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SPECIAL ISSUE

Application of Ultrasound Stimulation in Bone Tissue Engineering

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Many studies have been investigated on the effects of the low-intensity pulsed ultrasound (LIPUS) on bone healing, acceleration of bone mineralization and regeneration. Many researchers have focused on a more comprehensive understanding of the biological mechanism of the osteoblast by LIPUS because the osteoblast is an important cell in bone formation. The effects of LIPUS on the proliferation, gene expression of Runx2, Msx2, Dlx5, and AJ18, and the second messenger signaling of osteoblast were reported. Various parameters of LIPUS, such as intensity, frequency, duration and topology, were investigated to find appropriate conditions in osteoblast. Less than 120 mW/cm^2 of intensity and 1-3 MHz of frequency were considered good condition for regeneration of bone tissue. Increased osteoblast cells and higher mineralized nodule formation explain the enhancement of proliferation by LIPUS. In addition, LIPUS affects on differentiation of osteoblast cells, which is shown by increased ALPase, and transcriptional factors, Runx2. Ultrasound stimulates PEG2 and COX-2 in osteoblast, and the signals accelerates the bone regeneration in tissue engineering.

Keywords: Low-intensity pulsed ultrasound, Stimulation, Bone regeneration, Tissue engineering

Introduction

Bone is dynamic tissue, which is affected by many systemic and local factors (1). Bone regeneration, such as fracture healing and distraction osteogenesis, is generally a complex process that involves cell proliferation and differentiation, chemotaxis, and the synthesis of an extracellular matrix (2).

There is an increasing interest to apply therapeutic ultrasound for promotion of fracture healing since such a technique is enably easy and manageable use of non-invasive therapy that can improve the abnormal healing processes (3).

The ultrasound has been reported to improve stimulation of bone regeneration and bio-absorption (4,5). The use of ultrasound as a therapeutic approach in bone healing has a history of more than half a century. The ultrasound functions as stimulating on the growth of bone defects. The ultrasound is acoustic radiation at frequencies above the limit of human hearing. It is one of mechanical energies that can be transmitted into the body as high-frequency acoustical pressure waves.

Ultrasound is used at various intensities, with therapeutic or surgical applications as high as 1～300 W/cm^2, causing heat in tissues. Since high intensity can damage the organs, tissues, and cells, low-intensity pulsed ultrasound (LIPUS) has been used low enough to be considered neither thermal nor destructive. The LIPUS indicates less than 100 mW/cm^2 and the therapeutic LIPUS usually used range from 20 to 50 mW/cm^2.

The LIPUS influences all major cell types involved in

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bone healing, including osteoblasts, osteoclasts, chondrocytes and mesenchymal stem cells (6). *In vitro* studies suggested that LIPUS produced significant multifunctional effects that are directly relevant to bone formation and resorption. The primary cells involved are osteoblasts and osteoclasts. There is a close anatomic and functional relationship between resorption and formation of cells at remodeling sites (1).

Bone fracture healing and distraction osteogenesis consist of biological process that involves the spatial and temporal orchestration of various cell types, a large numbers of genes, and an extracellular matrix. The process of facture healing includes cell proliferation and differentiation, chemotaxis, and synthesis of the extracellular matrix. LIPUS treatment elevated Runx2 mRNA expression and progressively promoted osteocalcin mRNA expression in human osteoblasts (7).

Oseoblasts release cytokines in response to ultrasound stimulation, and the expression of transforming growth factor β, a protein known to have a role in bone growth and repair, is regulated by ultrasound (8).

The application of LIPUS to healing fractures has been shown to increase the mechanical strength of the callus and reduce the time to bone union.

In this review, effects of low-intensity pulsed ultrasound on the proliferation, gene expression, such as Runx2, Msx2, Dlx5, and AJ18, and second messenger signaling of osteoblast were explained, and the various parameters of LIPUS tested on osteoblast to find appropriate condition were covered.

Parameters of ultrasound stimulation

Intensity and frequency

Chen et al. reported the effect of low intensity ultrasound with two different frequencies of ultrasound, 1 MHz and 3 MHz, on osteoblast proliferation. The best rate of osteoblast proliferation was found at certain intensity of 100 mW/cm^2 with 1 MHz ultrasound insonation, where the best rate of proliferation with 3 MHz was at 50 mW/cm^2 (9).

Saito et al. evaluated different effects of two intensities of pulsed ultrasound, 30 and 120 mW/cm^2, on collagen post-translational modification and mineralization in osteoblastic MC3T3-E1 cells. The cross-link formation and calcium deposition in matrices showed resemble pattern between 30 mW/cm^2 LIPUS-treated cultures whereas 120 mW/cm^2 LIPUS-treated cultures showed increased calcium accumulation and no differences pattern of cross-links compared to control (10).

