Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

ARCHIVES OF ORAL BIOLOGY 54 (2009) 743–748

available at www.sciencedirect.com

journal homepage: www.intl.elsevierhealth.com/journals/arob

Anabolic effects of low-intensity pulsed ultrasound on human gingival fibroblasts☆

Nesrine Z. Mostafa [a,b], *Hasan Uludağ* [b,c,d], *Douglas N. Dederich* [a], *Michael R. Doschak* [b,d], *Tarek H. El-Bialy* [a,b,*]

[a] *Department of Dentistry, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada*
[b] *Department of Biomedical Engineering, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada*
[c] *Department of Chemical and Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, AB, Canada*
[d] *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada*

ARTICLE INFO

Article history:
Accepted 25 April 2009

Keywords:
Ultrasound
Acoustics
Gingival fibroblasts
Cell proliferation
Osteogenic differentiation
Periodontal regeneration

ABSTRACT

Objective: Low-intensity pulsed ultrasound (LIPUS) demonstrated anabolic effects on cementoblasts, odontoblasts, and periodontal ligament cells. However, LIPUS effect on human gingival fibroblasts (HGF) remains to be investigated. Therefore, we evaluated the *in vitro* effects of LIPUS on HGF proliferation and differentiation to test its feasibility for periodontal therapy.
Design: LIPUS treatment (1.5 MHz, 30 mW/cm^2) was applied to HGF in the experimental groups after 24-h of culture (5 or 10 min/day for 28 days) and omitted in the control. Changes in HGF activities were evaluated in response to LIPUS treatment in dose-dependent (5 and 10 min) and time-dependent (weeks 1–4) manner. The effects of LIPUS on HGF cell viability (MTT), proliferation (total DNA content and growth pattern), alkaline phosphatase (ALP) activity, and gene expression by reverse-transcriptase polymerase chain reaction (RT-PCR) were determined.
Results: Cell viability remained unchanged after LIPUS treatment during the 4 weeks of treatment as compared to the untreated control group which ensured a safe biological response. Both LIPUS treatments (5–10 min/day) did not yield any significant changes in the proliferation, and expression of proliferating cell nuclear antigen (PCNA) and collagen-I (COL-I). Conversely, LIPUS treatment enhanced osteogenic differentiation potential of HGF as determined by significant up-regulation of specific ALP activity and osteopontin (OPN) expression, with optimum effect following 3 weeks of 5 min/day LIPUS treatment.
Conclusion: LIPUS treatment at 30 mW/cm^2 selectively enhanced HGF differentiation but not proliferation. The ability of LIPUS to enhance HGF differentiation is promising for its application in cell-based periodontal therapy.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Ultrasound is a non-invasive therapeutic tool that is increasingly used to enhance bone fracture healing[1] and soft tissue repair.[2] LIPUS is an acoustic pulsed energy that demonstrated promising results in the dental field, as a form of non-invasive mechanical stimulation. Several *in vitro* studies demonstrated anabolic effects of different LIPUS intensities upon cementoblasts,[3] periodontal ligament and bone cells.[4] Clinical studies demonstrated the effectiveness of LIPUS at 30 mW/cm^2

☆ Based on a thesis submitted to the Faculty of Medicine and Dentistry, at the University of Alberta in partial fulfilment of the requirements for an MSc Degree.

* *Corresponding author at*: Faculty of Medicine and Dentistry, Room 4051C, Dentistry/Pharmacy Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2N8. Tel.: +1 780 492 2751; fax: +1 780 492 1624.
E-mail address: telbialy@ualberta.ca (T.H. El-Bialy).

0003–9969/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2009.04.012

Author's personal copy

intensity to accelerate periodontal wound healing and to increase cementum formation.[5] Moreover, it accelerated healing of orthodontically induced root resorption,[6] and enhanced bone formation at osteodistraction sites.[7–9] Recently, LIPUS at 30 mW/cm^2 significantly enhanced cellular proliferation, ALP activity and Col-I expression in cementoblasts *in vitro*,[3] which could explain the mechanism by which LIPUS accelerated repair of periodontal and root resorption defects *in vivo*.

