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Biological effects of ultrasound stimulus on cells derived from human ovarian follicular liquid

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***Abstract*— Low-Intensity Pulsed Ultrasound Stimulus (LIPUS) accelerates the bone fracture healing in animal models and in clinical studies. In this work, according to the literature, we have chosen the mesenchymal stem cells (MSCs) as precursors of bony tissue, in particular the MSCs derived from the human ovarian follicular liquid (FL), and we have investigated the effects of ultrasounds on their proliferation. We tested two different durations of ultrasound stimulus (2 and 5 min) and compared these data to the control without ultrasound treatment. To quantify the proliferation of these putative MSCs, we used the BrdU incorporation assay: in comparison with the control, the results showed that 5 min of ultrasound stimulus significantly increased the percentage number of cells in intensive proliferative activity; on the other hand, there was no significant difference using 2 min of stimulation, hypothetically because the transmitted energy was not sufficient to stimulate the cells and to consequently enhance their proliferation. In conclusion, the effects of LIPUS on putative MSCs derived from ovarian follicular liquid show potential developments in biotech or medical applications.**

I. Introduction

According to clinical practice, it is possible to enhance the bone fracture healing by applying an electrical current or ultrasound waves. Low-Intensity Pulsed Ultrasound Stimulus (LIPUS) accelerates the fracture healing in animal models [1,2] and in clinical studies [3,4]. In 1996, the FDA also approved the application of LIPUS as treatment for fracture non-unions. A LIPUS device is simply placed onto the skin over the fracture with the advantages of non-invasiveness and of short treatments (20÷30 min per day).

In vitro studies showed that ultrasounds increase the influx of calcium into bone cells, and, consequently, they can modulate the production of nitric oxide, PGE_2, c-fos, COX-2, osteopontin, and osteocalcin [5-8]. Besides, *in vivo* studies suggest that LIPUS does not affect the remodeling phase of fracture healing, but rather the inflammation phase and the callus formation, the angiogenesis and the restoration of the mechanical properties, such as the torsional stiffness [2,9-12]. From the point of view of bone tissue engineering, works on human SAOS-2 osteoblasts reported that such physical stimuli caused higher proliferation in comparison with control conditions [13-16].

In previous studies [17,18], we derived mesenchymal stem cells (MSCs) from the human ovarian follicular liquid (FL) that is usually wasted during the *in vitro* fertilization (IVF). These cells were positive for mesenchymal stemness markers (e.g., CD-90, CD-44, CD-105), but not for epithelial proteins (e.g., cytokeratins), and were also negative for CD-34 and CD-45. We also tested the multipotency of FL cells by *in vitro* differentiation (e.g., osteoblastic differentiation). In this study, we investigated the effects of LIPUS on these putative MSCs in terms of proliferation activity.

II. Materials and Methods

A. Human ovarian follicular liquids

By transvaginal ultrasound-guided aspiration, ovarian follicular liquids were collected during oocyte retrieval from 5 women of heterogeneous age (33.4 ± 4.7 years) (Prot. # 20080002153, Bioethics Committee of IRCCS Fondazione Policlinico San Matteo of Pavia). After the removal of the cumulus oophorous-oocyte complexes, follicular aspirates were centrifuged in density gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway) for 30 min at 1800 rpm in order to eliminate red blood cells and debris. In the middle layer is clearly visible the buffy coat containing the follicular liquid cells, which is recovered, twice washed in 10 ml of sterile PBS, and centrifuged for 10 min at 1200 rpm for the final cell collection.

B. Cell cultures

FL cells were seeded onto Petri dishes at 2.5×10^5 cell concentration in each Petri. Cells were grown in DMEM culture medium (Sigma-Aldrich, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, and 100 µg/ml streptomycin (EuroBio, France). Cultures were maintained at 37°C and 5% CO_2. After 48 h, non-adherent cells were discarded and the culture medium was changed twice a week.

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C. Ultrasound stimulus

After 24 h from cell seeding, for 4 days, we applied the ultrasound stimulus (average power, 149 mW; frequency, 1.5 MHz; FAST, Igea, Carpi, Italy): 2 min per day (i.e. transmitted energy equal to 17.88 joule per day) and 5 min per day (i.e. transmitted energy equal to 44.70 joule per day).

