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● *Original Contribution*

FOCUSED LOW-INTENSITY PULSED ULTRASOUND ENHANCES BONE REGENERATION IN RAT CALVARIAL BONE DEFECT THROUGH ENHANCEMENT OF CELL PROLIFERATION

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Abstract—A number of studies have reported the therapeutic potential of low-intensity pulsed ultrasound (LIPUS) for induction of bone repair. This study investigated whether bone regeneration might be enhanced by application of focused LIPUS to selectively stimulate fractured calvarial bone. To accomplish this, bone defects were surgically created in the middle of the skull of rats that were subsequently exposed to focused LIPUS. Bone regeneration was assessed by repeated computed tomography imaging after the operation, as well as histologic analysis with calcein, hematoxylin and eosin and proliferating cell nuclear antigen assay. At 6 wk after surgery, bone formation in the focused LIPUS-treated group improved significantly relative to the control. Interestingly, new bone tissue sprouted from focused LIPUS target points. Histologic analysis after exposure to focused LIPUS revealed that proliferating cells were significantly increased relative to the control. Taken together, these results suggest that focused LIPUS can improve re-ossification through enhancement of cell proliferation in calvarial defect sites. (E-mail: wchang1975@pusan.ac.kr or nm@catholic.ac.kr)

Key Words: **Focused low-intensity pulsed ultrasound, Calvarial bone fracture, Bone regeneration, Osteocyte proliferation.**

INTRODUCTION

Millions of bone fractures caused by congenital anomalies or bone loss from traumatic or neoplastic processes occur worldwide every year (Claes and Wilie 2007). Generally, most patients with a bone defect undergo self-regulating mechanisms that involve complex biological healing processes consisting of spatial and temporal orchestration of various cell types, cytokines and growth factors (Claes and Wilie 2007; Rubin et al. 2001; Sena et al. 2005). Although therapeutic strategies for re-ossification have been developed for many years, some patients still have problems healing fractured bone and require clinical intervention to regenerate bone. There are several methods for stimulating bone repair, including surgical implementation of osteoconductive bone graft substitutes (Walsh et al. 2008); addition of factors such as bone morphogenetic proteins, insulin-like growth factor 1 and transforming growth factor β (Gleizal et al. 2006; Harada et al. 1999; Reher et al. 1997); and elevation of gene expression (Harle et al. 2001). Although numerous clinical strategies can be applied to address bone defects, including defects with incomplete closure, known as non-union defects, they remain clinical challenges (Spicer et al. 2012), and orthopedic surgery can often cause failure of bone regeneration with complications.

One promising therapeutic method for bone regeneration is a non-operative treatment with ultrasound, the propagation of a pressure wave that transfers mechanical

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energy into the tissue (Gleizal et al. 2006; Reher et al. 1997). Some studies have proposed that ultrasound treatment enhances the repair of fractured bone (Hasuike et al. 2011; Ishihara et al. 2014; Lim et al. 2013; Yang and Park 2001); however, the energy from non-targeted exposure to ultrasound can diffuse, resulting in a decrease in positive effects. Therefore, in this study we investigated focused ultrasound (FUS), which can deliver highly focused acoustic energy to specific areas of biological tissues as small as a few millimeters in diameter (Kim et al. 2012). FUS can be classified as focused high-intensity pulsed ultrasound (focused HIPUS) or focused low-intensity pulsed ultrasound (focused LIPUS) (Noda et al. 2013; Tachibana et al. 1999). Focused HIPUS has been used in therapeutic research on hepatocellular carcinoma (Cheung et al. 2013a; Ni et al. 2012), prostate cancer (Blana et al. 2004; Pfeiffer et al. 2012) and other cancers. Focused HIPUS uses the mechanisms underlying high-intensity therapeutic ultrasound, including cavitation and temperature increase, to suppress tumor cells. Use of LIPUS at intensities as high as 30 W/cm^2 has been reported to result in transmission of mechanical energy into living tissue as acoustic pressure waves (Fávaro-Pípi et al. 2010). To date, the focused LIPUS technique has been investigated to determine if it has the potential for non-invasive neuromodulation and modulation of region-specific brain activity. Specifically, Kim et al. (2012) investigated the possibility of using focused LIPUS at 350 and 650 kHz to selectively stimulate the rat abducens nerve located above the base of the skull. In addition, Yoo et al. (2011) reported that neuromodulation by focused LIPUS is involved in cellular excitability through regulation of ion channels and mechanoreceptors embedded within the membrane. Previous studies have found that focused LIPUS successfully stimulates both brain tissue and cranial nerves in rat (Kim et al. 2012; Tyler et al. 2008; Yoo et al. 2011).

In this study, we used computed tomography (CT) imaging and histologic assays in a rat calvarial bone defect model to investigate whether focused LIPUS could stimulate re-ossification through proliferation of cells in the defect site.

METHODS

Animal model of calvarial bone defect

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Incheon Catholic University Medical School. Animals were housed in a light- and temperature-controlled environment and provided with food and water. Twelve 8-wk-old male Sprague–Dawley rats (290–300 g) were initially anesthetized with 5% isoflurane in 70% nitrous oxide and 30% oxygen using an induction chamber; anesthesia was maintained with a mixture of 2% isoflurane with temperature controlled (37 ± 0.1°C) using a rectal thermometer and a heating pad (Harvard Apparatus, Holliston, MA, USA). The rats were fixed to a stereotaxic apparatus, and the median skin over the head was incised. The periosteum was then removed, and a 4-mm calvarial bone defect was created using a trephine hand instrument without other damage (Fig. 1a). After the operation, skin was closed using 4-O silk suture.

Focused LIPUS setup

A spherical segment focused ultrasound transducer (diameter: 6 cm, radius-of-curvature: 7 cm) operating at a frequency of 650 KHz was used. A function generator (Agilent Technologies, Santa Clara, CA, USA) created sonication pulses that were amplified with 403 LA (Electronics & Innovation, Rochester, NY, USA), a frequency power amplifier, to drive the transducer. The electronic signals from the function generator were measured by a calibrated needle hydrophone (HNR500, ONDA, Sunnyvale, CA, USA) with the axial and lateral acoustic fields in a tank filled with degassed water. The cigar-shaped acoustic focus was 3.5 mm in diameter and 6.2 mm in length, measured at the full width at half-maximum of the acoustic intensity (Yoo et al. 2011). The optimum parameters determined from existing research (Kim et al. 2012) were as follows: 1 ms for the tone burst duration, 100 Hz for the pulse repetition frequency and 0.1 W/cm^2 (I_{spta}) for the acoustic intensity (expressed as power per unit area). The intensity value generated by these conditions corresponded to 1 W/cm^2 in terms of spatial-peak pulse-average intensity (I_{sppa}) (Fig. 1b).

Focused LIPUS sonication

The calvaria-fractured rats were randomly divided into two groups: a focused LIPUS treatment group and an untreated control group. Rats were anesthetized (isoflurane 1.5%) during the sonication process (Lavandier et al. 2009). The rats in the treatment group were exposed to focused LIPUS for 20 min twice a week (four points, 5 min per point). The same treatment was given to the untreated control group except the focused LIPUS machine was turned off (Cheung et al. 2013b). Application of focused ultrasound was started on day 1 and continued for 8 wk.

Computed tomography imaging

Computed tomography imaging was used to monitor the bone regeneration of the calvarial defect. Repeated CT imaging was performed biweekly after surgery. The anesthetic, a combination of zolazepam (10 mg/kg; Zoletil, Virbac AH, Carros, France) and 2% xylazine hydrochloride (2 mg/kg; Rumpun, Bayer, Leverkusen,