood supply and tissue regeneration. Recently, the effects of ESW therapy on skin flap tissue have been documented in a rat model, which showed that ESW enhances the distal area of the extended island skin flap survival [23, 24]. However, the biomechanisms of ESW rescue of ischemic skin flap survival remain unknown. This study used dorsal skin random flap in rodent model. The survival of an ischemia skin flap was tested using ESW. This study attempted to determine whether ESW therapy rescues compromised distal flap tissue via increasing tissue perfusion and regeneration and is associated with suppression of pro-inflammatory response.

MATERIALS AND METHODS

Animals were all treated humanely according to the guidelines in the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health. 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 for the rodents. Experiments were conducted under approval by the Institutional Animal Care and Use Committee (IACUC) at CGMH.

Skin Random Flap Model

A modified McFarlane skin flap model was used in this study [25]. Rat dorsum were shaved and a caudally based 10 × 3-cm dorsal extended random flap was drawn on the rats (Fig. 1A). Palpable hip joints were used as anatomical landmarks in defining the flap base. Under sterile conditions, incisions were made and the entire flap was undermined below the level of the dorsal fascia. The flap was elevated without perforating cutaneous blood vessels supplying the base to harvest a random skin flap. The skin flap was sutured back into its native position using 4-0 silk sutures. Following surgery, the rats were returned to their cages in the animal holding room after they came out of anesthesia. The necrotic area was well demarcated in the distal portion of the skin flap and was identified easily via gross observation at day 7 postoperatively.

Experimental Design

Thirty-six male Sprague Dawley rats weighing 250 to 300 g were evenly divided into three groups ($n = 12$ in each group). Anesthesia was administered via an intraperitoneal injection of 6% chlorohydrate (5 mL/kg; Riedel-de Haen, Schnelldorf, Germany) with an intramuscular injection of atropine (0.1 mg/kg) to reduce saliva secretion peri- and postoperatively.

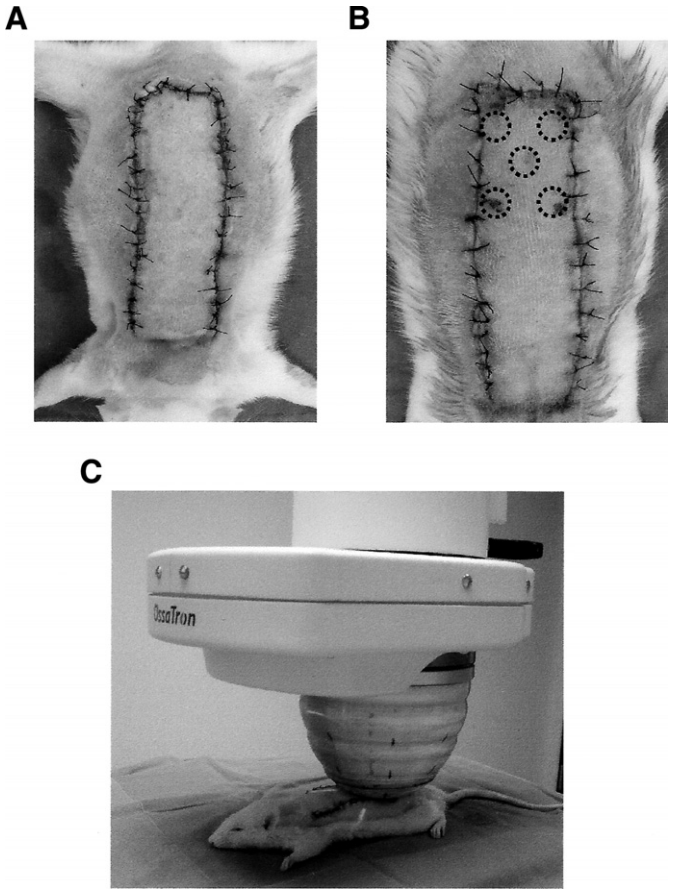

FIG. 1. (A) A modified McFarlane skin flap model was used. A caudally based 10 × 3-cm dorsal flap was elevated without perforation of cutaneous blood vessels supplying the base to ensure that the skin flap was random. The skin flap was sutured back to its original position. (B) Diagram of extracorporeal shock wave (ESW) therapy. Anesthetized rats were placed in a prone position. (C) Ultrasound transmission gel was used as contact medium between the ESW apparatus and the skin flap. The ESW strength was 500 impulses at 0.15 mJ/mm^2.