Little is known about LIPUS effects on the proliferation and differentiation of gingival cells and concomitantly its possible application for periodontal regeneration. Two studies reported significant increases in cellular proliferation of ultrasound treated HGF at 0.7 and 1.0 W/cm^2 and in collagen production at 0.1–0.7 W/cm^2.[10,11] However, the ultrasound intensities used in those studies[10,11] might be harmful to the dental pulp when applied *in vivo* since relatively lower intensity LIPUS (30 mW/cm^2) applied 20 min/day for 4 weeks was shown to produce dental pulp fibrosis in humans.[6] Ultrasound at 1.0 W/cm^2 also showed deleterious effects on bone fracture healing and was not recommended for clinical trials.[12] Therefore, expanding the use of LIPUS on HGF for periodontal therapy requires more optimization on LIPUS application timing to avoid adverse effects associated with LIPUS overdosage during wound healing.

To understand the *in vitro* effects of LIPUS on HGF proliferation and differentiation, we treated HGF with LIPUS at 30 mW/cm^2 and evaluated the subsequent changes in HGF activities. The effect of LIPUS on HGF proliferation and ALP activity were determined. The latter is a membrane bound enzyme that peaks in activity immediately before the onset of mineralization and is considered an early osteoblastic marker.[13] Expression of PCNA (a molecular marker expressed in the nuclei of cells during DNA synthesis),[14] and the extracellular-matrix proteins Col-I and OPN (associated with matrix anabolism and mineralization respectively[13]) were further determined as a function of LIPUS application. In mineralized tissues, type I collagen interacts with various non-collagenous proteins and constitutes a scaffold for the subsequent growth of mineral crystals (mediated by alkaline phosphatase activity), whereas OPN expression indicates potential differentiation towards osteoblast-like cells and/or bone forming events.[13]

2. Materials and methods

2.1. *Human gingival fibroblasts*

HGFs were derived from gingival biopsies obtained from healthy interdental papilla. Informed consent was obtained from donors and protocol was approved by the Health Research Ethics Board, University of Alberta, Edmonton, Canada. Gingival samples were placed in 'biopsy' medium, which consisted of Dulbecco's Modified Eagle Medium (DMEM), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Gingival tissues, were then cut into small pieces, dispersed on glass slides, placed in culture plates, and incubated with basic medium (DMEM, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/L streptomycin) at 37 °C in a humidified atmosphere of 5/95% CO_2/air. When the cells surrounding the tissue explants became confluent after 2–3 weeks of culture, they were transferred to 75 cm^2 flasks using 0.08% trypsin/0.04% ethylenediaminetetraacetic acid (EDTA).

2.2. *LIPUS treatment*

HGF at passage 4 were plated in 48-well plates at 2.5×10^3 cells/well. LIPUS treatment was applied after 24 h in the experimental groups (5 or 10 min/day for 28 days) and omitted in the control. The LIPUS machine used was Exogen Bone Healing System (Smith & Nephew, Memphis, TN, USA), which produced 1.5 MHz pulsed ultrasound with an output intensity of 30 mW/cm^2. Satisfactory operation of LIPUS transducers was checked before each experiment. The plates were placed on the LIPUS transducer and contact was ensured using Exogen coupling gel. HGFs activities were assessed at days 7, 14, 21 and 28 as described below.

2.3. *Cell viability*

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used as a measure for cell viability. Briefly, 100 μL of MTT solution (dissolved in Hank's Balanced Salt Solution (HBSS) at 5 mg/mL) was added into each well containing cells with 0.5 mL basic medium. After 2 h, the medium was replaced with 500 μL of dimethylsulfoxide to dissolve the MTT formazan crystals formed. The absorbance was then quantified at 570 nm and used as a relative measure of cell viability.[15]

