Original Article

Comparison of Low-Intensity Pulsed Ultrasound and Pulsed Electromagnetic Field Treatments on OPG and RANKL Expression in Human Osteoblast-like Cells

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ABSTRACT

Objective: To compare two clinically applied treatments to stimulate bone healing—low-intensity pulsed ultrasound (LIPUS) and pulsed electromagnetic field (PEMF)—for their effects on RANKL and OPG expression in osteoblast-like cells in vitro.

Materials and Methods: LIPUS or PEMF was applied to Saos-2 cells for 10 minutes or 3 hours. RANKL and OPG expressions were analyzed at 0, 4, 8, or 12 hours after treatment with real-time PCR. Secreted protein levels in culture supernatant were analyzed at the same posttreatment time points using specific ELISA assays.

Results: Neither LIPUS nor PEMF had an effect on RANKL protein expression. OPG protein was significantly increased by LIPUS after 0 and 4 hours (brief short-term effect) and was increased almost 2.5-fold by PEMF after 8 hours. The mRNA levels of OPG and RANKL were hardly affected by LIPUS treatment at any time point. PEMF induced a fivefold increase in RANKL mRNA expression at $t = 0$. A brief PEMF treatment of 10 minutes resulted in downregulation of RANKL expression after 0 and 4 hours and upregulation at 12 hours. OPG mRNA was downregulated after 8 hours.

Conclusion: The effects of LIPUS or PEMF expression on OPG and RANKL are limited. From our experiments, it seems that LIPUS treatment resulted in a quick protein response, while the response of cells to PEMF (3 hours) was delayed. The increase in OPG protein at 8 hours post PEMF treatment is indicative of reduction of osteolysis. (*Angle Orthod.* 2010;80:498–503.)

KEY WORDS: Bone turnover; Osteoblast; RANKL; OPG; LIPUS; PEMF

INTRODUCTION

One of the main goals after maxillofacial surgery, for example, distraction osteogenesis, is to achieve good bone growth. Fracture healing after distraction osteogenesis is a dynamic process that can be influenced by biophysical stimulation to enhance bone regeneration.[1–3] Bone consists of an osteoid matrix synthesized by osteoblasts. During its turnover, which is an ongoing process, osteoclasts (OCs) remove old bone and osteoblasts deposit new bone. OCs are derived from osteoclast-precursor cells, which mature toward OCs after binding of receptor activator of nuclear factor κ B Ligand (RANKL) to its receptor in the OC-precursor cell membrane, receptor activator of nuclear factor κ B (RANK). Osteoprotegerin (OPG) is a soluble form of RANK and acts as scavenger of RANKL, thus inhibiting OC-precursors to become mature OC. Both RANKL and OPG are synthesized by osteoblasts. The balance between RANKL and OPG expression determines whether bone is formed or removed.[4–7] Two methods of biophysical treatment to enhance bone regeneration have been used clinically. Low-intensity pulsed ultrasound (LIPUS) and pulsed electromagnetic field (PEMF) both have been found to accelerate bone