In Group I (control group), flaps were elevated and sutured back in place without ESW treatment. In Group II, flaps were elevated and sutured back in place and ESW treatment (Ossatron; HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was applied with ESW strength of 500 impulses at 14 kV (equivalent to 0.15 mJ/mm^2 energy flux density) once immediately following flap elevation and replacement. In Group III, animals were administered 500 impulses of 0.15 mJ/mm$^{2'}$ once immediately postoperatively and the day after surgery. It takes about 10 min to administer the 500 impulses ESW. The energy flux density describes the maximum amount of acoustic energy that is transmitted through an area of 1 mm^2 per pulse. The energy describes the total acoustical energy per released shock wave. Therefore, the total energy is the accumulated energy flux density as it integrated over the entire region. The reason for the dosages and timing that we applied to ESW strength of 500 impulses at 14 kV follows our pilot study and modified colleagues' previous experiences [15–18].

ESW Treatment Protocol

Immediately after surgical intervention, anesthetized rats were placed in a prone position. The ESW was applied to five areas from the mid-part of the dorsal flap to distal corner (Fig. 1B). These areas represent the ischemic portion of the flap that typically develops necrosis. The dosage was 14 kV and based on formal experimental experience that indicated this is an optimal dosage for tissue regeneration. Ultrasound transmission gel (Pharmaceutical Innovations, Inc., Newark, NJ) was applied as the contact medium between the ESW apparatus and skin (Fig. 1C).

Rats were observed daily and follow-up examination was performed on postoperative day 7. Blood pressure and flap circulation were determined by laser Doppler before and after ESW treatment on postoperative days 1 and 3. Tissue biopsy was performed on postoperative day 7 and examined via hematoxylin and eosin (H&E) stain. The survival flap area was checked for 7 d postoperatively. The animals were sacrificed with an overdose of intraperitoneal ketamine (100 mg/kg) on postoperative day 7.

Estimation of Flap Necrosis Area

Nonviable and viable skin in the flap was assessed at 7 d postoperatively using the template technique as described previously [11]. Gross observation identified a clear demarcation line between the living and necrotic tissue. The entire flap, including necrotic and living portions, were measured by tracing the flap onto transparent graph paper; the area traced was cut and its weight on the rat was estimated. Necrotic areas were presented as $A_7/A_0 \times 100\%$ calculated from the weight of the original flap area (A_0) and the weight of the necrotic area on day 7 (A_7).

Monitor of Flap Blood Perfusion Using Laser Doppler

The vascular flow was measured with a peripheral microvascular laser Doppler flow-perfusion imager (Lisca-PIMII, Järfälla, Sweden) [11]. Laser Doppler flowmetry was conducted using a camera-like device intended for two-dimensional mapping (imaging mode) and for continuous recording (perfusion monitor mode) of superficial tissue blood perfusion. In imaging mode, the low power (1-mW) laser beam at a 670 nm wavelength via an optic fiber successful scanned the tissue stepwise throughout several thousand measurement points. In tissue, the light is scattered and the frequency is shifted for interaction with moving blood cells based on the well-known Doppler principle. Sample depth was a few hundred micrometers. A fraction of the back-scattered and Doppler-broadened light was detected by a photo-detector in the scanner head. For each measurement point, the Doppler broadening and magnitude of the Doppler signal were calculated and a signal was generated that scales linearly with tissue perfusion defined as the product of RBC velocity and concentration. The results are presented as a two-dimensional color image on a computer monitor. The signal cannot be calibrated to an absolute value for blood flow; the output signal is therefore expressed as an arbitrary unit.