D. DNA synthesis by BrdU incorporation

The DNA synthesis was analyzed by measuring the BrdU incorporation at the end of the culture period. Briefly, cells were labeled by adding 30 µM BrdU (Sigma-Aldrich) to the medium during the last hour of culture. Then, the cells were washed in PBS and fixed in 70% ethanol. Incorporated BrdU was detected by immunostaining with anti-BrdU antibody. Briefly, the cells were washed with PBS and incubated in 2 N HCl for 30 min at room temperature. Samples were neutralized in 0.1 M sodium tetraborate (pH=8.5) for 15 min, washed in PBS for 15 min, and incubated for 20 min in PTA blocking solution (1% BSA and 0.02% Tween 20 in PBS). Cells were then incubated for 1 h with mouse anti-BrdU antibody (diluted 1:100 in PTA) (Amersham Bioscience), washed three times in PTA, and then incubated for 30 min with anti-mouse FITC-conjugated antibody (diluted 1:100 in PTA) (Sigma-Aldrich). Then, the cells were extensively washed in PBS, counterstained for DNA with 0.5 µg/ml Hoechst 33258 (Sigma-Aldrich), and mounted in Mowiol (Sigma-Aldrich). Finally, the cells were scored for immunofluorescence positivity with a fluorescence microscope (Zeiss Axiophot, Germany).

E. Statistics

Percentage numbers of cells in intensive proliferative activity are expressed by mean ± standard deviation (sample number=5). One-way Analysis of Variance (ANOVA) was performed to find statistically significant differences between the treatments studied (control or non-stimulated cultures, LIPUS stimulated cultures for 2 min and for 5 min). Bonferroni test was applied to perform multiple comparisons (p-value<0.05 was considered significant).

III. Results

To study the proliferation of the MSCs, at the end of the culture period, the cells were incubated for 1 h in the presence of BrdU, fixed, and immunostained (Fig. 1). The percentage number of cells showing intensive proliferative activity was 11.26 ± 5.19% in non-stimulated control cultures, 15.60 ± 1.70% in LIPUS stimulated cultures for 2 min, and 46.43 ± 15.04% in LIPUS stimulated cultures for 5 min (Fig. 2). Comparisons with Bonferroni test showed statistically significant differences between control and LIPUS stimulation for 5 min (p=0.003) and between the two LIPUS stimulations (p=0.021); on the other hand, the proliferation did not increase with the shorter ultrasound treatment.

IV. Discussion

Millions of fractures occur worldwide every year, with nearly 6.2 million fractures in the United States alone [19]. Bone fractures result from two general causes: trauma or pathological conditions. A trauma-induced fracture usually occurs when the normal range of loading is exceeded.

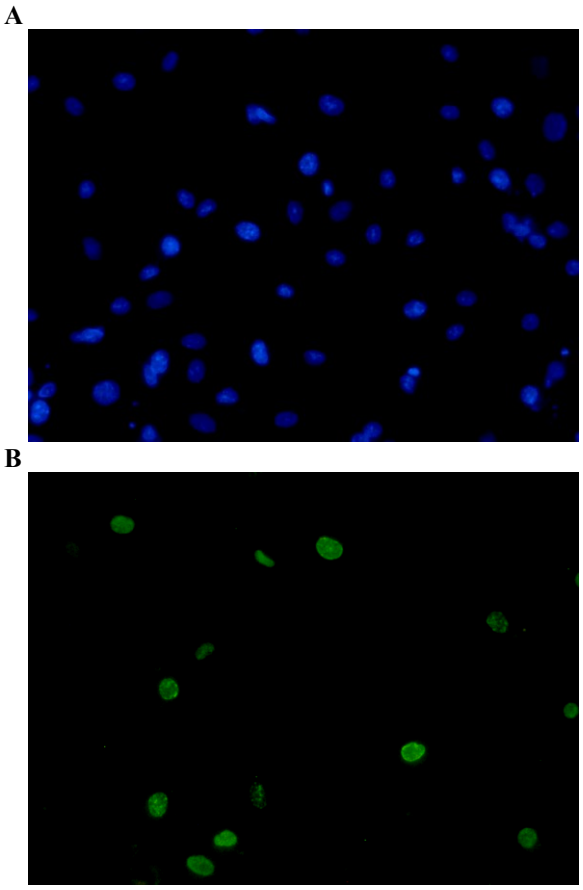

Figure 1. Proliferative activity of MSCs derived from human ovarian follicular liquid and stimulated for 5 min by LIPUS (*A*, cell nuclei in blue fluorescence; *B*, nuclei of proliferating cells in green fluorescence).