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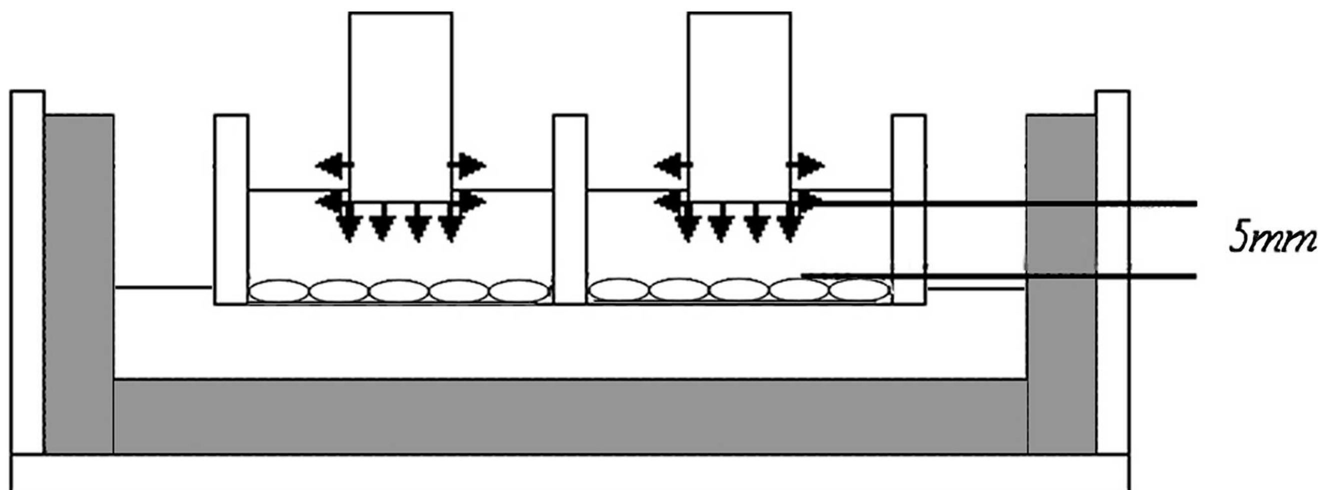

Figure 1. LIPUS exposure assembly. The culture plate filled with medium was positioned floating on water (37°C) with the transducers positioned 5 mm from the cells. The tank was covered with ultrasound-absorbing rubber. LIPUS was applied for 10 minutes.

maturation in distraction osteogenesis in animal models[8,9] and in clinical studies.[8,10,11]

In vitro osteoblasts can be stimulated by LIPUS or PEMF to increase OPG expression,[12,13] but which method is more effective is not known. In this study, we compared LIPUS and PEMF treatment of human osteosarcoma cells, Saos-2, for their effects on both mRNA expression and secreted protein levels of RANKL and OPG.

MATERIALS AND METHODS

Cell Line and Cell Culture

Human osteoblast-like Saos-2 cells were cultured in small tissue culture flasks for the PEMF experiment and on six well culture plates for the LIPUS experiment. The cells were seeded at 5×10^5 cells per flask/plate and were cultured until 80% confluence was attained in Dubeccos Modification of Eagles Medium (DMEM) w/o phenol red (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (both from Invitrogen bv, Breda, The Netherlands) in a humidified incubator at 37°C and 5% CO_2. To avoid the effects of fresh serum on gene induction, medium with 1% FBS was used 2 days before the experiment was begun.

LIPUS Exposure

A four-channel customized ultrasound unit with the same specifications as the Sonic Accelerated Fracture Healing System (SAFHS) (Exogen, Piscataway, NJ) previously described by Schortinghuis et al[14] was used. The specifications were as follows: pulse repetition frequency: 1 kHz, duty cycle: 20%, effective radiating area of transducer: 1.42 ± 0.11 cm^2, collimated beam. The temporal average power was set to 43 ± 6 mW, giving an effective intensity of 30 ± 6 mW/cm^2. The beam nonuniformity ratio was 2.3. A tank filled with distilled and demineralized water at 37°C was used for the experiment (Figure 1). The inside of the tank was covered with ultrasound-absorbing rubber. The six well culture plates with cell cultures were floating on the water surface. The setup (water bath and transducer head with stand) was placed in a sterile downflow cabinet. Before each experiment, the transducer heads were sterilized with 70% alcohol; they subsequently were lowered into the culture wells so that they just touched the surface of the medium. The distance between the transducer and the cells was 5 mm. LIPUS was applied for 10 minutes. Then the cells were incubated in a cell incubator at 37°C with 5% CO_2 for 0, 4, 8, or 12 hours. Duplicates of wells were treated for each time point, and controls were sham-treated before incubation. All experiments were repeated three times.