Immunohistochemical Staining for Tumor Necrosis Factor Alpha (TNF-α), PCNA, and VEGF Expression

Immunohistochemical (IHC) staining using a horseradish peroxidase-diaminobenzidine (HRP-DAB) system staining kit (R&D Inc., Minneapolis, MN) was performed as described previously [11, 12]. Flap tissues in the necrotic transitional zone were examined. Polyclonal TNF-α, PCNA, and VEGF antibodies (Santa Cruz, Santa Cruz, CA) at 1:100 dilutions in phosphate-buffered saline were used as the primary antibody for 1 h. Sections were then incubated with biotinylated goat antirabbit antibodies for 30 min. Visualization of specific binding to primary antibodies was developed by enzymatic conversion of chromogenic substrate 3,3′diaminobenzidine (DAB) into a brown precipitate using HRP. After mounting, cleared, and cover-slipped, slides were examined using a Zeiss fluorescence microscope (Zeiss, Jena, Germany).

Data Management and Statistics Analysis

Experimental results are presented as means ± SE. One-way analysis of variance. test was used to determine changes to the differences among the three groups in a normal distribution. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Optimal ESW Dosage Decreased Flap Tissue Necrosis

Analytical results revealed that the distal necrotic area in flaps treated once with ESW was significantly reduced compared with that in the control group (13 ± 2.6% *versus* 42.2 ± 5.7%, $P = 0.003$) (Fig. 2). There was

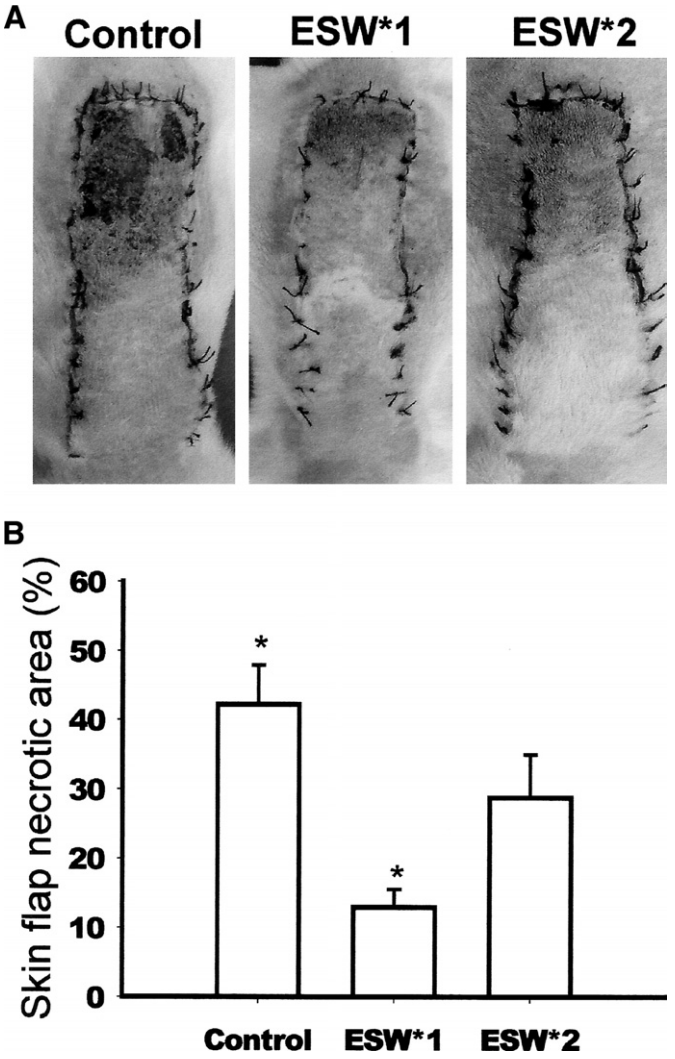

FIG. 2. Optimal dosage of ESW decrease flap tissue necrosis. The flap tissue area was examined on postoperative day 7. This diagram shows the percentage of necrotic tissue in different ESW dosages. Experimental results indicated the necrotic area of the flaps was significantly reduced after one ESW treatment compared with the controls or those treated with ESW twice. The indicated signals were statistically significant ($P < 0.05$). Abbreviations used are ESW*1: ESW treatment once; ESW*2: ESW twice.

smaller, but insignificant reduction in the necrotic area in flaps treated with ESW once a day for 2 d compared with that in the control group (28.8 ± 6.2% *versus* 42.2 ± 5.7%, $P > 0.05$). These analytical findings indicated that optimal ESW dosage has a positive effect on rescuing flap tissue.

