The effect of autologous endothelial progenitor cell transplantation combined with extracorporeal shock-wave therapy on ischemic skin flaps in rats

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Abstract

Background. Endothelial progenitor cells (EPCs) have been used to revascularize ischemic tissues, but only limited effect can be achieved. Extracorporeal shock-wave therapy (ESWT) is a promising angiogenic strategy. We hypothesized that EPC transplantation combined with ESWT would greatly benefit the survival of ischemic skin flaps.

Methods. Sixty-four male Sprague-Dawley rats were divided into 4 groups (n = 16 in each group): group 1 (serving as sham control), group 2 (treated with subcutaneous EPC implantation, 1.0 × 10⁶ cells), group 3 (treated with ESWT, 300 impulses at 0.10 mJ/mm²) and group 4 (treated with EPCs implantation combined with ESWT). Ischemic skin flaps were made on the backs of rats and treated accordingly. Blood flow of skin flaps was measured periodically after operation, and flap survival rates were compared. Tissue samples were harvested at 2 weeks postoperatively from each group. Results. The survival rate of skin flaps in group 4 was 87.5 ± 10.23%, which was statistically significantly higher than other groups. Histologic examination showed that the capillary density was higher in the dual-treatment group than in the two single-treatment groups. Compared with groups 2 and 3, blood perfusion increased significantly in group 4. A drastic increase of vWF⁺ cells was observed in the ischemic skin flaps on immunofluorescence staining in group 4. The expressions of chemotactic factors and angiogenic factors were higher in group 4.

Conclusions. Combined treatment with EPCs and ESWT is superior to either EPCs or ESWT alone in improving the survival of ischemic skin flaps in rats.

Key Words: angiogenic factors, apoptotic factors, chemotactic factors, endothelial progenitor cells, extracorporeal shock-wave therapy, ischemic skin flaps

Introduction

Random-pattern flaps are commonly used in plastic surgery to cover large defects (1,2). However, research has revealed that the incidence of skin flap necrosis after mastectomy is not insignificant, ranging from 10% to 30%, and is often difficult to predict (3). Partial necrosis remains a major clinical problem in its application because of inadequate blood supply (4). Although it is widely accepted that a length-to-width ratio of 2:1 is clinically safe for skin flaps (5,6), a random-pattern flap with a larger length-to-width ratio would widely extend its clinical indications in practice. How to reduce the necrotic area and increase the survival rate of the skin flap are important in reconstructive surgery.

Endothelial progenitor cells (EPCs) have been used to improve the revascularization of the ischemic skin flap (7,8), but the effect is still not satisfactory. Researchers are seeking various methods to improve the biological effect of EPCs. Studies have demonstrated that gene-modified or viral-transfected EPCs could overexpress angiogenic chemokines and offer additional benefits in ischemic disease (9–11). However, these studies cannot be applied directly in clinical practice because of the associated ethical problems. Therefore, a practicable strategy for cell therapy is needed in the clinic and is a direction for further research.

Shock wave is a longitudinal acoustic wave that propagates through water or soft tissue much as ultrasound does. In contrast to ultrasound, shock wave is a single-pressure pulse with a short needle-like positive spike <1 second in duration and up to 100 MPa in amplitude, followed by a tensile part of...
several microseconds with lower amplitude (12). Extracorporeal shock wave was initially used in lithotripsy. In the past decade, the value of extracorporeal shock-wave therapy (ESWT) to treat ischemic diseases has been recognized. Studies have demonstrated that ESWT is effective in the management of fracture nonunion, tendinitis and aseptic necrosis of the bone in orthopedics (13). It has also been used to treat ischemic diseases such as angina and ischemic heart failure in cardiology (14,15). Low-energy ESWT could improve the repair of the injured tissue, as demonstrated by the literature (16). However, whether EPCs transplantation combined with ESWT is superior to either one alone in improving the survival of ischemic skin flap is unknown.