Electromagnetic Field Exposure

A pulsed electromagnetic field generator (Physio-Stim, Orthofix Inc, McKinney, Tex) was used for stimulation (Figure 2). The magnetic field waveform consists of bursts of triangular pulses with a pulse frequency of 3.8 kHz, a burst duration of 5.56 ms, and a burst on-off period of 67 ms. The resulting burst on-off frequency is 15 Hz. The maximum amplitude of the magnetic field was approximately 2 mT (20 G). Electromagnetic field stimulation was applied for 10 minutes or 3 hours. The culture flasks were placed in central position of the generator. After PEMF stimulation, the cells were cultured for 0, 4, 8, or 12 hours. Duplicates of wells were treated for each time point, and controls were sham-treated before incubation. Each experiment was repeated three times.

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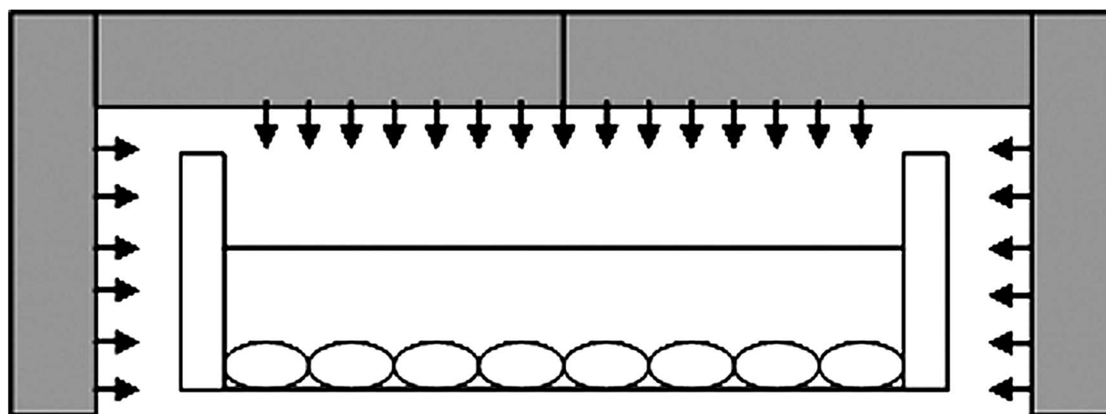

Figure 2. PEMF exposure assembly. The flask filled with medium was placed inside the PEMF machine. PEMF was applied for 10 minutes or 3 hours.

RNA Isolation and Real-Time PCR

Total RNA was isolated from cell cultures using the InVisorb Spin Cell RNA Mini Kit (InVitek, GmbH, Berlin, Germany), while following the manufacturer's instructions. The quantity and purity of RNA were determined using a spectrophotometer (NanoDrop, Wilmington, Del). RNA was reverse-transcribed using the I-script reverse transcription kit (Bio-Rad Inc, Hercules, Calif) while following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) amplification of cDNA was performed using the Sybr green mix from Abgene (Westberg bv Leusden, The Netherlands) and specific oligonucleotide primers (Table 1). The real-time PCR parameters were as follows: 95°C for 15 minutes, then 40 cycles at 95°C for 15 seconds, 52°C –60°C for 15 seconds, and 72°C for 15 seconds. Data were analyzed using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen,[15] using the housekeeping genes *GAPDH* and *β-actin* to calculate the ΔCT, and using the control at each time point to calculate the $\Delta\Delta$CT.

Enzyme-Linked Immunoabsorbent Assay (ELISA) for the Detection of RANKL and OPG

After exposures with LIPUS or PEMF, medium samples were collected at the different time points and used for OPG or RANKL detection at 1:10 dilution. ELISA assays for RANKL and OPG were performed with the Human sRANKL ELISA Development Kit (PeproTech, London, UK) and the OPG ELISA kit (R&D systems Europe, Ltd, Oxon, UK), respectively.

Statistical Analysis

Experiments were repeated three times. Statistical significance of differences among means was determined by one-way analysis of variance (ANOVA) and the post hoc Tukey-Kramer multiple comparisons test, electing a significance level of .05.

RESULTS

Osteoblasts were subjected to LIPUS for 10 minutes or PEMF for 10 minutes or 3 hours and then were returned to static culture for 0, 4, 8, or 12 hours, after which the culture medium was collected for ELISA and RNA was isolated from the cells.

