LOW INTENSITY PULSED ULTRASOUND STIMULATES OSTEOGENIC DIFFERENTIATION OF HUMAN GINGIVAL FIBROBLASTS

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1. INTRODUCTION:

Low intensity pulsed ultrasound (LIPUS) is widely used as a therapeutic tool in medicine. Several cell types have been reported to be sensitive to LIPUS exposure, including bone cells [1], cementoblasts [2], and odontoblast-like cells [3]. Certain clinical applications of LIPUS treatment were also studied. LIPUS was shown to enhance healing of periodontal defects in animals [4], and the repair of orthodontically induced tooth root resorption in humans[5]. However, little is known about the effects of LIPUS on the proliferation and differentiation of human gingival fibroblasts (HGF) and its possible application in periodontal therapy. Only two studies showed significant increases in cell proliferation and collagen production by HGF with LIPUS [6,7]. However, the effects of LIPUS on HGF differentiation were not examined. Therefore, our research tested the in-vitro effects of LIPUS on HGF osteogenic differentiation as a new tool for periodontal therapy.

2. METHODS:

HGF cells were cultured in 48-well plates at $2.5x10^3$ cells/well. HGF received LIPUS treatment for 5 or 10 min/day for 28 days (1.5-MHz frequency and 30mW/cm2 intensity). LIPUS treated and untreated HGF were analyzed for different cell activities at weeks 1, 2, 3 and 4.

2.1. Cell viability assay:

The MTT (4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used as a measure of cell viability. Yellow MTT is reduced to purple formazan in the mitochondria of living cells, which is then quantified at $\lambda_{absorbance}$: 570nm. Cell survival after LIPUS treatment was expressed relative to untreated control HGF [8].

2.2. Differentiation assay (Alkaline phosphatase "ALP"):

ALP enzyme cleaves the phosphate group from ALP substrate p-nitro phenol phosphate to produce p-nitrophenol, which is measured at $\lambda_{absorbance}$: 405. ALP activity was reported in terms of the p-nitrophenol/ well and normalized to the DNA content (µg DNA/well) in each lysate to obtain specific ALP activity (ALP/DNA) [9].

2.3. Proliferation assay (total DNA content):

The remaining cell lysates from the ALP assay were subsequently used to measure DNA content. They were analyzed with the CyQUANT DNA kit following the manufacturer's instructions using a fluorescent plate reader ($\lambda_{excitation}$ at 480 nm, $\lambda_{emission}$ at 527 nm). [9]

2.4. Reverse-transcriptase polymerase chain reaction (RT-PCR):

Gingival fibroblasts were harvested using Trizol® Reagent. The total RNA was extracted using the RNeasy Mini Kit following the manufacturer's instructions. Then, RNA was quantified fluorometrically ($\lambda_{excitation}$: 480 nm, $\lambda_{emission}$: 527 nm). 0.3 µg of RNA was then used for the RT reaction using the Omniscript kit [9]. The resulting cDNA was used as a template for PCR amplification of osteopontin (OPN) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

3. RESULTS:

3.1. Cell viability:

Both LIPUS treatments did not affect cell viability at any time point.

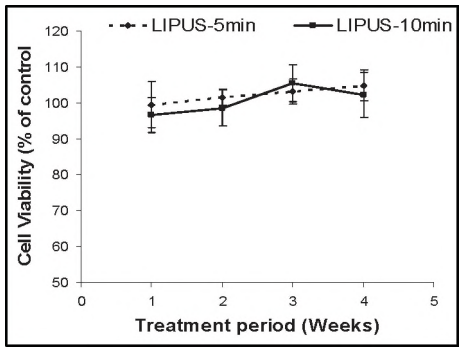

Figure (1): Cell viability for LIPUS treated HGF. Each point represents the percentage of the value to untreated control HGF.

3.2. Specific ALP activity (ALP\DNA):

ALP activity increased with 5min of LIPUS treatment compared to the other groups ($p<0.05$) by week 3. After week 4, ALP activity was increased with both LIPUS treatments 5 min ($p<0.005$) and 10 min ($p<0.05$) when compared to untreated HGF.