Reher et al. reported the results of nitrite production and prostaglandin E2 (PGE2) production by manibular-osteoblasts after four different intensities (5, 15, 30 and 50 mW/cm^2) by 45 kHz and four different intensities (0.1, 0.4, 0.7, and 1 W/cm^2) by 1 MHz. All intensities with the 1 MHz and 45 kHz machine showed significantly more prostaglandins (PGs) than control (11).

Duration

Monici et al. investigated whether mechanical stress caused by ultrasound exposure affected osteoclastic precursor cells, thus addressing the hypothesis that mechanical strain-induced perturbation of preosteoclastic cell machinery can contribute to the occurrence of bone turnover alterations. The result showed an increase in cell proliferation at both 24 and 48 h after the ultrasound treatment less than 25 min with 1 Hz pulse repetition frequency whereas cell growth at 24 h was slightly increased and decreased at 48 h for exposure time longer than 15 min (12).

Yang et al. reported that long-term ultrasound stimulation at proper intensity enhanced the differentiation and maturation of osteoblasts. The alkaline phosphate (ALP) activity was also enhanced on days 4 to 14 at 125 mW/cm^2 (13).

Cui et al. reported the effects of low-intensity ultrasound on chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BM-MSC) at a frequency of 0.8 MHz, and intensity of 200 mW/cm^2 for 10 min per day up to 4 weeks. Total collagen, glycosaminoglycan (GAG), and compressive strengths were increased compared to control whereas no significant differences of total DNA was observed (14).

Topology

3-D structure of scaffold may influence integrin adhesion and subcellular protein distribution thus changing biological response (15).

Appleford et al. tested the effect of LIPUS treatment on osteoblast precursor cell signaling and adhesion behavior was examined in 3-D culture on hydroxyapatite (HA) and B-tricalcium phosphate (TCP) scaffolds. Calcium release from the scaffolds was partially involved in the activation of PERK 1/2 when cell response was compared between culture on 2-D surfaces and three-dimensional (3-D) HA and TCP scaffolds. Calcium media extracts with cells in HA and TCP was observed greater than in 2-D (16).

Hsu et al. demonstrated that pulsed ultrasound at 1MHz with 67 mW/cm^2 for 10 min per day enhanced cell proliferation and matrix deposition in 2-D as well as 3-D

Table 1. Parameters of ultrasound stimulation

Cell sources	Intensity (mW/cm^2)	Frequency	Exposure time	Reference	Effect
Human mandibular osteoblasts	5-50, 100～1000	1 MHz, 45 kHz	5 min	Reher et al. (2002) (11)	Stimulates the production of No and PGE2 in osteoblasts
Mouse osteoblastic cell line	125	1MHz	10 min/day	Yang et al. (2005) (13)	Increased surface expression of integrins and caused actin reorganization
Mouse osteoblastic cell line	50～150	1 MHz, 3 MHz	3 min/ day	Chen et al. (2007) (15)	Increased the proliferation rate of cells
Human chondrocyte	67	1 MHz	10 min/day	Hsu et al. (2006) (17)	Promoted cell proliferation and matrix deposition
Murine MC3T3-E1	30, 120	1 MHz	20 min/day	Saito et al. (2004) (10)	Promoted calcium deposition as well as the synthesis of the collagenous matrix
Human bone marrow cell line	10, 100	1 Hz, 100 Hz	15 min, 30 min, 60 min	Monici et al. (2007) (12)	depresses the expression of cytoskeletal components and proliferation and differentiation
Human embryonic palatal mesenchyme cells	30	1 kHz	20 min	Appleford et al. (2007) (16)	Effect stress-signaling mediators and adhesion proteins in cells

chondrocyte cultures. Cell number, GAG, and collagen in 3-D constructs significantly increased in control, ultrasound, and rotator groups during the culture period compared to culture in monolayer (17).

Recent studies on several parameters on conditions of ultrasound stimulation for bone tissue engineering were summarized in Table 1.

Effects of LIPUS on cellular behaviors

Adhesion

Integrins are a family of transmembrane cell adhesion molecules that are responded to mechanical stimuli by initiating intracellular signaling and organization of the cell cytoplasm. It has a function as mechanoreceptors that notice mechanical stimuli from the extracellular matrix and convert them to chemical signaling pathways that regulate cell viability (18, 19).

Yang et al. reported that ultrasound stimulation at 125 mW/cm^2 for 10 min temporarily increased the surface expression of $\alpha 2$, $\alpha 5$, and $\beta 1$ integrins in both MC3T3-E1 and primary osteoblasts. In addition, the reorganization of actin cytoskeleton was found in response to ultrasound stimulation by fluorocytochemistry (13).