Optimal Dosage of ESW Increase Flap Tissue Blood Perfusion

Flap blood perfusion was detected by laser Doppler. Doppler signal was generated as RBC velocity, which expressed as an arbitrary unit and correlated to flap tissue perfusion. No significant differences existed in flap tissue blood perfusion between the control group and ESW-treated groups at day 1 postoperatively. However, the group treated with ESW once had a significant increase in flap tissue perfusion on day 3 postoperatively compared with that in the control group (0.4 ± 0.04 *versus* 0.21 ± 0.03, $P = 0.005$) (Fig. 3). Flap tissue blood perfusion was not significantly improved for the group treated twice with ESW compared with that in the control group (0.21 ± 0.02 *versus* 0.21 ± 0.03, $P = 0.45$).

ESW Suppress Flap Tissue Inflammatory Response

The transitional ischemic zone of flap tissues underwent histological examination. The H&E staining indicated that one ESW treatment markedly reduced leukocyte infiltration from the dermis to subcutaneous-muscular layers of ischemic zone of flap tissue compared with that in controls (Fig. 4). Conversely, the group treated with ESW immediately postoperatively and the following day had a minimal decrease in leukocytes inflammatory response compared with that in the controls. Meanwhile, the TNF-α expression in flap tissue ischemic zone was examined using HRP-DAB immunohistochemical staining. Staining results indicated that TNF-α expression was decreased on day 1 and day 7 in the group treated once with ESW compared with that in controls. There was mild decrease but no significant differences in TNF-α expression of the flap tissue ischemic zone between rats treated with ESW twice and the control group (Fig. 5).

ESW Up-Regulates Tissue Angiogenesis and Regeneration

Cellular proliferation and regeneration was analyzed as demonstrated by PCNA expressions in the flap tissue ischemic zone using HRP-DAB immunohistochemical staining. Staining results indicated that PCNA expression was increased, especially that of fibroblasts in the basal layers of epidermis and subcutaneous layers in the group treated once with ESW compared with that in controls (Fig. 6). However, no