2.4. *Hexosaminidase assay*

A chromogenic substrate for the lysosomal enzyme hexosaminidase was used to estimate cell numbers.[16] The substrate for hexosaminidase enzyme, p-nitrophenol glucoseaminide (Sigma, St. Louis, MO, USA), was dissolved in citrate buffer, mixed with equal volume of 0.5% Triton X-100 in water and added to HGFs in flat bottom microtitre wells. After 4 h, the coloured product was formed and the enzyme activity was blocked by the addition of 50 mM glycine buffer with 5 mM EDTA. Absorbance was measured at 405 nm and a calibration curve prepared with different cell numbers was used to calculate cell number/well at each time point. The nonlinear equations were fitted to growth data by nonlinear regression using GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA). The specific growth rate was calculated in the exponential growth phase.[17]

2.5. *ALP assay*

ALP is a membrane bound enzyme that peaks in activity immediately before the onset of mineralization. ALP is considered as an early marker for osteoblast differentiation.[18] HGFs were washed with HBSS and lysed with ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton X-100; pH 10.5). Two hours after lysis, 100 μL of lysed solution (in duplicate) was added to 96-well plates, and 100 μL of 2 mg/mL ALP substrate p-nitrophenol phosphate (pNPP) (Sigma, St. Louis, MO) was added to lysed cells to give a final concentra-

Author's personal copy

tion of 1 mg/mL pNPP. The absorbance was monitored at 405 nm at periodic intervals for up to 20 min. The ALP activity was reported in terms of the p-NPP product formed (p-nitrophenol; in mmol/min/mL), and normalized with the DNA content to obtain a specific ALP activity (ALP/DNA). The DNA content of the lysates was determined using CyQUANT DNA kit (Molecular Probes, Portland, OR, USA) following the manufacturer's instructions.[19]

2.6. *RNA extraction and RT-PCR*

HGFs were harvested by using Trizol® Reagent and stored at −20 °C. The RNA was extracted using the RNeasy Mini Kit. Freshly isolated RNA was quantified fluorometrically using the RNA stain SYBR Green, and 0.3 μg of RNA was then used for reverse transcription reaction using the Omniscript kit.[19] The resulting cDNA was used as a template for the polymerase chain reaction amplification of the genes of interest: Col-I, PCNA, OPN and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with primers' sequence as follow:

GAPDH (158 bp[20])	Forward: 5′-CTGAACGGGAAGCTCACTGG-3′; backward: 5′-TAGCCCAGGATGCCCTTGAG-3′
COL-I (182 bp[20])	Forward: 5′-AGACACTGGTGCTAAGGGAGAG-3′; backward: 5′-GACCAGCAACACCATCTGCG-3′
PCNA (320 bp[14])	Forward: 5′-CTGCAGAGCATGGACTCGTC-3′; backward: 5′-GTAGGTGTCGAAGCCCTCAGA-3′
OPN (126 bp[21])	Forward: 5′-CCAAGTAAGTCCAACGAAAG-3′; backward: 5′-GGTGATGTCCTCGTCTGTA-3′

2.7. *Statistical analysis*

Results were expressed as mean ± standard deviation (S.D.). Means were analysed by one-way analysis of variance (ANOVA) using SPSS12.0 software package (SPSS, Chicago, IL, USA). The inter-group differences ($p < 0.05$) were determined by Boneferroni post hoc testing in accordance with the software instructions.

3. Results

3.1. *Effect of LIPUS on cell viability*

The effect of LIPUS on HGF viability was assessed by the MTT assay. Both LIPUS treatments (5 and 10 min/day) did not yield any obvious changes in cell viability as compared to untreated HGFs (Fig. 1A). The DNA content of LIPUS-treated and untreated HGFs showed gradual increase as a function of time without any significant differences amongst the study groups (Fig. 1B). The cell growth was investigated by plotting the cell numbers as a function of time (Fig. 2). Typical sigmoidal curves were obtained indicating the lag, exponential and stationary phases of growth.[22] As with the MTT results, there were no obvious changes in the HGF growth pattern as a result of LIPUS treatment. There were no apparent differences in the growth rates of LIPUS-treated HGFs as compared to the control: 12.4, 9.9, 10.8 h for control, 5 min/day LIPUS and 10 min/day LIPUS, respectively. The final cell numbers obtained at the end of cell expansion were also similar in all groups.