Focal adhesion functions as a bridge to link integrin cytoplasmic domain to the cytoskeleton and activates integrin-associated signaling pathways such as mitogen-activated protein kinase (MAPK) pathway (20). Focal adhesion kinase (FAK) is the protein responsible for integrin-mediated mechanically induced bone formation. Carlos et al. found that the LIPUS increased FAK expression at 7 days, extracellular signal-regulated kinase-1/2 (ERK-1/2) at 14 days, and insulin receptor substrate-1 (IRS-1) at 7 days, but the expression decreased 7 days later, indicating a noncumulative effect of LIPUS. The ultrasound stimulation increased the phosphorylation of FAK, ERK, p85 subunit of phosphoinositide 3-kinase (PI3K), and serine 473 of Akt (21). Tang et al. also demonstrated that ultrasound stimulation advanced bone formation in osteobalsts via the integrin/FAK/PI3K/Akt and ERK signaling pathways and increased cyclooxygenase-2 (COX-2) expression.

Proliferation

Many studies demonstrated the cell proliferation of osteoblasts by LIUPS. Takayama et al. reported that the rate of rat osteoblast cell proliferation was not affected by 30 mW/cm^2 with 1.5 MHz for 20 min LIPUS stimulation. However, the ALPase activity increased gradually through day 7 of culture both with and without transient LIPUS stimulation, and it decreased at day 10 (22).

Monici et al. reported that the proliferation of the rat osteoblast cells was determined with and without transient LIPUS stimulation for up to 14 days of culture. Cell proliferation was increased at both 24 and 48 h when the ultrasound stimulation of the cells was performed with the lowest pulse repetition frequency at 1 Hz and short exposure time, which is less than 15 min (12).

Sun et al. showed that the osteoblast cells increased to

114.91% of the control whereas the osteoclasts decreased to 36.15% of the control cells after 7 days of stimulation. The ultrasound stimulation was 1.0 MHz in frequency and 68 mW/cm^2 in intensity 20 min per day for 7 days (23).

Nodule formation elucidates the osteoblast maturation. After ultrasound stimulation for 11 days on 10 min per day, calcium deposition was determined using alizarin red-S staining. It was found that the difference was statistically significant. Sun et al. reported that in 10 days of co-cultures of alveolar mononuclear cells with osteoblast cells, more tartrate-resistant acid phosphatase (TRAP)-positive cells were seen with a tendency to form clusters in the control specimen. When alveolar mononuclear cells were co-cultured with rat-calvarial osteoblasts in the presence of 1α, 25-dihydroxyvitamin D3 (1α, 25$(OH)_2D_3$) for 10 days, both osteoclasts and osteoblasts increased gradually; mostly, multinucleated TRAP positive cell clusters were frequently observed (23).

Mineralized nodule formation was determined at day 14 of culture with and without transient LIUPS stimulation. The staining of mineralized nodules by alizarin red was clearly higher in the rat osteoblast cells transiently stimulated with LIPUS compared to the control cell staining (22).

Differentiation

ALPase and osteocalcin are the classical osteoblast-specific differentiation markers, and they are upregulated by the ultrasound exposure of the osteobalstic cells (3).

ALPase activity was increased after 10 days of culture with daily LIPUS stimulation (24). Takeyama et al. determined ALPase activity in cells up to day 14 of culture with and without transient LIPUS stimulation. The ALPase activity was increased gradually through day 7 of culture both with and without transient LIPUS stimulation, and it decreased at day 10. The activity was significantly increased at days 5, 7, 10, and 14 of culture in the presence of transient LIPUS stimulation compared to the control (22).

Sukuki et al. reported that Runx2 increased early stage osteoblast differentiation and inhibited the late stage of osteoblast maturation, suggesting that Runx2 appears to be a crucial transcriptional factor for osteoblast differentiation (24). Ikeda et al. also studied the mRNA expression of cellular phenotype-specific markers characterizing osteobasts using real-time polymerase chain reaction analysis (25).

Other transcriptional factors, such as Dlx5, Msx2, and AJ18, are important for osteogenesis. Dlx5 expression is related with osteoblast differentiation, and occurs in the final stages of osteoblast differentiation *in vitro* (26). Msx2 is predominantly expressed by proliferating osteoblasts and preosteoblasts, but its expression decreases according to terminal osteoblast differentiation (26-28).

LIPUS significantly increased the expression of mRNAs encoding Runx2, Msx2, Dlx5, osterix, bone sialoprotein, and bone morphogenetic protein-2, whereas it significantly reduced the expression of mRNA encoding the transcription factor AJ18 (24).

Gene expression signal

Erdogan et al. explained that ultrasound signals induce conformational changes in the cell membrane and alter ionic permeability and second messenger activity. Also, changes in second messenger activity lead to downstream alterations in gene expression, and resulting in an acceleration of the fracture repair process by up-regulating bone specific genes (1).

