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The dose–effect relationship in extracorporeal shock wave therapy: the optimal parameter for extracorporeal shock wave therapy

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ABSTRACT

Background: Extracorporeal shock wave therapy (ESWT) has been demonstrated to have the angiogenic effect on ischemic tissue. We hypothesize that ESWT exerts the proangiogenesis effect with an energy density–dependent mode on the target cells.

Materials and methods: Endothelial progenitor cells (EPCs) of rats were obtained by cultivation of bone marrow–derived mononuclear cells. EPCs were divided into five groups of different energy densities, and each group was furthermore subdivided into four groups of different shock numbers. Thus, there were 20 subgroups in total. The expressions of angiogenic factors, apoptotic factors, inflammation mediators, and chemotactic factors were examined, and the proliferation activity was measured after ESWT.

Results: When EPCs were treated with low-energy (0.04–0.13 mJ/mm^2) shock wave, the expressions of *endothelial nitric oxide synthase, angiopoietin (Ang) 1, Ang-2, and B-cell lymphoma 2* increased and those of *interleukin 6, fibroblast growth factor 2, C-X-C chemokine receptor type 4, vascular endothelial growth factor a, Bcl-2-associated X protein, and caspase 3* decreased. *stromal cell-derived factor 1* changed without statistical significance. When cells were treated with high-energy (0.16 mJ/mm^2) shock wave, most of the expressions of cytokines declined except the apoptotic factors and *fibroblast growth factor 2*, and cells lead to apoptosis. The proliferation activity and the ratio of *Ang-1/Ang-2* reached their peak values, when cells were treated with ESWT with the intensity ranging from 0.10–0.13 mJ/mm^2 and shock number ranging from 200–300 impulses. Meanwhile, a minimal value of the ratio of *Bax/Bcl-2* was observed.

Conclusions: There is a dose–effect relationship in ESWT. The shock intensity ranging from 0.10–0.13 mJ/mm^2 and shock number ranging from 200–300 impulses were the optimal parameters for ESWT to treat cells *in vitro*.

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1. Introduction

Extracorporeal shock wave is a longitudinal acoustic wave that propagates through water or soft tissue as ultrasound does [1]. Extracorporeal shock wave was first used in lithotripsy. Studies have demonstrated that low-energy extracorporeal shock wave therapy (ESWT) is effective for nonunion of fractures, tendinitis, and aseptic necrosis of bone in orthopedics [2]. It is also used to treat ischemia disease such as angina and ischemia heart failure in cardiology [3,4]. We have reported that ESWT could improve the healing of diabetic wound and the survival of ischemia skin flap in rats [5,6]. ESWT has been considered as a promising noninvasive angiogenic strategy [7].

However, the mechanism of ESWT is still not clear [8]. Some researchers have reported that ESWT could improve the expression of angiogenic factors [6,9] in stem cells and protect cells from apoptosis [9]. But the dose–effect relationship between the intensity and the biological effects of shock wave is poorly investigated.

Endothelial progenitor cells (EPCs) are the progenitors of endothelial cells with robust angiogenic activity [10]. Researches revealed that EPCs circulate from bone marrow to incorporate into and contribute to postnatal physiological and pathologic neovascularization [11,12]. Furthermore, EPCs have recently been suggested to represent a promising cellular tool [13,14]. Therefore, in the present study, EPCs were used as the targets of ESWT.

We hypothesize that the ESWT exerts its proangiogenesis effect with an energy density–dependent mode on the target cells. Accordingly, the present study aimed to reveal the dose–effect relationship in ESWT, and determine the optimized parameter (including energy flux density and shocks) for ESWT to treat cells cultured *in vitro*.

2. Material and methods

2.1. EPCs cultivation

All animal procedures were approved by the Laboratory Animal Ethics Committee of Shanghai Jiaotong University and conformed to the National Guidelines for Care and Use of Laboratory Animals. A total of 12 male Sprague-Dawley rats (Laboratory Animal Research Center, Shanghai), each weighing 250–300 g, were used in the experiment. According to the method described in the literature [15], bone marrow tissue was aspirated from bone marrow cavity of femurs and tibias of rats. The bone marrow tissue was flushed out several times with low glucose Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Beijing, China) supplemented 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 Histopaque (1.083 g/mL; Sigma-Aldrich, St. Louis, MO) density–gradient centrifugation. *Ex vivo* expansion of EPCs was performed as previously described [16,17]. Briefly, these mononuclear cells were plated on rat fibronectin (Calbiochem, Merck, Darmstadt, Germany)–coated culture dishes and maintained in an Endothelial Basal Medium-2 (EBM-2) (Lonza, Basel, Switzerland) supplemented with Endothelial Cell Growth Media 2 (EGM-2) MV SingleQuots (Lonza, Basel, Switzerland) (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). After 4 d, nonadherent cells were removed by washing, new media were added, and the culture maintained through 16 d, and plenty of cells ($\sim 1.0 \times 10^6$) were found to grow on the plate. After all the operations, the animals were returned to animal center and kept as usual.

2.2. Identification of EPCs

To identify the population of EPCs cultivated, the cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and immunostained for 30 min on ice with the following antibodies: polyclonal sheep antibody against rat CD34 (R&D, Minneapolis, MN), DyLight 488–conjugated monoclonal antibody against kinase insert domain receptor (KDR) (Novus, Littleton, CO), fluorescein isothiocyanate (FITC)-conjugated antibody against Von Willebrand factor (vWF) (Abcam, Cambridge, MA). Cells labeled with nonfluorescence-conjugated antibodies were then incubated with Alexa Fluor 488–conjugated antibodies specifically against sheep immunoglobulin G (Jackson, West Grove, PA). Isotype-identical antibodies (immunoglobulin G) served as controls. Flow cytometric analyses were performed using fluorescence–activated cell sorter (Navios, Bechman Coulter, Brea, CA). DiI-Ac-Low-density lipoprotein (LDL) (Molecular Probes; Invitrogen, Eugene, OR) uptake test and FITC-Ulex europaeus (UEA) (Sigma, St. Louis, MO) binding test were also performed. Cells staining positively for both markers were considered to be differentiating EPCs as reported previously [17,18].

2.3. Group division and shock wave treatment

EPCs of third passage were used in the experiment. Cell suspension was collected in a centrifuge tube by trypsinization, and the concentration was adjusted to 1.0×10^5/mL. EPCs were divided into five groups with energy flux densities: 0.04, 0.07, 0.10, 0.13, and 0.16 mJ/mm^2. Each group was subdivided into four subgroups with shock numbers: 140, 200, 300, and 500 impulses. Thus, there were 20 subgroups in total. ESWT with different parameters was applied on cell suspension in a centrifuge tube accordingly. The centrifuge tubes were smeared with ultrasound transmission gel (Pharmaceutical Innovations Inc, NJ) as contact medium between the tube and the ESW apparatus (Orthospec, Medispec Ltd, Yehud, Israel). After shock wave treatment, the EPCs were reseeded on plates to continue cultivation separately according to different subgroups. After 48 h, Methyl Thiazolyl Tetrazolium (MTT) assay and real-time quantitative polymerase chain reaction (PCR) were performed to examine the proliferation activity and expression of cytokines.

2.4. MTT assay for proliferation activity

Treated with shock waves of different intensity, 2000 cells of third passage per well were seeded in fibronectin–coated