RANKL and OPG mRNA Expression After 10 Minutes of LIPUS (Figure 3A)

RANKL mRNA expression was enhanced 1.7× by LIPUS at 4 hours posttreatment compared with expression at $t = 0$ ($P < .05$). After prolonged culturing,

Table 1. Oligodeoxynucleotide Primers Used for Real-Time PCR

Target cDNA	Primer Sequence (5′→3′)	Product Size, bp	Annealing Temperature, Celsius
β-Actin[a]	CACCACACCTTCTACAATGAG GTCTCAAACATGATCTGGGTC	118	52
GAPDH[a]	ACTTTGTGAAGCTCATTTCCTGGTA GTGGTTTGAGGGCTCTTACTCCTT	107	54
OPG[a]	GCAGCGGCACATTGGAC CCCGGTAAGCTTTCCATCAA	69	60
RANKL[a]	AGAGCGCAGATGGATCCTAA TTCCTTTTGCACAGCTCCTT	180	56

[a] Purchased from Osimum Biosolutions, IJsselstein, The Netherlands.

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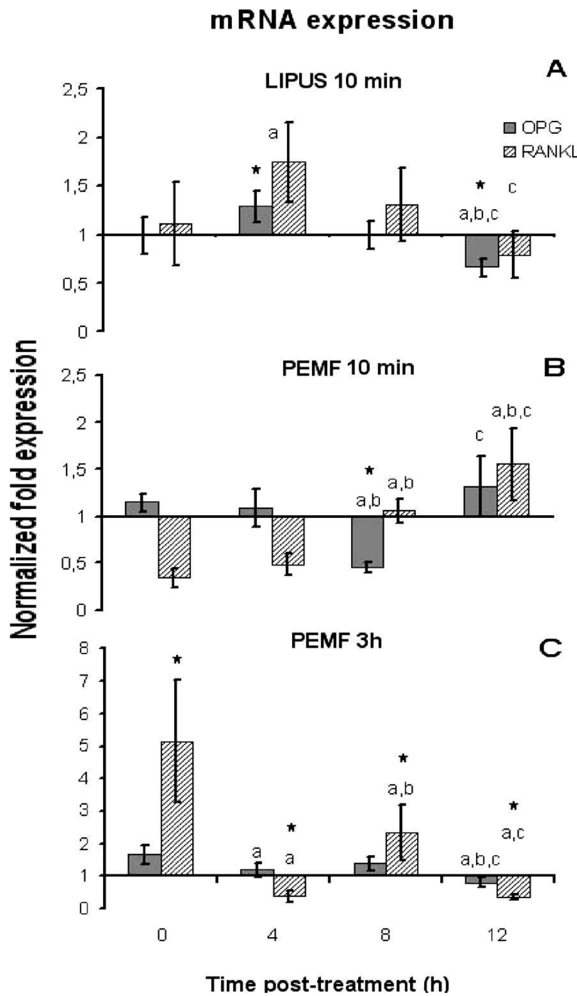

Figure 3. Expression of mRNA of RANKL and OPG normalized to GAPDH and β-actin after (A) 10 minutes of LIPUS, (B) 10 minutes of PEMF, or (C) 3 hours of PEMF. ([a] Significant difference from t = 0; [b] significant difference from 4 hours; [c] significant difference from 8 hours ($P < .05$) * Significant difference from control ($P < .05$).

the RANKL expression declined to the control level or slightly below. The difference in RANKL expression between 8 hours (1.3× control level) and 12 hours (0.8× control level) was statistically significant ($P <$ .05). OPG mRNA expression was not increased immediately after LIPUS but was 1.3× the expression of the control at 4 hours after LIPUS. RANKL mRNA level was 0.8× control level after 12 hours; this was significantly lower than the level at 4 hours ($P < .05$).

RANKL and OPG mRNA Expression After 10 Minutes of PEMF (Figure 3B)

Compared with the untreated control samples, RANKL mRNA expression was decreased at $t = 0$ and at 4 hours after treatment. At 8 hours after treatment, RANKL expression was back at the control level, and at 12 hours this treatment resulted in a 1.5× increase in expression.