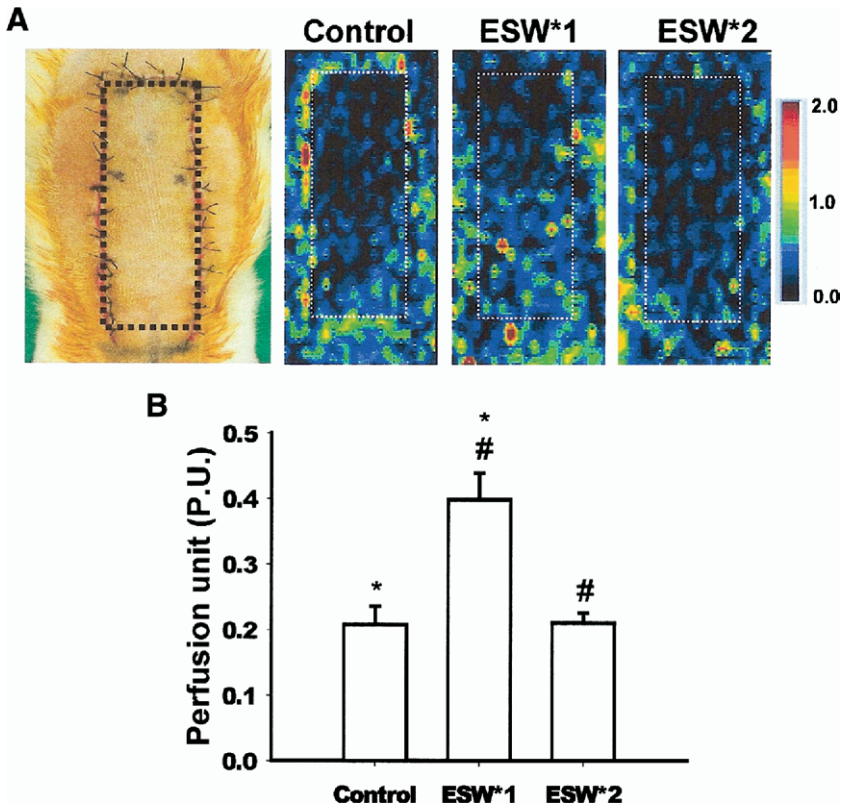

FIG. 3. Optimal ESW dosage promotes flap tissue blood perfusion. One ESW treatment significantly increased flap tissue perfusion on postoperative day 3 compared with that in controls ($P = 0.005$) or two ESW treatments ($P = 0.005$). Abbreviation used are ESW*1: ESW treatment once; ESW*2: ESW treatment twice. The indicated signals were statistically significant ($P < 0.05$).

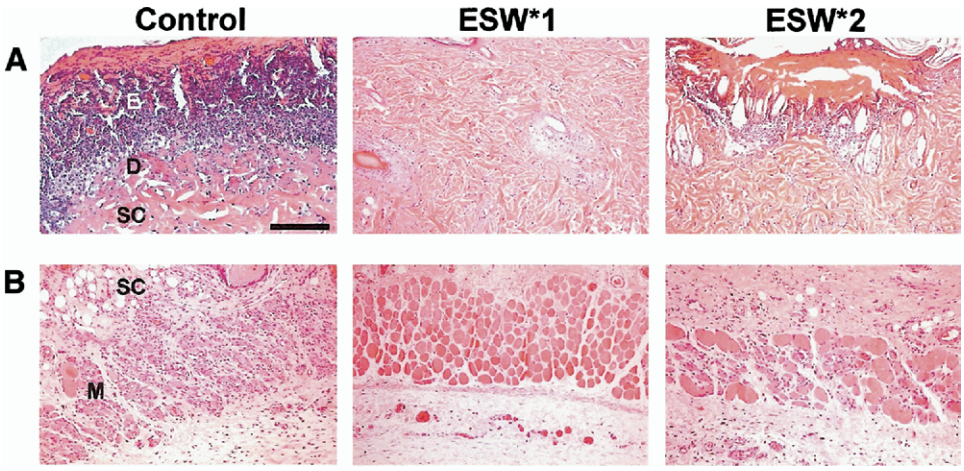

FIG. 4. The ESW treatment suppresses flap tissue inflammatory response. Histological examination was performed in the transitional zone of flap tissues. Staining by H&E revealed that one ESW treatment immediately postoperatively markedly decreased leukocyte infiltration between the dermis to subcutaneous-muscular layers in the ischemic zone of flap tissue compared with that in controls and ESW twice. Photo magnification is 100×. Scale bar is 100 μm.

significant differences existed for PCNA expressions in the flap tissue ischemic zone between rats treated with ESW twice and the control group.

Immunohistochemical study determined angiogenesis as demonstrated by VEGF expressions in the flap tissue ischemic zone. Expression of VEGF was significantly increased, particularly in fibroblasts and endothelial cells in the group treated once with ESW, compared with that in the controls (Fig. 7). No obvious increase in VEGF expression of flap ischemia zone was observed for group treated twice with ESW compared with that in the control group.