PGs induce bone formation and they are necessary for remodeling of bone by mechanical stimuli. Especially, PEG2 is a messenger molecule produced by osteoblasts and is up-regulated at sites of fractures. Reher et al. reported the effect of the therapeutic range of ultrasound on PGE2 production *in vitro* in human osteoblasts. More PGE2 was synthesized at both 1 MHz and 45 kHz compared to the control culture (11).

PGE2 production is regulated by COX-2. Tang et al. reported the effect of ultrasound stimulation on the COX-2 expression in MC3T3-E1 or primary osteoblast cells with 30 mW/cm^2. The ultrasound exposure of the osteoblasts for 20 min increased PGE2 production. Transient ultrasound exposure increased the membrane expression of integrins and led the expression of COX-2 and the formation of PGE2, suggestion of a role of integrin in the transduction of the acoustic pressure that leads to the expression of COX-2 and the enhanced maturation of osteoblasts (29).

The LIPUS stimulated the expression of COX-2 genes and elevated mRNA levels for the bone matrix protein ALPase and osteocalcin *in vitro*. The ultrasound stimulated osteoblast differentiation by increasing ALPase, osteocalcin and vascular endothelial growth factor (VEGF) expression and mineralization (30).

The mechanism of LIPUS on osteoblasts growth and upregulation of osteoclasts formation might be explained by cytokine release (31). Li et al. reported that ultrasound stimulation would inhibit interleukin-6 (IL-6) secretion of osteoblasts significantly, suggesting that it down-regulated the formation of osteoblasts and prevented bone resorption. Also, specific pulsed ultrasound exposure enhanced osteo-

blasts population together with increase in transforming growth factor-β1 (TGF-β1) secretion and diminished in concentration of IL-6 and tumor necrosis factor-α (TNF-α) in the culture medium (31).

Applications

In bone tissue engineering, many studies on effects of ultrasound stimulation have been conducted. In the first clinical application of ultrasound on the healing of cortical fractures, a 24% reduction in the time to clinical was resulted by ultrasound treatment at 30 mW/cm^2 for 20 min per day, and a 38% decrease in the time to overall healing. The daily use of the ultrasound device on patients was high, and there were no complications related to its use, suggesting that ultrasound exposure not only accelerates healing but also may help to ensure healing (32).

Studies examining various animal models indicated that LIPUS (49, 57 mW/cm^2) treatment resulted in accelerated callus formation compared to untreated controls. Duarte reported that the callus area increased rapidly in the LIPUS treated bones during the first 12 days of treatment whereas callus area increased rapidly in untreated bones after 12 days post-operation. Qualitative radiographic and histological analysis demonstrated that LIPUS treatment resulted in a significant increase in bone growth inside femoral cortical bone defects (36% at day 15) and at a rabbit fibulae osteotomy site (27% at day 18) compared to control (33).

Clases et al. performed the experiment with significantly greater mineral apposition rates at 2 and 3 weeks postfracture in rabbit fibulae treated with 500 mW/cm^2 of ultrasound. The results indicated that larger callus area might be attributed to increased endochondral bone formation processes after LIPUS treatment. Histological analysis showed more advanced endochondral ossification and a smaller fracture gap in LIPUS treated compared to untreated rat femora after 14 days of healing (30).

Shimazaki et al. reported the effects of LIPUS on distraction osteogenesis in rabbits. Half of the 64 rabbits were treated to right leg at 30 mW/cm^2 for 20 min per day by ultrasound, while other half was maintained by rigid fixation. The ultrasound treated group showed significant greater value with normal distraction, the hard callus area, and the findings on mechanical testing (34).

Conclusion

Several biological mechanisms have been demonstrated the influence of the ultrasound stimulation on bone tissue engineering. Many studies investigated the effect of physical parameters, such as intensity, duration of stimulation, and topology, on complex biological levels. Cellular adhesion, proliferation, differentiation, and gene expression were shown to explain regeneration of bone tissue. Less than 120 mW/cm^2 of intensity and 1∼3 MHz of frequency were considered good condition for regeneration of bone tissue (9-11). Increased osteoblast cells and higher mineralized nodule formation explain the enhancement of proliferation by LIPUS (12, 22, 23). In addition, LIPUS affects on differentiation of osteoblast cells, which is shown by increased ALPase, and transcriptional factors, Runx2 (3, 22, 24-28). Ultrasound stimulates PEG2 and COX-2 in osteoblast cells, and the expression of signals accelerates the bone regeneration in tissue engineering (1, 11, 29-31). Animal experiments and clinical applications were reported to show the possibility of therapeutic use. Much research for ultrasound stimulation in bone tissue engineering is required for clinical strategies, and hope for the development of cell based treatment in the future.

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Potential conflict of interest

The authors have no conflicting financial interest.

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