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Biphasic electric current stimulates proliferation and induces VEGF production in osteoblasts

In Sook Kim [c,1], Jong Keun Song [b,1], Yu Lian Zhang [c], Tae Hyung Lee [b], Tae Hyung Cho [c], Yun Mi Song [c], Do Kyun Kim [a], Sung June Kim [b,*], Soon Jung Hwang [a,c,*]

[a] *Department of Oral & Maxillofacial Surgery, College of Dentistry, Seoul National University, Brain Korea 21 2nd Program, Korea*
[b] *School of Electrical Engineering and Computer Science, Seoul National University, Korea*
[c] *Dental Research Institute, Brain Korea 21 2nd Program, Seoul National University, Korea*

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Abstract

This study investigated biphasic electric current (BEC) functions as a new type of electrical stimulation to induce rat calvarial osteoblasts to proliferate, differentiate and synthesize cytokines. The culture system was designed so that biphasic current flowed between upper and lower gold plates. BEC helps to minimize the net charge accumulation during cell exposure to the electrical stimulation. Osteoblasts were exposed to electrical stimulation of 1.5 $\mu A/cm^2$ at 3000 Hz, and the effect of BEC was assessed in the interrupted mode (6 h daily) and in the continuous mode (24 h daily), depending on the interval of stimulation. Whereas proliferation increased by 31% after stimulation in the continuous mode for 2 days, it was unaffected in the interrupted mode. The transcriptional expression of osteogenesis-related genes such as alkaline phosphatase (ALP), osteopontin, and type I collagen was unchanged 4 days after stimulation in both modes, while cbfa1 was decreased under the same conditions. There was no detectable change in mRNA expression of growth factors (BMP-2, -4, IGF-2 and TGF-β1) that promote osteoblast differentiation. However, real-time RT-PCR and ELISA demonstrated that vascular endothelial growth factor (VEGF) was markedly up-regulated by BEC. Induction of VEGF by BEC was not hypoxia driven. In conclusion, the present *in vitro* study demonstrates that BEC increases cell proliferation and induces the production of VEGF. The BEC was more effective with continuous stimulation than with interrupted stimulation. To confirm whether BEC can enhance osteogenesis, further *in vivo* studies are needed.

Keywords: Biphasic electric current (BEC); VEGF; Osteoblast differentiation; Proliferation; Bone formation

1. Introduction

It has long been acknowledged that physical stress such as mechanical loading and electric stimulation promote osteogenesis and healing of bone fractures [1–5]. Since the discovery of the natural electrical property of bone in the 1950s and 1960s, the concept of coupling endogeneous and exogeneous electric currents has been introduced as an important tool in bone remodeling [6]. Based on this concept, various kinds of electrical stimulations have been investigated for their effects on bone regeneration in animal models and *in vitro* studies.

In vitro studies have demonstrated that electrical direct current injection, capacitive coupling and inductive coupling electric fields enhance proliferation of cells with a subsequent increase in prostaglandin E and extracellular matrix synthesis such as collagen [7–9]. Pulsed electromagnetic field (PEMF), as a method of inductive coupling, exerts stimulating effects on bone mineralization, the proliferation and differentiation of osteoblasts, and the production of cytokines such as BMP-2,-4 or TGF-β1 [10–13]. Several animal experiments show that electrical stimulation can increase calcification and enhance radiographic and mechanical strength in healing bone [14,15]. Supported by a substantial number of *in vitro* and *in vivo* animal

* Corresponding authors. S. J. Kim is to be contacted at School of Electrical Engineering and Computer Science, Seoul National University, San 56-1 Shinlim-Dong Kwanack-Gu, Seoul, Korea. Tel.: +82 2 880 1812; fax: +82 2 882 4658; S. J. Hwang, Department of Oral & Maxillofacial Surgery, College of Dentistry, Seoul National University, 28 Yeongun-Dong, Chongro-Gu, Seoul 110-749, Korea. Tel.: +82 2 2072 3061; fax: +82 2 766 4948.
E-mail addresses: kimsj@snu.ac.kr (S.J. Kim), sjhwang@snu.ac.kr (S.J. Hwang).

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studies, electric energies have been used clinically to treat fresh fractures and osteotomies, spine fusions, and delayed and nonunion fractures [16–18]. These preclinical and clinical studies demonstrate that electrical energy is an important tool for enhanced bone formation.

Electrical stimulation can be done invasively using implants. In this case, the electrical current can be injected as a constant or as a pulsatile type, which can be either monophasic or biphasic. On the other hand, electrical stimulation can be conducted noninvasively using inductive or capacitive coupling. Each of these types of electric currents has some shortcomings in its application. It was suggested that electromagnetic fields are not themselves carcinogenic but promote tumor progression via increased angiogenesis, although clinical data suggest that PEMF is safe [19,20]. In addition, electric stimulation using PEMF requires large equipment and space for application. Animal studies and clinical applications with direct current injection are restricted because of the risk of postoperative infection with the surgical implantation of electrodes [21]. Moreover, new bone formation is limited to areas near the electrode tip and does not cover the wide extent of the damaged or fractured bone tissue. However, this feature can be advantageous when treating a restricted region such as a dental implant because a continuous stimulation in a specific restricted area can maximize the efficacy of bone formation without being limited by space and the burden of large equipment.