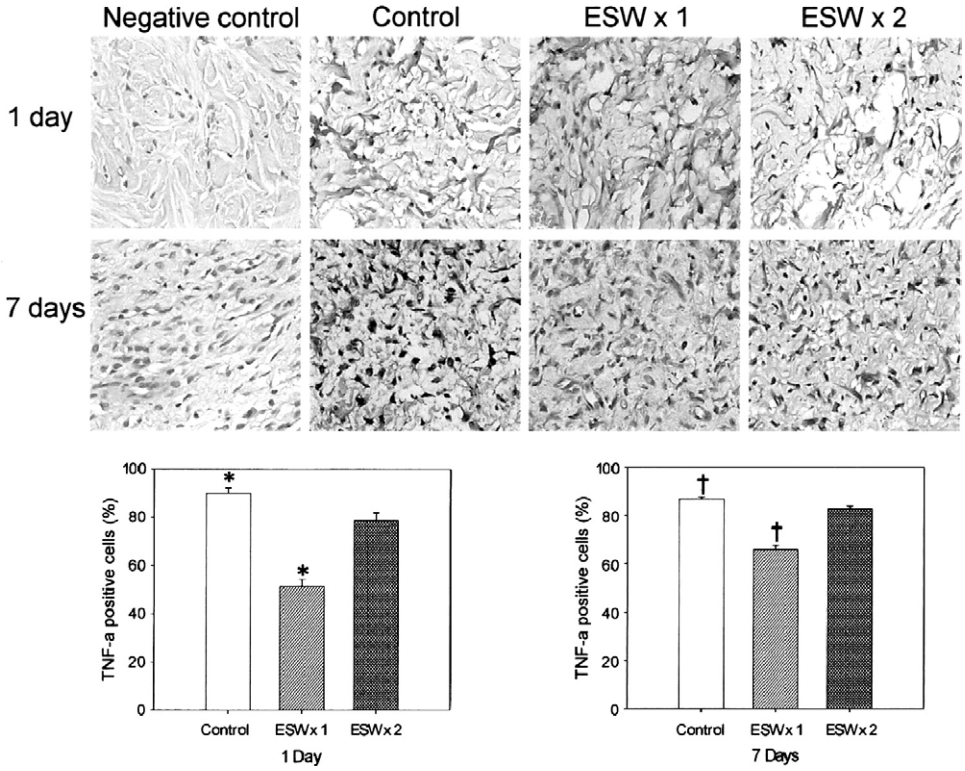

FIG. 5. The ESW treatment suppressed pro-inflammation as demonstrated by down-regulation of the TNF-α expressions in the ischemia zone of flap tissue by HRP-DAB IHC staining. The TNF-α expression was significantly decreased in the dermis and subcutaneous tissue in the group treated once with ESW for day one (*$P < 0.001$) and day seven (+$P < 0.001$), compared with that in controls. No significant differences existed for TNF-α expression of flap tissue between the group treated with ESW twice and the controls. Abbreviations used are ESW*1: ESW treatment once; ESW*2: ESW treatment twice. Photo magnification is 400×.