OPG mRNA levels were hardly affected by PEMF for 10 minutes. Only at 8 hours, the OPG mRNA level was significantly reduced (0.4× the control value; $P <$ .05 compared with $t = 0$, $t = 4$ hours, and $t = 12$ hours).

RANKL and OPG mRNA Expression After 3 Hours of PEMF (Figure 3C)

Compared with the control samples, RANKL mRNA expression was at all time points significantly different: 0 hours: 5.2×; 4 hours: 0.4×; 8 hours: 2.3×, and 12 hours: 0.4×.

The OPG mRNA expression at $t = 0$ was significantly higher compared with control (1.7×). At 4 hours, the level of OPG mRNA was comparable with the untreated control and remained so until 12 hours after treatment.

Protein Levels of OPG and RANKL (Figure 4)

RANKL and OPG protein levels were assessed in the collected culture media by sandwich-ELISA. RANKL protein levels revealed no change after LIPUS exposure. The OPG protein levels after 10 minutes LIPUS exposure at $t = 0$ and 4 hours were significantly higher compared with the control (0 hours: 1.7×; 4 hours: 1.4×) (Figure 4A). After 10 minutes of PEMF exposure, no change in RANKL or OPG protein levels was noted (Figure 4B). After 3 hours of PEMF, no change in RANKL protein levels was seen. OPG protein expression did not increase immediately but was 2.5× the expression of the control at 8 hours (Figure 4C).

DISCUSSION

In the present study, we investigated the expression of RANKL and OPG, two important regulators of bone remodeling, in human osteoblasts after the application of LIPUS or PEMF. For the LIPUS and PEMF treatments, we have chosen to use two devices that are common in clinical practice—the SAFHS for LIPUS and the Physio-Stim for PEMF.

Although OPG mRNA expression after LIPUS was 1.3× the expression of the control at 4 hours and 0.7× that at 12 hours, this might not be relevant biologically. The increase at 4 hours and the decrease at 12 hours were not reflected at the OPG protein levels. The RANKL mRNA expression of 1.7× the control was also not reflected at the protein levels. The high protein levels of OPG at $t = 0$ and at 4 hours post treatment were not expected and may have several explanations that warrant further research: LIPUS may have an effect on OPG mRNA stability, which in itself is sufficient to increase protein levels without extra

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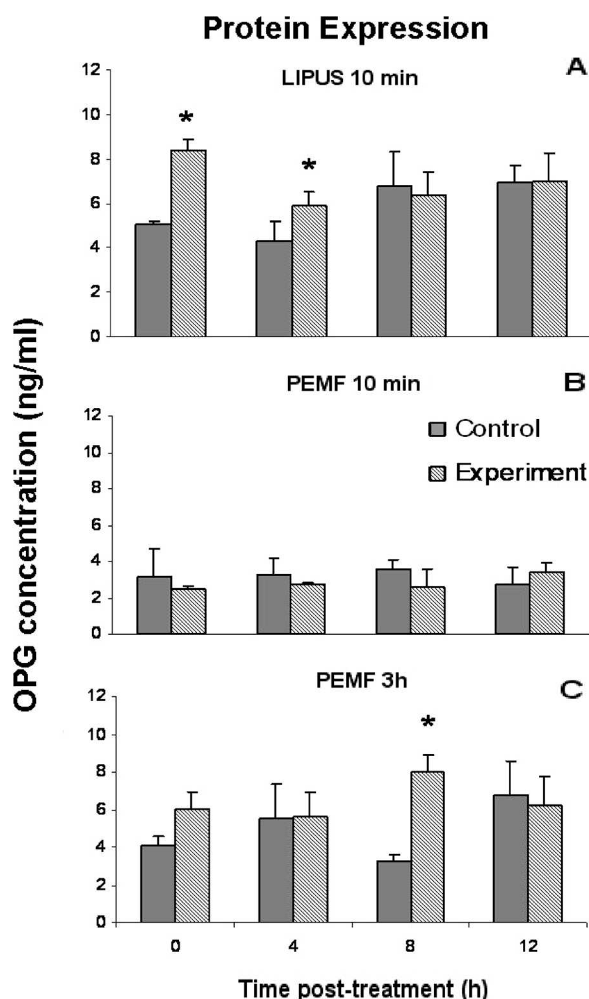