Whereas the *in vitro* and *in vivo* effects of PEMF have been well studied, the effect of direct current injection on the function of osteoblasts has scarcely been investigated. Most previous studies used constant type stimulation [9,22–24] and only a few employed pulsatile stimulation [25,26]. Constant direct current stimulation has shown several problems. During the electrical stimulation, charged proteins in the supernatant can accumulate on the surfaces of electrodes of opposite charge, and this accumulation can impede the flow of current at those locations. This local change of impedance can cause cells to receive inconstant current. In addition, during electrical stimulation, faradic products including hydrogen peroxide, hydroxyl and oxygen ions, free radicals, or other intermediates can be created [27]. It has been reported that reactive oxygen species, such as hydrogen peroxide, can indeed modulate bone resorption *in vitro* [28,29]. Moreover, constant direct current results in increased media pH. Uncontrolled elevation of pH can be toxic to cells and tissues, although some studies have reported that elevated pH stimulates osteoblastic activity [24,30–32]. However, under stimulation using BEC, charged proteins do not accumulate on the surface of electrodes because the current is charge balanced. Therefore, the charges are modulated in a more continuous and regular fashion [33].

In the present study, we analyzed the effects of BEC as one type of pulsatile direct electrical current injection on the proliferation, differentiation and cytokine synthesis of rat calvarial osteoblasts *in vitro*. We built a custom-designed Integrated Circuit (IC) and *in vitro* culture system to satisfy these requirements for our study.

2. Materials and methods

2.1. Design and implementation of BEC system

We designed the biphasic current stimulator chip using a 0.8-μm high voltage complimentary metal-oxide semiconductor fabrication process at Austria Micro-systems Corporation. The circuit configuration to form the biphasic pulses is shown in Fig. 1A. Briefly, the complementary D1 and D2 signals are delivered to switches M1 to M4, while another switch, M5, is turned on by the D3 signal (D1–D3 were designated as input signals and M1–M6 were designated as transistor switches in the following text). The current capability of the stimulator is precisely controlled by the geometry of M6 and by the amplitude of the bias voltages applied to its gate terminal. M1 and M2 are p-type transistors while M3 and M4 are n-type transistors. During the first phase of a period, the D1 signal turns on both the M1 and M3 switches, while the M2 and M4 switches stay off and the electric current flows from the channel to the reference electrode. This forms the positive phase of the biphasic current. During the second half of the period, the D1 signal turns off M1 and M3, while the D2 signal turns on M2 and M4, and the current flows in the reverse direction, from the reference to the channel electrode, forming the negative phase.

A culture dish was designed such that osteoblasts could be cultured on its conductive surface with electrical stimulation. The dish needed to contain electrodes for electrical stimulation while maintaining biocompatibility. To accomplish these goals, only materials known to be biocompatible were used for this system. As shown in Fig. 1B, individual culture wells were made with Teflon®, a material well known for its excellent chemical endurance, biocompatibility and mechanical stabilities. Gold was chosen as a conductive culture substrate material because of its several properties of biocompatibility, excellent cell attachment, and high conductivity. Six wells were made with their respective anodes and cathodes connected to form an electrical shunt configuration, as shown in Fig. 1C. The electrodes in the culture dish were connected to the biphasic current stimulator chip.

The electrodes were gold-deposited on 30-mm × 30-mm silicon plates. To enhance adhesion of the 3000-Å thick Au film to the silicon plate, a 300-Å thick titanium layer was deposited. The upper plates were used as cathodes while the lower plates were used as anodes. These wells and gold deposited glass plates were cleaned in 4:1 H_2SO_4:H_2O_2 solutions for 10 min, and then rinsed in acetone and methanol solvent for 10 min. The culture dish was then washed in 70% ethanol and deionized water overnight before being autoclaved for 1 h.

The fabricated biphasic current stimulator chip is presented in Fig. 2A. An electrostatic discharge protector was built at all input and output pads of the chip to protect the circuit from external shock or electrostatic discharge. The chip can deliver biphasic stimulation currents with amplitudes ranging from 2 μA to 1024 μA in 2-μA steps, and with pulse duration varying from 16 μs to 496 μs in 16-μs steps. The stimulation rate can be changed from 10 Hz to 3000 Hz with 64 levels. Fig. 2B shows the captured biphasic current waveform induced between the channel electrode and the reference electrode with a 20-μA amplitude and 32-μs duration at 3000 Hz. The amplitude, duration and pulse rate were precisely controlled by setting the pad connection.

2.2. Preparation of rat calvarial osteoblasts

Rat calvarial osteoblasts from Sprague–Dawley rats were isolated by sequential collagenase digestion. The cranial bone from 1-day-old rats was submerged in sterile PBS and chopped into pieces in a solution containing 0.1% Collagenase type I (Worthington Biochemical Corporation, USA) and 0.2% Dispase (Boehringer Mannheim, Germany). The calvarial pieces were subjected to collagenase digestion two times at 37 °C for 30 min. Cells from the first two digestions were washed, pelleted, and plated at a density of 2×10^6 cells/90-mm culture dish in minimal essential media modified form (α-MEM; JBI, Korea), supplemented with 10% heat inactivated fetal bovine serum (HIFBS), 100 unit/ml penicillin G and 100 μg/ml streptomycin (GIBCO BRL, USA) under cell culture conditions of 37 °C in a 5% humidified CO_2 air environment. The media were changed at every 3–4 days, and the cells were