



ADIPOSE DERIVED CELLS

Defocused low-energy shock wave activates adipose tissue-derived stem cells *in vitro* via multiple signaling pathwaysLINA XU¹, YONG ZHAO¹, MUWEN WANG¹, WEI SONG¹, BO LI¹, WEI LIU^{2,3}, XUNBO JIN¹ & HAIYANG ZHANG^{1,4}

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Abstract

Background aims. We found defocused low-energy shock wave (DLSW) could be applied in regenerative medicine by activating mesenchymal stromal cells. However, the possible signaling pathways that participated in this process remain unknown. In the present study, DLSW was applied in cultured rat adipose tissue-derived stem cells (ADSCs) to explore its effect on ADSCs and the activated signaling pathways. **Methods.** After treating with DLSW, the cellular morphology and cytoskeleton of ADSCs were observed. The secretions of ADSCs were detected. The expressions of ADSC surface antigens were analyzed using flow cytometry. The expressions of proliferating cell nuclear antigen and Ki67 were analyzed using western blot. The expression of CXCR2 and the migrations of ADSCs *in vitro* and *in vivo* were detected. The phosphorylation of selected signaling pathways with or without inhibitors was also detected. **Results.** DLSW did not change the morphology and phenotype of ADSCs, and could promote the secretion, proliferation and migration of ADSCs. The phosphorylation levels were significantly higher in mitogen-activated protein kinases (MAPK) pathway, phosphoinositide 3-kinase (PI-3K)/AKT pathway and nuclear factor-kappa B (NF-κB) signaling pathway but not in Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Furthermore, ADSCs were not activated by DLSW after adding the inhibitors of these pathways simultaneously. **Conclusions.** Our results demonstrated for the first time that DLSW could activate ADSCs through MAPK, PI-3K/AKT and NF-κB signaling pathways. Combination of DLSW and agonists targeting these pathways might improve the efficacy of ADSCs in regenerative medicine in the future.

Key Words: adipose tissue-derived stem cells, cell proliferation, cell signaling, defocused low-energy shock wave, Janus kinase, mitogen-activated protein kinase, regenerative medicine

Introduction

Since the early 1990s, shock wave therapy has been clinically implemented as an effective treatment to disintegrate urinary stones in human patients. As a successful case in the area of mechanical biomechanics, shock wave therapy has made great progress in clinical applications. In 1998, Rompe *et al.* [1] first proposed the concept “energy flux density,” which was used to indicate the shock wave energy flowing through an area perpendicular to the direction of propagation. The authors concluded that low-energy shock

wave (<0.1 mJ/mm²) was powerless for stones, but a promising strategy for regeneration medicine. In the last decade, lots of literature confirmed that defocused low-energy shock wave (DLSW) therapy was beneficial for various disorders from benchside to bedside, including soft tissue wounds [2], erectile dysfunction [3,4], Peyronie’s disease [5], injury and ischemia of bone [6,7].

However, the underlying mechanisms of the therapeutic effect of DLSW are not yet completely understood. Previous studies suggested that up-regulated neovascularization [8,9], neuroregeneration

[4], enhanced cell proliferation [10], enzymatic activities [11] and anti-inflammatory [12] and anti-microbial [2,13] effects might be involved in the process. Furthermore, we also found that DLSW could lead to a recruitment of endogenous mesenchymal stromal cells (MSCs) to the disease sites [4]. Hence, activation of stem cells may be a new mechanism of DLSW in regenerative medicine. To test this hypothesis, bone marrow stromal cells (BMSCs) were treated by DLSW, and results demonstrated that DLSW could enhance the secretion and proliferation of BMSCs and promote angiogenesis and nerve regeneration *in vitro* [14]. This conclusion was further supported by some other research groups [15,16].

