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Original article

The effect of low-intensity pulsed ultrasound on wound healing using scratch assay in epithelial cells

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ABSTRACT

Purpose: Low-intensity pulsed ultrasound (LIPUS) is widely used in medical fields because it shortens the time required for biologic wound healing in fracture treatment. Also, in dental fields, LIPUS should be effectively employed for implant treatment.

However, most of the relevant reports have been published on its effects on bone formation around implants, and the effects of LIPUS on soft tissue healing remain unclear. In the present study, we examined the effects of LIPUS on soft tissue healing using gingival epithelial cells.

Methods: Gingival epithelial cells were cultured on a dish, followed by LIPUS exposure at a frequency of 3 MHz for 15 min. The cells were counted with a hemocytometer, and a scratch assay was conducted by measuring the closing area of the scratch wound using a microscope. Following LIPUS exposure, total RNA was collected for microarray analysis. In addition, real-time PCR was performed to examine the mRNA expression level of integrin α6β4. Furthermore, total protein was collected to examine the protein expression level of integrin α6β4 by western blotting.

Results: The cell count and scratch assay demonstrated that LIPUS exposure promoted cell proliferation and scratch-wound closure. Microarray analysis demonstrated the increased expression levels of adhesion-related genes, including integrin. Real-time PCR analysis demonstrated that LIPUS exposure significantly up-regulated the mRNA expression level of integrin α6β4. Western blotting showed intense staining of integrin α6β4.

Conclusion: LIPUS exposure promotes wound closure in the scratch assay and up-regulates the expression level of integrin α6β4 as compared with the control.

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1. Introduction

Free gingival graft is an effective surgical treatment in cases with an insufficient width of keratinized gingiva around natural teeth [1] or implants [2–4]. However, this technique causes an open secondary wound with post-surgical pain and bleeding at a donor site [5,6]. Hence, in cases for which multiple grafts or procedures are needed, shortening the time required for wound healing is critical for reducing the patient burden, such as postoperative discomfort and pain.

Physical therapy by applying physical energy to a wound site to promote healing has attracted attention [7]. Physical therapy is a therapeutic approach to utilize physical actions, such as heat, electricity, light, X-ray, and air. The energy used is roughly classified into electromagnetic and mechanical energies [8,9]. In addition, ultrasound therapy has attracted attention for decades as physical therapy.

Low-intensity pulsed ultrasound (LIPUS) is a medical device, developed as a therapeutic approach to promote fracture healing in the 1950s. This device applies acoustic waves as physical energy to living tissue [10,11].

An *in vivo* study demonstrated that LIPUS exposure promoted wound healing at all stages during fracture healing [12–15] and increased mechanical strength in a fibula fracture model [16]. Moreover, LIPUS up-regulated the expression levels of aggrecan mRNA in a rat femur fracture model [17]. An *in vitro* study showed that Cbfa-1/Runx2, Alk-3, alkaline phosphatase, osteopontin, TGF-β1 and BMP-7 in the rat bone marrow stromal cells [18], up-regulated osteoblasts and down-regulated osteoclasts in the rat alveolar mononuclear cells [19].

Thus, LIPUS is a noninvasive and safe device for fracture healing, and has been employed in daily clinical practice in orthopedic fields.

Reportedly, in dental fields, LIPUS exposure prevents insufficient salivary secretion due to autoimmune sialadenitis [20] and enhances bone formation around miniscrew implants [21], gradually demonstrating its usefulness.

Only a few studies have been conducted on soft tissue healing. For example, an *in vivo* study demonstrated that LIPUS enhances palatal mucosa wound healing in rats [22] and accelerates periodontal wound healing after flap surgery [23]. An *in vitro* study indicated that the expression of connective tissue growth factor (CTGF) in gingival cells (GE1) was enhanced by LIPUS exposure [24]. However, the detailed mechanisms remain unclear.

Integrin is a transmembrane glycoprotein that forms 24 different heterodimers by combining any of 18α and 8β subunits. It is a bidirectional signaling receptor located at the interface between the extracellular matrix and the intracellular milieu, a major receptor in keratinocyte adhesion in the basement membrane that underlies the skin epidermis [25]. Recently, the role of integrin in wound healing has attracted attention. Wound healing occurs in the soft tissue not through cell division and blood clot formation, but through resealing by the active movement of surrounding cells. As previously reported, integrin α6β4 controls cell migration by regulating the transcription and translation of different integrin subunits in a wound site [26]. In addition, integrin plays a central role in wound healing by inducing tissue remodeling and controlling various cell functions (cell proliferation and survival and extracellular matrix remodeling).

Thus, in the present study, the effects of LIPUS exposure on wound healing and integrin α6β4 expression were examined using gingival epithelial cells (GE1).

2. Materials and methods

2.1. Cell culture

A GE1 cell line collected from the gingival epithelial tissue of an SV40 large T-antigen transgenic C57BL/6 mouse [27], purchased from RIKEN BRC CELL BANK (Tsukuba, Japan), was cultured in a chemically-defined medium SFM101 (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 mg/mL streptomycin, and 10 ng/mL murine epidermal growth factor (mEGF) in a humidified atmosphere of 5% CO_2 at the optimal temperature of 33 °C.

The cells were cultured in 3.5-cm culture dishes, with the media exchanged every other day.

2.2. Ultrasound treatment

Ultrasonic irradiation was performed with a LIPUS exposure system (BR-Sonic PRO, ITO, Tokyo, Japan). LIPUS exposure was carried out from the lower surface of the 3.5-cm culture dish through an ultrasound gel placed between a LIPUS probe (L size) and the dish.

LIPUS exposure was conducted at a frequency of 3 MHz, a temporal average intensity of 160 or 240 mW/cm^2, and exposure time of 15 min [22,24,28]. Ultrasound was delivered with pulse ratio of 1:4 in order to deliver only non-thermal effect to the cells [29]. During the LIPUS exposure, GE1 cells were maintained in a humidified atmosphere of 5% CO_2 and at an optimal temperature of 33 °C. To prepare a control, the same conditions were employed, except for the absence of LIPUS exposure.

2.3. Cell counts

For cell counting, cells were detached from the dish with trypsin–EDTA (0.05% trypsin and 0.02% EDTA in Hanks Balanced Salt Solution) (SAFC Bioscience, KS, USA). GE1 cells were seeded at 1.0×10^5 cells/mL, and were collected every other day from the day after seeding (1, 3, 5 and 7 days after the seeding), followed by measurement using a standard microscope with a hemocytometer. LIPUS exposure at 3 MHz frequency, 160 mW/cm^2 intensity was performed every 24 h from the day after seeding.

2.4. Scratch assay

GE1 cells were seeded at 4.0×10^5 cells/mL. Two days later, confluent cells were linearly scratched using a 20-μL pipette chip. The scratched region was photographed immediately and every 12 h after scratching using a microscope equipped with a camera.

The photograph was traced to tracing paper, followed by the coloring of cells with image editing software (FireAlpaca).

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