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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, 1.0×10^6 cells), group 3 (treated with ESWT, 300 impulses at 0.10 mJ/mm^2) 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 $87.5 \pm 10.23\%$, 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

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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 sterilely 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 of 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 ($\sim 1.0 \times 10^6$) 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