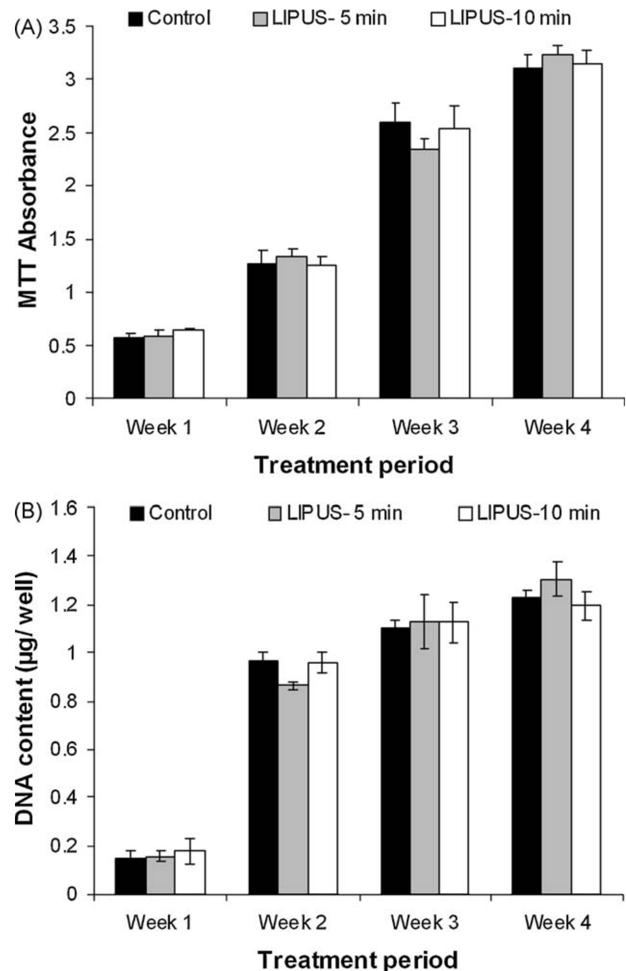

Fig. 1 – (A) Changes in MTT absorbance for HGF after 1–4 weeks of LIPUS treatment. Each point represents mean ± S.D. of triplicate wells. (B) Changes in the total DNA content after 1–4 weeks of LIPUS treatment. Each bar represents the mean ± S.D. of triplicate wells.

3.2. *Effect of LIPUS on specific ALP activity*

The specific ALP activity progressively increased with culture time in all groups (Fig. 3). ALP activity in the LIPUS-treated groups was similar to the control groups during the first 2 weeks of culture and was significantly higher after 3 weeks with 5 min/day LIPUS treatment as compared to other groups ($p < 0.05$). After 4 weeks, ALP activity was significantly higher in both LIPUS-treated HGF ($p < 0.005$ for 5 min/day and $p < 0.05$ for 10 min/day) as compared to the control group.

3.3. *Effect of LIPUS on the expression of PCNA, Col-I, and OPN by HGF*

The levels of mRNA expression for PCNA, Col-I, and OPN are shown in Fig. 4A and their relative intensities (normalized with GAPDH) are illustrated in Fig. 4B–D, respectively. The RT-PCR analyses for PCNA gene expression did not reveal any significant differences between LIPUS-treated and untreated

Author's personal copy

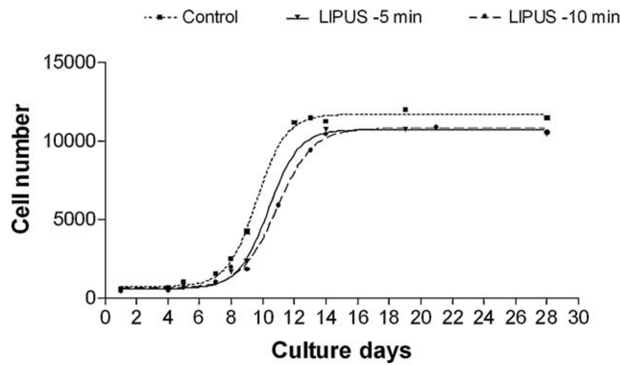

Fig. 2 – Changes in cell numbers of HGF with LIPUS treatments over a 28-day time period. Three phases of cellular growth: lag, exponential and stationary phases, were evident for each group. Each point represents the mean from six wells for each treatment group.