We hypothesized that dual treatment using ESWT and bone marrow–derived EPCs may provide extra protection for ischemic skin flap in rats. Furthermore, we also measured the expressions of relevant cytokines such as angiogenic factors and chemotactic factors in the ischemic skin flap tissue for exploring the mechanism of the combined therapy.

**Methods**

**Ethics**

All experimental animal procedures were approved by the Institute of Animal Care and Use Committee of School of Medicine, Shanghai Jiao Tong University, and performed in accordance with the Regulations of Laboratory Animal Care.

**Groups division**

Sixty-four male Sprague-Dawley rats (Slac Laboratory Animal Corporation, Shanghai, China), weighing 250–300 g each, were randomly assigned to four groups with 16 rats in each: group 1 (treated with phosphate-buffered saline [PBS]), group 2 (treated with autologous EPCs), group 3 (treated with ESWT) and group 4 (treated with ESWT combined with autologous EPCs). Mononuclear bone marrow cells were harvested from each rat, but only groups 2 and 4 received cells implantation. ESWT was applied to groups 3 and 4. To compare the experiment results with normal data, tissue samples were obtained from five extra rats as normal control.

**EPCs cultivation**

Every rat and the sample of EPCs extracted were carefully labeled for the next autotransplantation step. Bone marrow cells were harvested according to a previously described method (17). The rats were anesthetized by pentobarbital intraperitoneal administration (40 mg/kg). After knee joint hair was shaved, all rats were steriley prepped and draped. After carefully separating the ligament from the patella, a 1.5-mm diameter electric rotablator was used to drill into bone marrow cavity of femurs from the distal end and that of tibias from the proximal end. A sterile 22-gauge needle syringe was then used to aspirate the bone marrow. Bone marrow tissues were flushed several times with low-glucose Dulbecco’s modified Eagle’s medium (HyClone, Beijing, China) with 20% fetal bovine serum (HyClone). The bone marrow cell suspension was filtered through a 40-µm strainer, and the fraction of mononuclear cells was separated by centrifugation on Histopaque density gradient (1.083 g/mL, Sigma-Aldrich, St. Louis, MO, USA). *Ex vivo* expansion of EPCs was performed as previously described (18,19). Briefly, mononuclear cells were plated on rat fibronectin-coated (Calbiochem, Merck, Darmstadt, Germany) culture dishes and maintained in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with EGM-2 MV SingleQuots (including 5% fetal bovine serum, hydrocortisone, vascular endothelial growth factor (VEGF), human fibroblast growth factor-B, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid). Four days later, non-adherent cells were removed by washing, new substrate was applied and the culture was maintained through 16 days; endothelial cells (~1.0 × 10⁶) were found to grow abundantly on the plates.

**Identification of EPCs by cell fluorescence label and flow cytometry based on surface markers**

To identify the population of EPCs cultivated, cells were trypsinized, washed twice with PBS, and immunostained for 30 min on ice with the following antibodies: sheep anti-CD34 (R&D, Minneapolis, MN, USA), fluorescein isothiocyanate (FITC)-conjugated antibody against kinase insert domain receptor (Novus, Littleton, CO, USA) and FITC-conjugated antibody against c-kit (Abcam, Cambridge, MA, USA). Cells labeled with nonfluorescence-conjugated antibodies were further incubated with AlexaFluor 488-conjugated antibodies specifically against sheep immunoglobulin G (Jackson, West Grove, PA, USA). Isotype-identical antibodies (immunoglobulin G) served as controls. Flow cytometric analyses were performed by using fluorescence-activated cell sorter (Navios, Beckmann Coulter, USA). Cells adherent to the flask were incubated with 10 µg/mL DiI-acetyl-LDL (Molecular Probe, Invitrogen, Eugene, OR, USA) at 37°C for 24 hours and then washed three times with PBS and incubated with 10 µg/mL fluorescein isothiocyanate–conjugated lectin (Sigma-Aldrich) for 1 h (19,20). Cells were then observed under fluorescent microscope (Nikon, Japan). Cells staining positively for...
both markers were considered to be differentiating EPCs, as reported previously (21,22).