Figure 4. Expression of OPG protein after (A) 10 minutes LIPUS, (B) 10 minutes PEMF, and (C) 3 hours PEMF. (* Significant difference from control [$P < .05$]).

mRNA expression. 1α,25-dihydroxyvitamin D3 has been shown to do just the opposite and reduce the half-life of OPG mRNA.[16] Another explanation could be that OPG protein secretion is enhanced by the LIPUS treatment. Maddi et al[12] found that ultrasound could raise the levels of OPG mRNA at $t = 0$ and at 12 hours in MG63 osteosarcoma cells, and that the OPG protein levels were raised at 3 hours.[12] The RANKL mRNA levels did not differ significantly from those of the control, and the RANKL protein levels decreased at 6 hours and at 12 hours. Besides the other cell line, these authors used another ultrasound system, long-wave continuous ultrasound (45 kHz, intensity 30 mW/cm^2) for 5 minutes, which may explain the differences with our data.

To our knowledge, the present study is the first to describe the effects of PEMF on OPG and RANKL expression in human osteoblasts in vitro. Chang et al[13] showed that the levels of OPG mRNA in murine osteoblasts were enhanced after PEMF and the levels of RANKL mRNA were downregulated. Their study had a completely different design by stimulating cells during 8 h/d for 14 days. Also their PEMF stimulation differed from ours in the magnitude of the magnetic field. For the PEMF, we used two exposure times: 10 minutes of exposure to compare with the LIPUS exposure time and 3 hours as the normal exposure time for PEMF in the clinical setting.[17] For the downregulation of RANKL mRNA expression after 0 and 4 hours and the upregulation at 12 hours after a 10-minute treatment with PEMF, we have no plausible explanation. This finding may be biologically relevant when subsequent (each 6 hours) 10-minute treatments of PEMF manage to inhibit RANKL expression for a longer period. However, RANKL protein expression then should also be inhibited. This hypothesis requires further investigation.

The other significant change after 10 minutes of PEMF occurred at 8 hours. OPG mRNA was 0.4× the control level, but OPG protein did not change after 10 minutes of PEMF. On the other hand, 3 hours of PEMF resulted in significant changes. During the 3 hours of PEMF treatment, both mRNA and protein can be synthesized as well as degraded. So it is not surprising to find a strong stimulation of RANKL mRNA at $t = 0$ (5.2× the control). RANKL protein was never detected, possibly because of the detection limit of the assay, although this assay was used in other studies with human osteoblasts as well.[12,18] Schwartz et al[19] did not detect changes in RANKL protein level after application of PEMF. These authors used another ELISA assay for RANKL protein levels. This may suggest that PEMF does not mediate RANKL protein secretion.

OPG mRNA at $t = 0$ was also significantly enhanced (1.7× the control). OPG protein levels at 8 hours were 2.5× enhanced compared with $t = 0$, which again was not reflected by mRNA expression. Again, both mRNA stability and OPG secretion may have been affected, but mRNA levels for OPG may have been shortly raised between 4 and 8 hours.

This study did not show any effect on RANKL protein levels, but both LIPUS and PEMF modulate OPG protein expression. Our data on effects of LIPUS on OPG protein levels were corroborated by Dalla-Bona et al.[20] These authors demonstrated that ultrasound stimulation at 30 mW/cm^2 did not raise the protein levels of OPG in cementoblasts. Only with ultrasound stimulation of 150 mW/cm^2 was the protein level of OPG raised. In our study also, the OPG level was raised by PEMF, but only after 3 hours of stimulation. With 10 minutes of PEMF, the protein levels did not change. The RANKL protein levels in both studies were unaffected. Although direct comparison between cementoblasts and osteoblasts is not possible, in both

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studies a minimum stimulus was needed for the protein level of OPG to rise. In both studies there appeared to be a threshold in stimulation, either in the magnitude of energy (Dalla-Bona) or in the duration of treatment (the present study), before the OPG protein levels were raised.