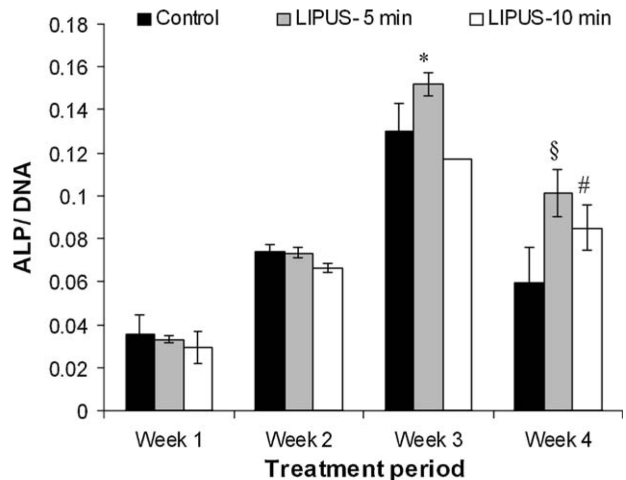

Fig. 3 – Changes in the specific ALP activity (ALP/DNA) of HGF after 1–4 weeks of LIPUS treatment. The results are presented as the mean ± S.D. of specific ALP activity of triplicate wells for each treatment group. (*$p < 0.05$ as compared to other groups, §$p < 0.005$ as compared to control, and #$p < 0.05$ as compared to the control).

HGF at any time point. Col-I expression was markedly increased in all groups after 2 weeks of culture; however there were no statistically significant differences at any time point between the different treatments. Alternatively, LIPUS at low dose (5 min/day) consistently induced significant up-regulation of OPN expression starting from week 2 ($p < 0.05$). The highest stimulation was observed for OPN at weeks 3 and 4 with 5 min/day LIPUS treatment compared to other groups ($p < 0.005$).

4. Discussion

LIPUS was shown to induce cellular responses in cementoblasts,[3] periodontal ligament and bone cells.[4] It has clinical potential related to healing of periodontal defects,[5] repair of