**Ischemic skin flap animal model establishment and ESWT**

According to the method described in the literature (23) and our previous experience (24), a cranially based random-pattern flap with 3 cm in width and 10 cm in length was designed and elevated from the back of rat. Its pedicle was located on the infrascapular line. The midline vascular bundle, emerging from the interscapular fat pad, was cauterized at the base of the flap. The flap was then sutured back to its original site with 4-0 nylon suture. The ratio of width to length of the flap was 1:3.3, much beyond the safe ratio (1:1.5), and thus the distal portion of the flap was subjected to necrosis. The middle portion, which was distal to the pedicle by 4 cm and proximal to the free edge by 3 cm, was considered to be relatively ischemic. This is the position where the autologous EPCs would be implanted and ESWT applied. After surgery, EPCs (1 × 10⁶) labeled with DiI-ac-LDL were immediately injected equally into the skin flap subcutaneously at the four corners of the middle third of the flap. The ESWT probe was positioned perpendicularly to the surface of the flap, and the skin of the flap was smeared with ultrasound transmission gel (Pharmaceutical Innovations, Newark, NJ, USA) as contact medium between the skin and ESW apparatus (Orthospec, Medispec, Yehud, Israel). On the basis of our own pilot study (25), ESWT was applied with a dose of 300 impulses at 0.13 mJ/mm² (2 impulses per second) on the middle third of the flap without any movement (Figure 1F).

After the operation, the animals were housed separately in cages in an air-conditioned room (24°C) during the measurement. For consistency, each measurement lasted at least 30 seconds. The results were expressed as blood perfusion unit.

**Blood flow measurement by laser Doppler flowmetry**

Tissue blood perfusion in skin flap was measured with laser Doppler flowmetry (Peri-Flux system 5000; Perimed, Jarfalla, Sweden) on postoperative days 1, 3, 5, 7 and 10. The probe was placed on the median line of the flap, and the testing points were on the median portions. The room temperature was maintained at approximately 24°C during the measurement. For consistency, each measurement lasted at least 30 seconds. The results were expressed as blood perfusion unit.

**Skin flap survival**

Photographs of the flaps were taken postoperatively at day 10 when the borderline of necrotic area on the flap was clear. Standardized digital pictures of the flaps were taken using a digital camera (T200, Sony, Japan) at the same distance and with the same focus, and then transferred to the computer for analysis. The necrotic regions were defined as zones with full-thickness flap necrosis as well as unhealed areas (covered with scab or not yet epithelialized). The surviving area (defined by the survived skin borders) and total flap areas (defined by the surgical borders) were delineated, and surface areas were calculated in square centimeters by investigators blinded to the research using ImageJ Software (version 1.46; National Institutes of Health, Bethesda, MD, USA). The results were expressed as percentage of the surviving area relative to the total surface area of the flap (survival rate = surviving area/total area × 100%) (4,24).

**Gene expression measurements of angiogenic factors, apoptotic factors, hypoxia indicator and chemotactic factors**

Four rats were sacrificed randomly from each group at days 3, 7 and 14. A 1.5 × 1.5-cm full-thickness skin specimen from the middle portion of the flap was harvested and homogenized immediately at 4°C. Total RNA from skin samples was isolated using Trizol reagent (Invitrogen), according to the manufacturer’s protocol. A 1-µg RNA sample was reverse-transcribed with oligo dt₁₅ by avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Real-time polymerase chain reaction (7500 Sequence Detection System, ABI, Invitrogen) amplifications were performed by using the Power SYBR Green PCR Master Mix reagent (Invitrogen). Primer pairs used are listed in Table I. β-actin messenger RNA (mRNA) was used as an internal control.