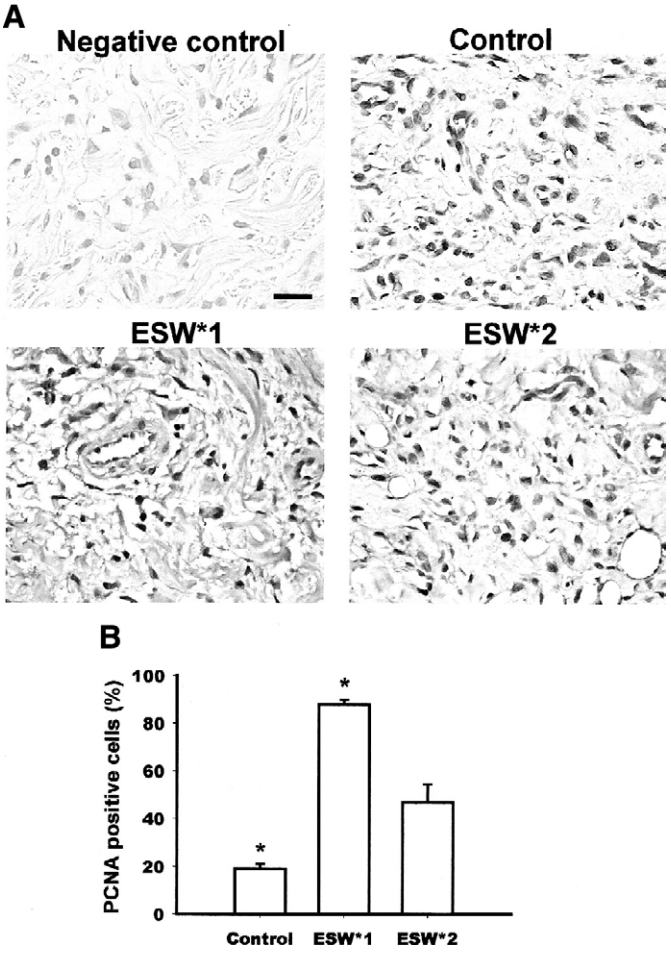

FIG. 6. The ESW treatment up-regulated the PCNA expressions in the ischemia zone of flap tissue by HRP-DAB IHC staining. Cellular proliferation and regeneration were examined as indicated by PCNA expressions of ischemia zone of flap tissues and revealed that PCNA expression was significantly increased, especially in fibroblasts, in the group treated once with ESW, compared with that in controls. No significant differences existed for PCNA expression of flap tissue between the group treated with ESW twice and the control group. Indicated signals were statistically significant ($P < 0.05$). Abbreviations used are ESW*1: ESW treatment once; ESW*2: ESW treatment twice. Photo magnification is 400×.

DISCUSSION

Random-pattern skin flaps are still widely used as a reconstructive option in plastic surgery. Necrosis of flaps remains a serious problem with high morbidity in reconstructive surgical procedures [26]. Ischemia is particularly likely in the distal part of a random flap and this may lead to necrosis. Although distal skin ischemic necrosis is a common complication of skin flap surgery, the pathogenic mechanism remains unclear. Tissue ischemia induced by leukocyte inflammation and inadequate blood flow was believed to be the principal factors predisposing a patient to flap tissue necrosis [1, 4]. No effective clinical treatment exists for rescuing skin flap ischemic necrosis. Several approaches have been developed to reduce ischemic necrosis in failed skin flaps [13, 27–30]. Although several methods exist for augmenting tissue perfusion in flap ischemia, suppression of leukocyte inflammation and induction of neovascularization and regeneration are thought to be the primary factors involved in flap tissue survival [28, 31, 32].

Several studies have proposed that ESW treatment may have beneficial effects in bone fracture and tendon healing [19, 21]. In a previous study, a significant increase of growth factors, such as endothelial nitric oxide synthase, VEGF, and PCNA, induced in-growth of neovascular formation [15–17, 19]. Recently, Meirer *et al.* indicated that ESW therapy has a rescue effect on extended skin epigastric artery island flaps in a rodent model [23, 24]. However, the exact biological mechanism of shock wave therapy in ischemic flap tissue remains unknown.