To reveal more details of mechanisms of DLSW on stem cells, especially the participated signaling pathways, the effect of DLSW on activation of adipose tissue-derived stem cells (ADSCs) was investigated in the present study. ADSCs are routinely isolated from the stromal vascular fraction (SVF) of homogenized adipose tissue. The report from Bourin *et al.* [17] provided initial guidance to academia, industry and regulatory authorities regarding the properties of cell surface markers expected for ADSCs and SVF. ADSCs have shown great advantages and promise in the field of regenerative medicine. Moreover, they can be readily harvested in large numbers with low donor-site morbidity [18]. In the present study, the morphology of ADSCs, the expression profile of ADSCs surface markers, the proliferation features and the migratory function were compared before and after being shocked by DLSW. The secretion ability of ADSCs was also examined, based on our previous conclusions that the therapeutic effect of ADSCs was achieved not by their ability of differentiation, but by their paracrine release of cytokines and growth factors [14,19–21]. In particular, several signaling pathways related to the activation of stem cells in previous reports were selected and examined to determine their roles in the process.

Materials and methods

Isolation and culture of ADSCs and shock wave treatment

All animal care, treatments and procedures were approved by the Ethics Committee of Shandong Provincial Hospital. Isolation and expansion of ADSCs were performed according to a previously standardized protocol [22]. Female Sprague-Dawley rats (8 weeks old) were under anaesthetic with pentobarbital and fat around the ovary was removed to a centrifuge tube filled with cold phosphate-buffered saline (PBS). After digestion and centrifugation, ADSCs were harvested and seeded in specialized basal medium (RASMD-90011,

Cyagen Biosciences Inc.) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine onto culture flasks and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Two days later, non-adherent cells were removed and fresh culture medium was added. The culture medium was changed every 3 days. Cells were passaged when they reached approximately 90% confluence.

The shock wave treatment on cultured ADSCs was performed by an extracorporeal shock wave machine (derma-PACE device, SANUWAVE). The shock wave probe was kept in contact with the culture flask containing adherent ADSCs by means of a water-filled cushion covered with common ultrasound gel. The cells were subjected to 800 impulses of DLSW at an energy flux density of 0.1 mJ/mm² with a frequency of 120/min. The shock wave treatment was performed before each passage. ADSCs without shock wave treatment (untreated ADSCs) were cultured at the same time and served as the control.

To test the possible signaling pathways activated by DLSW, ADSCs were also cultured in 6 parallel experiments in a different medium for 1 h with the following: (1) 10 µmol/L U0126 (the mitogen-activated protein kinase kinase [MEK] inhibitor, 9903; Cell Signaling Technology); (2) 50 µmol/L SP600125 (the Jun N-terminal kinase [JNK] inhibitor, 8177; Cell Signaling Technology); (3) 20 µmol/L SB202190 (the p38 mitogen-activated protein kinases [MAPK] inhibitor, 8158; Cell Signaling Technology); (4) 50 µmol/L LY294002 (the inhibitor of phosphoinositide [PI-3K], 9901; Cell Signaling Technology); (5) 20 µmol/L pyrrolidine dithiocarbamate (PDTC, the nuclear factor-kappa B [NF-κB] inhibitor, ab141406, Abcam); and (6) the five inhibitors above. Then, the cells were treated by DLSW before each passage. Treated ADSCs without combination of inhibitors of signaling pathways served as control.

Cell morphology test

The cultured ADSCs above were examined using a microscope with the Axiovision image analysis system (Carl Zeiss).

To visualize the actin cytoskeleton, ADSCs were incubated with phalloidin (fluorescein isothiocyanate [FITC]-conjugated) (P5282, Sigma-Aldrich). The ADSCs of passage 1–passage 5 (P1–P5) were fixed for 5 min in 3.7% formaldehyde solution in PBS, and stained with a 50 g/mL fluorescent phalloidin conjugate solution for 40 min at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (D8417, Sigma-Aldrich). The images were captured using a fluorescence microscope (Nikon Ti-S, Nikon Inc.) with the Axiovision image analysis system.

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