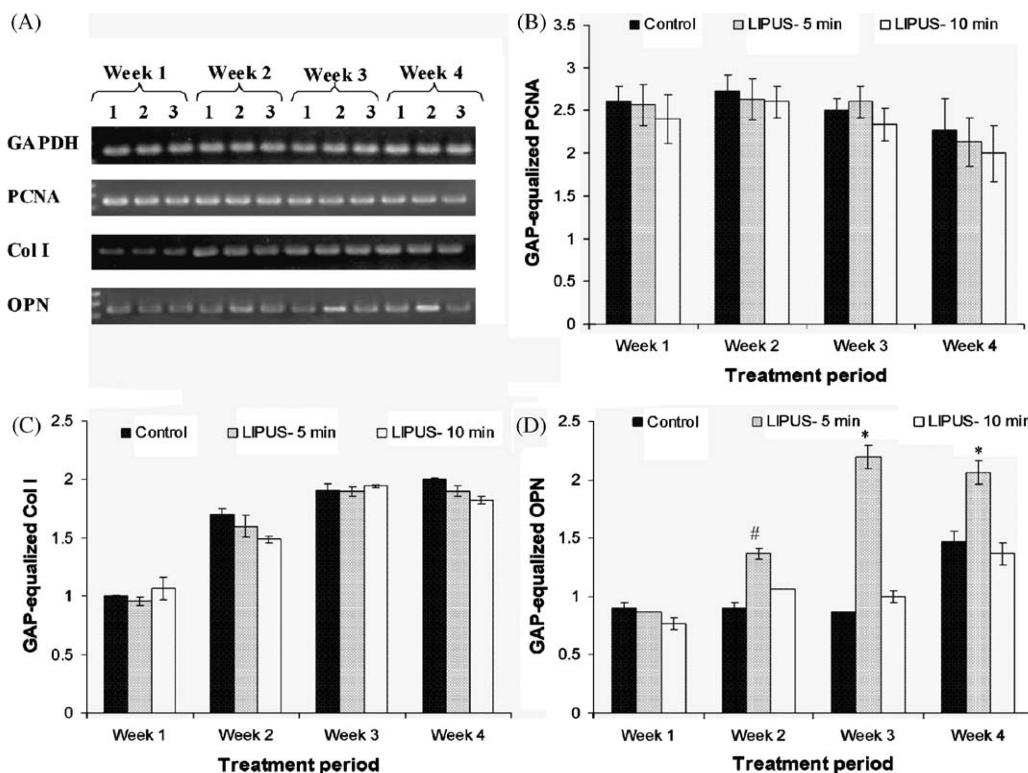

Fig. 4 – (A) Changes in expression of PCNA, Col-I, OPN, and GAPDH in HGF after 1–4 weeks of LIPUS treatment as analysed by RT-PCR and agarose gel electrophoresis. RT-PCR band from a representative well for each treatment group is shown. Densitometric analyses of PCNA (B), Col-I (C) and OPN (D) expression at weeks 1–4 after normalization with GAPDH (#$p < 0.05$ and *$p < 0.005$ as compared to other groups).

Author's personal copy

root resorption,[6] and bone formation at osteodistraction sites.[9,23] However, LIPUS effects on gingival cells and its applications for periodontal regeneration remain to be investigated. We evaluated the effectiveness of LIPUS treatment on HGF proliferation and osteogenic differentiation to test its feasibility for periodontal therapy. HGFs were selected as 'target' cells because they are accessible autologous cell sources that can be harvested with little donor-site morbidity. LIPUS frequency (1.5 MHz) and intensity (30 mW/cm^2) used in our study were identical to clinically applied ultrasound[5,6] and had a lower intensity than the studies yielding an anabolic effect on HGFs (Doan et al.[10]; Reher et al.[11]) to avoid deleterious effects reported at high intensities.[12] LIPUS doses used in our study (5 and 10 min/day) were adopted from a previous study on the effect of LIPUS on skin fibroblasts,[24] which reported significant increase in cell proliferation with 6 and 11 min/day of LIPUS treatment. Our rationale for using LIPUS doses lower than the clinical therapy was based on the concern about undesirable effects of LIPUS when applied for 20 min/day over 4 weeks on the dental pulp.[6]

LIPUS treatments should not inflict damage to HGFs during cell growth, as this could negatively affect cell proliferation and differentiation. Our results indeed showed that cell viability and growth pattern remained unchanged after LIPUS treatment during the 4 weeks of treatment, indicating the safety of the applied LIPUS doses. The proliferation of HGFs was assessed by evaluating growth rate, total DNA content and PCNA gene expression. These assays consistently indicated no significant differences in HGF proliferation with LIPUS treatments. It was reported that HGF treated with 5 min ultrasound at 0.7–1.0 W/cm^2 significantly increased DNA synthesis and collagen production, unlike our results.[10,11] The much higher ultrasound intensity used in those studies was likely the reason for the differences in the results. However, we did not explore such higher doses, given the deleterious effects reported at the 1.0 W/cm^2 intensity on bone fracture healing *in vivo*.[12] Zhou et al.[24] conversely, used similar LIPUS intensity, frequency and exposure technique as reported in our study, and obtained a significant increase in DNA synthesis of primary human skin fibroblasts. It is possible that primary skin fibroblasts are more responsive to LIPUS and display proliferation at this low LIPUS intensity unlike adult HGF used in our study.