CONCLUSIONS

- Both LIPUS and PEMF affect RANKL and OPG expression in osteoblast-like cells such that osteoclastogenesis can be expected to be reduced after LIPUS treatment at $t = 0$ and after 8 hours with PEMF treatment.
- Gene expression levels were not compatible with protein expression.
- The increase in OPG protein at 8 hours post PEMF treatment is indicative of reduction of osteolysis.

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REFERENCES

1. Fredericks DC, Nepola JV, Baker JT, Abbott J, Simon B. Effects of pulsed electromagnetic fields on bone healing in a rabbit tibial osteotomy model. *J Orthop Trauma*. 2000;14:93–100.
2. Duarte LR. The stimulation of bone growth by ultrasound. *Arch Orthop Trauma Surg*. 1983;101:153–159.
3. Claes L, Willie B. The enhancement of bone regeneration by ultrasound. *Prog Biophys Mol Biol*. 2007;93:384–398.
4. Khosla S. Minireview: the OPG/RANKL/RANK system. *Endocrinology*. 2001;142:5050–5055.
5. Boyce BF, Xing L. The RANKL/RANK/OPG pathway. *Curr Osteoporos Rep*. 2007;5:98–104.
6. Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res Ther*. 2007;9(suppl 1):S1.
7. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med*. 2006;12:17–25.
8. Zhu WQ, Wang X, Wang XX, Wang ZY. Temporal and spatial expression of osteoprotegerin and receptor activator of nuclear factor-kappaB ligand during mandibular distraction in rats. *J Craniomaxillofac Surg*. 2007;35:103–111.
9. El-Bialy TH, Elgazzar RF, Megahed EE, Royston TJ. Effects of ultrasound modes on mandibular osteodistraction. *J Dent Res*. 2008;87:953–957.
10. Eyres KS, Saleh M, Kanis JA. Effect of pulsed electromagnetic fields on bone formation and bone loss during limb lengthening. *Bone*. 1996;18:505–509.
11. Malizos KN, Hantes ME, Protopappas V, Papachristos A. Low-intensity pulsed ultrasound for bone healing: an overview. *Injury*. 2006;37(suppl 1):S56–S62.
12. Maddi A, Hai H, Ong ST, Sharp L, Harris M, Meghji S. Long wave ultrasound may enhance bone regeneration by altering OPG/RANKL ratio in human osteoblast-like cells. *Bone*. 2006;39:283–288.
13. Chang WH, Chen LT, Sun JS, Lin FH. Effect of pulse-burst electromagnetic field stimulation on osteoblast cell activities. *Bioelectromagnetics*. 2004;25:457–465.
14. Schortinghuis J, Ruben JL, Raghoebar GM, Stegenga B. Therapeutic ultrasound to stimulate osteoconduction: a placebo controlled single blind study using e-PTFE membranes in rats. *Arch Oral Biol*. 2004;49:413–420.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–408.
16. Kondo T, Kitazawa R, Maeda S, Kitazawa S. 1-Alpha,25 dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. *J Bone Miner Res*. 2004;19:1411–1419.
17. Midura RJ, Ibiwoye MO, Powell KA, et al. Pulsed electromagnetic field treatments enhance the healing of fibular osteotomies. *J Orthop Res*. 2005;23:1035–1046.
18. Evans CE, Galasko CS, Ward C. Effect of donor age on the growth in vitro of cells obtained from human trabecular bone. *J Orthop Res*. 1990;8:234–237.
19. Schwartz Z, Fisher M, Lohmann CH, Simon BJ, Boyan BD. Osteoprotegerin (OPG) production by cells in the osteoblast lineage is regulated by pulsed electromagnetic fields in cultures grown on calcium phosphate substrates. *Ann Biomed Eng*. 2009;37:437–444.
20. Dalla-Bona DA, Tanaka E, Inubushi T, Oka H, Ohta A, Okada H, et al. Cementoblast response to low- and high-intensity ultrasound. *Arch Oral Biol*. 2008;53:318–323.

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