**Histologic evaluation and micro-vessel density**

Fourteen days after operation, the rest rats were sacrificed, and the middle portion of the flaps were harvested and divided into two parts: one for histologic examination, the other for molecular biological examination. The specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 5-µm-thick sections, and stained with hematoxylin and eosin. The numbers of infiltrating neutrophile granulocytes were evaluated in 20 random fields by two reviewers blinded to the research. Furthermore, fixed tissues were stained with anti-CD31 (Abcam) and colored by diaminobenzidine staining. The vascular density was confirmed by calculating the CD31+ capillaries in 20 random fields at 400× magnification. The counting was performed by two reviewers blinded to the research.
Figure 1. The survival rate and blood perfusion of the ischemic skin flap in the four groups at postoperative day 10. (A) Control group; (B) EPC group; (C) ESWT group; (D) combined group; (E) design of the skin flap on the animal. To keep the same size of skin flap, the curving surface of the animal’s back should be taken into consideration. The four circles indicate the points where cells were injected subcutaneously. (F) Application of ESWT; (G) comparison of the flap survival among the four groups; (H) blood perfusion of the skin flap in the four groups at different time points. Symbols (*, †, ‡, §) indicated significant difference (at .05 level) by Tukey multiple comparison procedure. * versus †, * versus ‡, † versus §, P ≤ 0.05. There was no significant difference between groups labeled with same symbol.
**Immunofluorescence staining**

Immunofluorescence staining was performed to examine the numbers of vWF+ cells in skin flap tissues ($n = 6$ for each group) using FITC-conjugated primary antibodies (Abcam). Irrelevant antibodies were used as controls in the current study. Slices were observed using laser confocal microscopy (A1; Nikon, Tokyo, Japan) at 200× magnification. The numbers of vWF+ cells were evaluated in 20 random fields by two reviewers blinded to the research.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

To investigate the apoptosis in the flap, flap tissues were fixed overnight in 4% paraformaldehyde in 0.1 M PBS (pH 7.4), embedded in paraffin wax, cut into 5-μm sections and deparaffinized according to the standard procedures. Manifestations of cell death were sought using fluorescent in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, performed according to the manufacturer’s manual (Roche Diagnostics, Mannheim, Germany). Dead cells within the flap tissue were counted using a fluorescence microscope (Eclipse 80i, Nikon). Cells were counted manually by two blinded investigators.

**Statistical analysis**

All experiments were performed in triplicate and repeated three times. Data were expressed as mean ± SD. Statistical analysis was adequately performed by analysis of variance followed by Tukey multiple comparison procedure. Statistical analysis was performed using SPSS for Windows version 13.0 (SPSS, Chicago, IL, USA). A probability value of $P \leq 0.05$ was considered statistically significant.

**Results**

**EPC cultivation and identification**

Approximately 4—6 days after seeding, spindle-shaped cells appeared. Gradually, spindle-shaped cells disappeared, and colonies of cobblestone-like cells emerged after 7—9 days. After cultivation for 16 days, cobblestone-like cells grew in the culture dish. Flow cytometric analysis showed strong expressions of CD34, kinase insert domain receptor and weak expression of c-kit on cell membrane, along with the ability of being double stained by DiI-ac-LDL and FITC-lectin, indicating the EPC phenotype (Figure 2).

**Flap survival**

Postoperative day 10, the edges of the necrotic flaps became evident (Figure 1A-D). The survival rate in group 1 was 34.72 ± 7.35%, which was the lowest among the four groups. Survival rate in group 4 was 87.5 ± 10.23%, which was notably higher than the other groups. There was no significant difference between groups 2 and 3 in survival rate (Figure 1G).