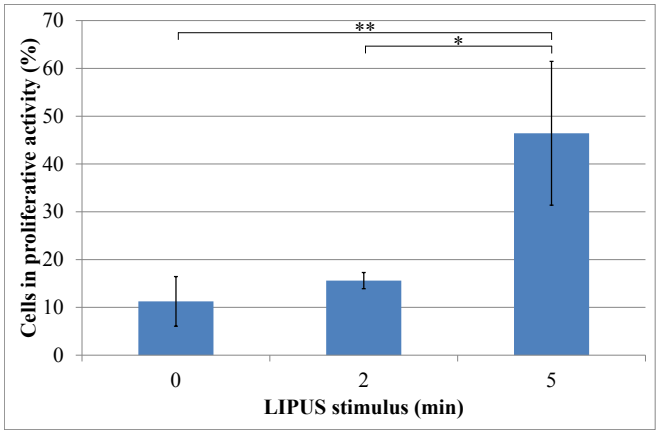

Figure 2. Percentage number of cells in proliferative activity (* p<0.05, ** p<0.01).

The other kind of fracture usually occurs under normal loading in a bone weakened by a disease (e.g., osteoporosis or tumors). In both cases, the continuity of the bone tissue is lost. When a fracture occurs, the bone has a self-healing mechanism to recover its mechanical function. Even if the clinical methods were improved over the last decades, 5÷10% of fractures still show delayed healing. Many of these

delayed healings persist for more than nine months and are termed non-unions. Many patients would benefit from improved methods to heal these non-unions and delayed unions.

One promising method is LIPUS. LIPUS generally entails a 20 min treatment per day (frequency, 1 MHz; average intensity, 30 mW/cm^2; pulse width, 200 ms). LIPUS transmits energy through and into living tissues. It has been theorized that the micromechanical strains produced by LIPUS pressure waves may result in biochemical events that regulate the fracture healing [20]. The ability of LIPUS to stimulate changes in tissues and in cells may be due to the temperature increase associated with energy absorption [21,22,23]. Changes observed after LIPUS treatment may also be associated with non-thermal processes such as acoustic streaming and cavitation [24,25]. Moreover, it is known that ultrasound stimulation can cause higher cell proliferation in human SAOS-2 osteoblasts [15].

Our previous studies [17,18] showed that FL cells have an interesting ability to differentiate into osteoblasts under appropriate stimulus. In this study, we used FL cells as model to investigate the ultrasound effects on proliferation.

Our results showed that the application of LIPUS for 5 min per day was sufficient to induce a significantly enhanced cell proliferation (Fig. 2). We also observed the inefficacy of 2 min of LIPUS. To explain this evidence, we would consider that the efficient ultrasound frequency is 1.5 MHz, but it is more crucial the amount of transmitted energy: during 2 min of LIPUS the transmitted energy is low and the cells do not activate the biochemical mechanisms to increase the proliferation, whereas the energy provided in 5 min is 2.5-fold the previous and the data showed an enhancement of cell proliferation.

The pilot study of this work opens perspectives. In fact, we strongly confirmed the possibility to use FL cells as model for *in vitro* studies thanks to their proliferation activity and differentiation potential. Moreover, we also suggest that LIPUS promotes the proliferation activity and not only the protein synthesis [26-28]. In the future, it will be certainly interesting to develop these observations in the field of biochemical and molecular analyses to better explain the effects induced by LIPUS.

Studies also suggest the use of this mechanical stimulus to promote the osteoblastic differentiation. We are going to develop a model of osteoblastic differentiation which includes a 3D scaffold (e.g., gelatin cryogel) designed to support the cell culture. FL cells grown on the biomaterial could be stimulated not only by appropriate culture medium, but also by means of a mechanical stimulus in order to obtain enhanced cell proliferation, cell differentiation, and extracellular matrix production.

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