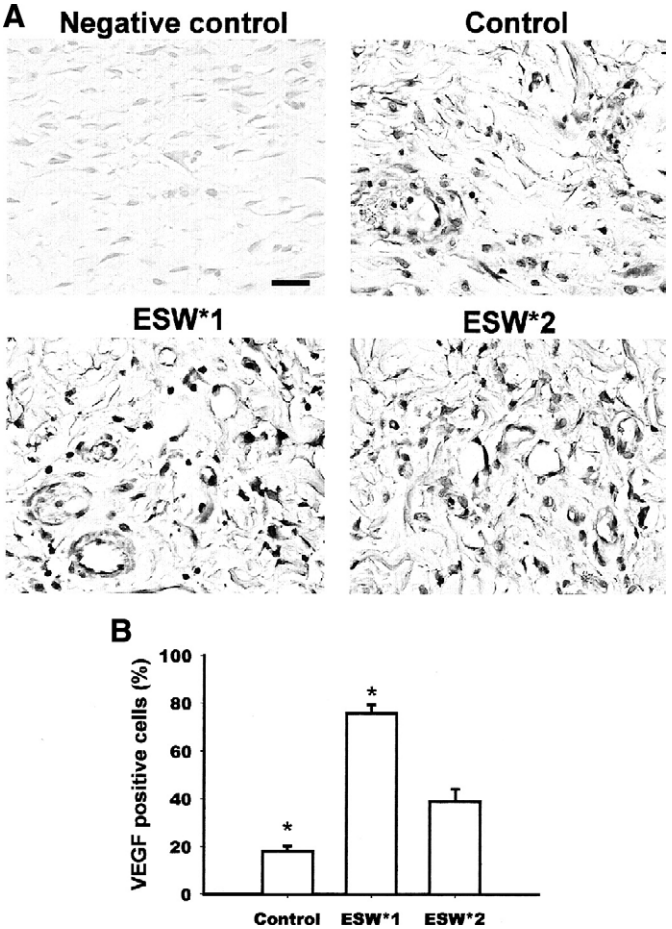

FIG. 7. The ESW treatment up-regulated the VEGF expressions in the ischemia zone of flap tissue by HRP-DAB IHC staining. The IHC study to detect angiogenic ability demonstrated by VEGF expressions showed a marked increase in the ischemia flap tissue, especially in fibroblasts and endothelial cells, in rodents treated on with ESW, compared with that in controls. Indicated signals were statistically significant ($P < 0.05$). Abbreviations used are ESW*1: ESW treatment once; ESW*2: ESW treatment twice. Photo magnification is 400×.

This study assessed the effectiveness and biomechanisms of ESW treatment on dorsal random skin flaps in a rodent model. Experimental results demonstrated that a significant reduction existed in the necrotic area of flaps under optimal dosages of ESW treatment compared with that in controls. Additionally, flap tissue blood circulation detected by laser Doppler flowperfusion imager determined a significant increase in tissue perfusion in the immediate postoperative ESW-treated groups compared with that in the untreated group. This finding indicates that optimal single dosage of ESW treatment has enhanced flap survival, likely by increasing topical flap tissue blood perfusion. However, extra ESW for the two-times therapy indicated it was harmful for flap tissue survival. The reasons why there was a difference in therapy between both groups might be that extraphysical shock waves induced too much energy density, and pressure distribution resulted in localized flap tissue damage and blockade of blood perfusion and neovascularization.

Literature has reported that leukocyte inflammation is an important factor predisposing a flap to ischemic necrosis [1, 5, 33, 34]. In this study, histological analysis of the flap tissue ischemia zone demonstrated the inflammatory cell infiltration was attenuated in immediate postoperative ESW treatment compared with that in controls. The immunohistochemical study analyzed TNF-α expression in skin flap ischemic zone also indicated a significant decrease in immediate postoperative ESW treatment for the one time group compared with controls. These data demonstrated that TNF-α expression and inflammatory cell infiltration were attenuated with immediate postoperative ESW. We could not prove whether this is a secondary response or a mechanism for attenuating necrosis in our limited analysis. However, it is possible that ESW improved flap survival by enhancing the blood flow and innate immune response was attenuated secondary to the decreased necrotic tissue. This demonstrates that enhanced flap survival after immediate postoperative ESW is associated with suppression of pro-inflammatory response.

Nevertheless, angiogenic factors play an important role in preventing tissue ischemic necrosis. In this work, immunohistochemical study analyzed ischemic zone tissue after ESW treatments. Experimental results indicated that VEGF expression was significantly elevated in the flap tissue ischemic area, particularly in endothelial cells and fibroblasts. This finding suggests that the optimal dosage of ESW for enhancing flap survival is via increasing VEGF expression and induction of neovascularization in the ischemic transitional zone.

Cellular proliferation and regeneration was examined by PCNA expressions of flap tissues ischemia zone. Experimental data indicated that marked PCNA expression, particularly in fibroblasts and basal layers of the epidermis, was found for the optimal ESW dosage. This finding indicates that topical ESW reduced tissue necrosis by increasing cellular proliferation, resulting in attenuation of flap tissue ischemic injury. Nevertheless, the mechanism by which ESW enhances ischemic flap tissue healing remained to be determined. This is a very early study in the application of ESW for ischemic flap tissue. There is much more that needs to be performed because of limitation of our experimental design. Further studies are required to elucidate the mechanical effect in the changes of cell function, the vascular and immunological changes in ischemic tissue after ESW.

In summary, this rodent study indicated that optimal ESW dosage has a positive effect in rescuing ischemic skin flaps. Shock wave treatment increased tissue perfusion and regeneration and is associated with suppressed topical tissue inflammation. Further investigations are required to determine the biological responses following ESW treatment for skin flap survival. Nevertheless, this technique represents a feasible method for improving compromised tissue circulation and may be suitable for clinical application, such as distal circulation compromise of flap tissue transfer, ischemic chronic wounds, etc.

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