**Regional blood perfusion in flap (laser Doppler flowmetry)**

During first 3 postoperative days, blood perfusion unit of the flap in all four groups decreased below normal, but the blood perfusion unit in groups 3 and 4 was a little higher than that in groups 1 and 2. From day 4 on, blood perfusion of the skin flap in group 4
increased significantly. However, the recovery of blood perfusion in group 1 was slower than in the other three groups, and it was even undetectable in some middle portions of the flaps where necrosis occurred at that stage. Ten days postoperatively, blood perfusion of the flap in group 4 was much higher than that in the other three groups and close to normal (Figure 1H).

**Histologic examination**

Two weeks postoperatively, few blood vessels could be observed in group 1, with many infiltrating neutrophil granulocytes in the ischemic tissue. In group 2, some small blood vessels with dilated lumen were observed, and granulocyte infiltration was less than in group 1. Group 3 was similar to group 2, and the number of granulocytes did not differ between the two groups. The vessel density in group 4 was highest among the four groups, and only slight granulocyte infiltration was observed. The number of infiltrating granulocytes did not differ between group 4 and normal control (Figures 3 and 4).

In the immunofluorescence staining examination, the number of vWF+ cells was remarkably lower in group 1 than in the other groups. In groups 2 and 4, plenty of vWF+ cells could be detected in one field. More than 90% of them were also labeled with DiI. There were more vWF+ cells aggregating together in group 4 than in group 2. However, the number of vWF+ cells was lower in group 3 than in groups 2 and 4 but was still higher than in group 1 (Figure 5).

**TUNEL assay in tissues**

Nearly 40% nucleuses stained with FITC were observed in sham group, indicating a high apoptosis rate. Nearly 30% of nucleuses in the EPCs or ESWT group were FITC-positive, while the FITC-positive nucleuses were less than 5% in the dual treatment group (Figure 6).
mRNA expressions of angiogenic factors, chemotactic factors and apoptotic markers

Most of the cytokine expressions were sharply reduced at postoperative day 3. The expression of these cytokines recovered gradually with the time progression. Generally speaking, at a same time point, the cytokine expressions were higher in group 4 than in the other three groups and lowest in group 1.
However, stromal cell-derived factor 1 (SDF-1) was an exception. The expression of SDF-1 increased at postoperative day 3 in all four groups and reached its highest point at postoperative day 7. SDF-1 levels were higher in groups 3 and 4 than in groups 1 and 2 at day 3. At the end of first week, this situation changed, and the SDF-1 level in groups 1 and 3 increased drastically, becoming highest in group 1 and lowest in group 4; it then decreased the following week in all four groups.

The mRNA expressions of BAX and BCL-2 were also reduced in the four groups at day 3. It was significantly higher in group 4 than in the other three groups and did not differ among the groups 1–3. The mRNA expression of BCL-2 was similar to BAX but reduced more sharply than BAX. Because the decreased expression of BCL-2 was out of proportion with the expression of BAX, the BAX/BCL-2 ratio, which is closely related to apoptosis, was significantly higher in group 1 than in the other groups and was lower in group 4 than in groups 2 and 3. With the time progression, the BAX/BCL-2 ratio decreased with the recovery of BCL-2 expression (Figure 7).

Discussion

The aim of the study was to investigate the compound effects of EPCs implantation combined with ESWT on the survival of ischemic skin flaps in an animal model. In the present study, we found that (i) ischemia inhibited cytokine expression except for SDF-1, (ii) expressions of angiogenic markers and chemotactic factors could be substantially augmented by EPC therapy combined with ESWT and (iii) application of the dual treatment could prevent over-dimensioned skin flap from distal necrosis. ESWT is a promising non-invasive angiogenic strategy (12). Although the exact mechanism of shock-wave therapy remains uncertain (26), some studies have shown that shock-wave treatment could significantly up-regulate expression of the chemotactic and angiogenic cytokines (26,27). In our pilot study (24,28), we also demonstrated that shock wave could avail diabetic wound healing and avoid necrosis in free skin flap in rats by up-regulating the expression of endothelial nitric oxide synthase (eNOS) and VEGF, as well as the expression of transforming growth factor-β (TGF-β).