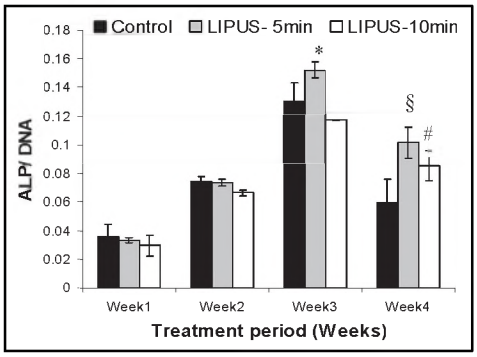

Figure (2): Changes in the specific ALP activity (*: p<0.05 as compared to other groups, §: p<0.005 and #: p<0.05, compared to the control).

3.3. Total DNA Content:

All treatments demonstrated an increase in total DNA content as a function of time, yet there were no significant differences between different treatments at any time point

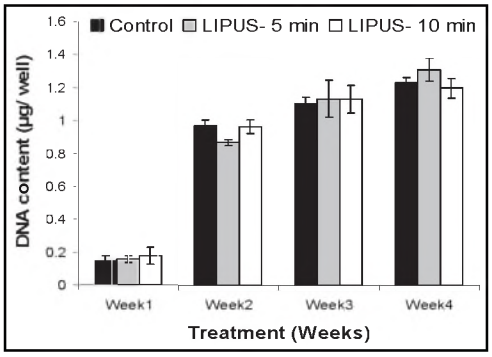

Figure (3): Changes in DNA content.

3.4. RT-PCR:

LIPUS treatment (5min/day) consistently induced significant upregulation of OPN gene expression compared to other groups starting from week 2 ($p<0.05$), with the highest stimulation observed at week 3 and 4 ($P<0.005$).

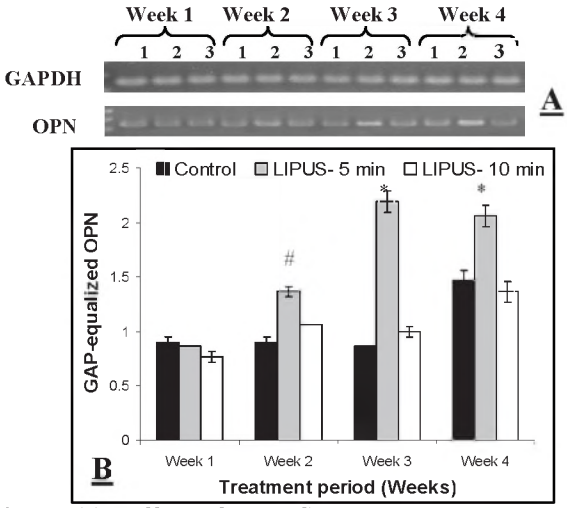

Figure (4): Effect of LIPUS treatment on expression of OPN in HGF, as analyzed by RT-PCR. A. cDNA levels of OPN for 1: control, 2: LIPUS- 5min, and 3: LIPUS-10min group at 1, 2, 3 and 4 weeks. B. Densitometric analyses of OPN expression for LIPUS treated and untreated HGF after equalization with GAPDH. (#: p<0.05 and *: p<0.005 as compared to other groups).

4. DISCUSSION

Our study is the first to examine the effects of LIPUS on the differentiation of HGF. LIPUS stimulation (5 min/day) enhanced the differentiation of HGF, as evidenced by the significant increase of ALP activity and OPN expression. ALP is considered an early marker for osteoblast [1] and cementoblast [2] differentiation. Interestingly, our results are consistent with other studies, which reported significant increases in ALP activity with LIPUS treatment in osteoblasts [1], cemetoblasts [2] and odontoblast-like cells [3]. OPN is an extracellular matrix protein that is expressed in the early stages of mineralized tissue development. LIPUS stimulation of odontoblast–like cells resulted in the significant increase of OPN expression [3]. Our results suggest that furthering the therapeutic usage of LIPUS stimulation would be beneficial for periodontal therapy.

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