The LIPUS treatment, conversely, enhanced the osteogenic differentiation of HGFs, based on increased specific ALP activity and OPN deposition. ALP is considered an early marker for differentiation of osteoblasts.[13] It plays a vital role in mineralization since the enzyme hydrolyses pyrophosphate and ATP that inhibit calcification and produce local phosphates needed for crystallization of hydroxyapatite. Our results are in agreement with a previous study, which reported significant increase in ALP expression in LIPUS-treated cementoblasts at 30 mW/cm^2 for 15 min.[3] OPN is an extracellular-matrix protein that is expressed in early stages of mineralization. It contains an arginine–glycine–aspartate motif, which binds to cell surface integrins and enables bone cells to adhere to mineralized matrix.[25] LIPUS effects on OPN expression by HGF was not reported before but Scheven et al.[26] reported a dose-dependent increase in the OPN gene expression by ultrasound treated odontoblasts. Moreover, mechanical stimulation (i.e., centrifugal force) was also shown to increase the levels of OPN expression in HGFs without any effect on osteonectin synthesis.[27] The authors suggested that the response of gingival tissue to mechanical stimulation is primarily osteoblastic activation, which is consistent with our findings. HGFs were also reported to express transcripts associated with mineralization (ALP and OPN) under normal culture condition[28] that could explain why these transcripts are expressed by HGFs in the control group. Therefore, LIPUS treatment at 30 mW/cm^2 and the current exposure technique may selectively enhance HGF differentiation but not proliferation.

To the best of our knowledge, this is the first study that demonstrated LIPUS effects on osteoblastic differentiation potential of HGF, as determined by up-regulation of ALP activity and OPN expression—although those events may be at different stages in different cells within the explanted HGF population. A robust effect on HGF cells was seen following 3 weeks of 5 min/day LIPUS treatment. Conversely, LIPUS did not stimulate cellular proliferation or Col-I gene expression by HGF. Establishment of these cellular responses with further optimization of LIPUS dose and application timing could stimulate further development of LIPUS-treated HGFs for periodontal regenerative therapies.

Acknowledgements

This research was supported by NSERC I2I project and a grant from the Faculty of Medicine and Dentistry, University of Alberta (Grant # N031000426). The authors declare this article is free of conflict of interest and there are no financial disclosures to be made. The authors would like to thank Professor Dr. Paul Scott for providing the HGF cells and for productive discussions. Authors also would like to thank Mrs. Carol Dodd for her technical help.

Competing interests: None declared

Funding: NSERC I2I project, Fund for Dentistry (Grant # 2007-08), and a grant from the Faculty of Medicine and Dentistry, University of Alberta (Grant # N031000426).

Ethical approval: Health Research Ethics Board, University of Alberta, File #6738.

REFERENCES

1. Parvizi J, Vegari D. Pulsed low-intensity ultrasound for fracture healing. *Foot Ankle Clin* 2005;**10**(4):595–608. [vii].
2. Uhlemann C, Heinig B, Wollina U. Therapeutic ultrasound in lower extremity wound management. *Int J Low Extrem Wounds* 2003;**2**(3):152–7.
3. 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**(4):318–23.
4. Harle J, Salih V, Mayia F, Knowles JC, Olsen I. Effects of ultrasound on the growth and function of bone and periodontal ligament cells in vitro. *Ultrasound Med Biol* 2001;**27**(4):579–86.
5. Ikai H, Tamura T, Watanabe T, Itou M, Sugaya A, Iwabuchi S, et al. Low-intensity pulsed ultrasound accelerates periodontal wound healing after flap surgery. *J Periodontal Res* 2008;**43**(2):212–6.