Research has revealed that ESWT could reduce apoptosis in tissue (29,30). In our pilot research (25), we demonstrated that ESWT could reduce the BAX/BCL-2 ratio in vitro. This phenomenon reappeared in the present study in vivo. However, whether the decreased BAX/BCL-2 ratio actually reduced apoptosis was unknown. Therefore, we performed a TUNEL assay to investigate the apoptosis in the skin flap tissue. We observed that there were fewer FITC-stained nucleuses in the dual-treatment group than in the two single-treatment groups, indicating decreased apoptosis in the dual-treatment group.
Figure 6. TUNEL assay in tissue (400× magnification). FITC-labeled nucleus (green channel) indicates an apoptotic cell. 4',6-diamidino-2-phenylindole (DAPI, blue channel) was used to locate the nuclei of the cells. Fewer FITC-labeled nuclei were observed in the dual-treatment group. Scale bars represent 50μm.
We also examined the expression of hypoxiainducible factor 1α (HIF-1α) to investigate tissue response to ischemia. HIF-1α is a central regulator of the hypoxia-induced response (31). In hypoxia, HIF-1α translocates to the nucleus and promotes the transcription of a variety of effectors such as VEGF and FGF-2, promoting angiogenesis and vasculogenesis (31,32). Low oxygen pressure is a common characteristic symptom of hypoxia and ischemia. However, the expression of HIF-1α reduced in all four groups when the blood supply decreased despite the concomitant low oxygen pressure. It seemed that the inhibitive effect of ischemia on the expression of HIF-1α was stronger than the inducible effect of hypoxia. Thus, HIF-1 is not an indicator of ischemia.

Under the ischemic condition, expression of most cytokines, with the exception of SDF-1, decreased, then recovered with the alleviation of ischemia. The expression of SDF-1 increased with the aggravation of ischemia and decreased with its alleviation. It seemed that SDF-1 might serve as an ideal indicator for ischemia. However, why the expression of SDF-1 increased in ischemic tissue when expression of most cytokines decreased is still not clear.

SDF-1 is the ligand of CXCR4 and plays an important role in the migration of stem cells to ischemic site (33,34). The SDF-1 mRNA level was higher in ischemic tissue, and SDF-1 expression was further elevated in the dual-therapy group in the early stage, which may provide an advantageous condition for EPCs to reside in ischemic tissues. Histologic examination indicated that shock-wave therapy could increase the recruitment of vWF+ cells at the site of ischemic injury. Furthermore, most of the vWF+ cells were labeled with DiI, indicating the implanted cells. Thus, we believe that ESWT could increase the expression of SDF-1 and facilitate the recruitment of the implanted EPCs. This hypothesis corresponds with previous research (27).

EPCs have been shown to have potent angiogenic ability in ischemic tissues (35,36). There are three explanations for the effect of the EPC transplantation. First, EPCs are capable of homing to the
sites of neo-vascularization and are thus able to exert their effects in areas most in need of new blood vessel growth (37). Second, EPC therapy is able to deliver both the substrate (endothelial cells) and the cytokines and growth factors necessary for neo-vascularisation (38—41). Third, EPCs could differentiate into mature endothelial cells and incorporate into newly formed vessels (42,43).

According to the results of the experiment, we believe that single treatment (EPCs or ESWT alone) may provide protection for the skin flap with a length-to-width ratio lower than 2:1 in rats. Dual treatment with EPCs and ESWT is effective for the skin flap with a length-to-width ratio greater than 2:1 in rats. However, this result is based on an animal model. The human dermal structure is different from that of rats, so the clinical application of this technique needs further study.

Conclusion
Treating ischemic skin flap with low-energy shock wave can improve the recruitment of EPCs by increasing the expression of chemotactic factors. Thus, ESWT may facilitate the efficacy of cell therapy and augment its effect. This technology could be used clinically without ethical problems.

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