Author's personal copy

6. El-Bialy T, El-Shamy I, Graber TM. Repair of orthodontically induced root resorption by ultrasound in humans. *Am J Orthod Dentofacial Orthop* 2004;**126**(2):186–93.
7. Chan CW, Qin L, Lee KM, Cheung WH, Cheng JC, Leung KS. Dose-dependent effect of low-intensity pulsed ultrasound on callus formation during rapid distraction osteogenesis. *J Orthop Res* 2006;**24**(11):2072–9.
8. Chan CW, Qin L, Lee KM, Zhang M, Cheng JC, Leung KS. Low intensity pulsed ultrasound accelerated bone remodeling during consolidation stage of distraction osteogenesis. *J Orthop Res* 2006;**24**(2):263–70.
9. El-Bialy TH, Elgazzar RF, Megahed EE, Royston TJ. Effects of ultrasound modes on mandibular osteodistraction. *J Dent Res* 2008;**87**(10):953–7.
10. Doan N, Reher P, Meghji S, Harris M. In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. *J Oral Maxillofac Surg* 1999;**57**(4):409–19. [discussion 420].
11. Reher P, Doan N, Bradnock B, Meghji S, Harris M. Therapeutic ultrasound for osteoradionecrosis: an in vitro comparison between 1 MHz and 45 kHz machines. *Eur J Cancer* 1998;**34**(12):1962–8.
12. Tsai CL, Chang WH, Liu TK. Preliminary studies of duration and intensity of ultrasonic treatments on fracture repair. *Chin J Physiol* 1992;**35**(1):21–6.
13. Bosshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? *J Dent Res* 2005;**84**(5):390–406.
14. Pagliarulo V, George B, Beil SJ, Groshen S, Laird PW, Cai J, et al. Sensitivity and reproducibility of standardized-competitive RT-PCR for transcript quantification and its comparison with real time RT-PCR. *Mol Cancer* 2004; **3**:5.
15. Issa Y, Brunton P, Waters CM, Watts DC. Cytotoxicity of metal ions to human oligodendroglial cells and human gingival fibroblasts assessed by mitochondrial dehydrogenase activity. *Dent Mater* 2008;**24**(2):281–7.
16. Ilesanmi AO. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: applications to cell adhesion assays in endometrial and ovarian carcinoma cell lines. *Afr J Med Med Sci* 1997;**26**(1–2):87–9.
17. Zwietering MH, Jongenburger I, Rombouts FM, van't Riet K. Modeling of the bacterial growth curve. *Appl Environ Microbiol* 1990;**56**(6):1875–81.
18. Anderson HC. Mechanism of mineral formation in bone. *Lab Invest* 1989;**60**(3):320–30.
19. Varkey M, Kucharski C, Doschak MR, Winn SR, Brochmann EJ, Murray S, et al. Osteogenic response of bone marrow stromal cells from normal and ovariectomized rats treated with a low dose of basic fibroblast growth factor. *Tissue Eng* 2007;**13**(4):809–17.
20. Karner E, Unger C, Sloan AJ, Ahrlund-Richter L, Sugars RV, Wendel M. Bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. *Stem Cells Dev* 2007;**16**(1):39–52.
21. Shimonishi M, Hatakeyama J, Sasano Y, Takahashi N, Uchida T, Kikuchi M, et al. In vitro differentiation of epithelial cells cultured from human periodontal ligament. *J Periodontal Res* 2007;**42**(5):456–65.
22. Schaller G, Meyer-Hermann M. Continuum versus discrete model: a comparison for multicellular tumour spheroids. *Philos Transact A Math Phys Eng Sci* 2006;**364**(1843):1443–64.
23. Schortinghuis J, Bronckers AL, Stegenga B, Raghoebar GM, de Bont LG. Ultrasound to stimulate early bone formation in a distraction gap: a double blind randomised clinical pilot trial in the edentulous mandible. *Arch Oral Biol* 2005;**50**(4):411–20.
24. Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J Biol Chem* 2004;**279**(52):54463–9.
25. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. *FASEB J* 1993;**7**(15):1475–82.
26. Scheven BA, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ. Short-term in vitro effects of low frequency ultrasound on odontoblast-like cells. *Ultrasound Med Biol* 2007;**33**(9):1475–82.
27. Jeon YM, Kook SH, Son YO, Kim EM, Park SS, Kim JG, et al. Role of MAPK in mechanical force-induced up-regulation of type I collagen and osteopontin in human gingival fibroblasts. *Mol Cell Biochem* 2009;**320**(1–2):45–52.
28. Ivanovski S, Li H, Haase HR, Bartold PM. Expression of bone associated macromolecules by gingival and periodontal ligament fibroblasts. *J Periodontal Res* 2001;**36**(3):131–41.