Electromagnetically Stimulated SAOS-2 Osteoblasts inside a Porous Hydroxyapatite Scaffold *in Vitro*

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Abstract: Several studies suggest that the modification of a biomaterial surface play an important role in bone tissue engineering. In this study we have followed a biomimetic strategy where electromagnetically stimulated SAOS-2 osteoblasts, from a human osteosarcoma cell line, proliferated and built their extracellular matrix inside a porous hydroxyapatite scaffold. In comparison with control static conditions, the electromagnetic stimulus (magnetic field, 2 mT; frequency, 75 Hz) increased the cell proliferation and the production of bone proteins (decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen), with a consequent surface coating of the scaffold. The physical stimulus was aimed at obtaining a biomimetic modification of the internal porous surface of the hydroxyapatite scaffold. The cell-biomaterial construct could be used as an implant for bone repair in clinical applications.

1. *Introduction*

One of the key challenges in reconstructive bone surgery is to provide living constructs that possess the ability to integrate in the surrounding tissue. Bone graft substitutes, such as autografts, allografts, xenografts, and biomaterials have been widely used to heal critical-size long bone defects and maxillofacial skeleton defects due to trauma, tumor resection, congenital deformity, and tissue degeneration.

Specific biomaterials used to build 3D scaffolds for bone tissue engineering are hydroxyapatite [1], partially demineralized bone [2], and biodegradable porous polymer-ceramic matrices [3]. The preceding osteoinductive and osteoconductive biomaterials are ideal to follow a typical approach of tissue engineering, an approach that involves the seeding and the *in vitro* culturing of cells within porous scaffolds before the implantation [1-3].

To overcome the drawbacks associated with the standard culture systems, such as limited diffusion and inhomogeneous cell-matrix distribution, several bioreactors have been designed: a rotating vessel bioreactor [4], a perfusion bioreactor [5], or an electromagnetic bioreactor [6], for instance. The ideal feature of a bioreactor is the supplying of suitable levels of oxygen, nutrients, cytokines, growth factors, and appropriate physical stimuli (e.g. shear stress [5] or electromagnetic field [6]), to populate, with living bone cells and mineralized extracellular matrix, the volume of a porous biomaterial for reconstructive bone surgery: this living and biocompatible tissue-engineering construct could be implanted together with the insertion of a vascular pedicle [7].

Gorna and Gogolewski [8,9] have drawn attention to the ideal features of a bone graft substitute: it should be porous with interconnected pores of adequate size allowing for the ingrowth of capillaries and perivascular tissues; it should attract mesenchymal stem cells from the surrounding bone and promote their differentiation into osteoblasts; it should avoid shear forces at the interface between bone and bone graft substitute; and it should be biodegradable.

In this study, following the preceding "golden rules" of Gorna and Gogolewski, we have elected porous hydroxyapatite [10-12] as cancellous bone graft substitute and, using an electromagnetic bioreactor [6], we have attempted to populate it with extracellular matrix and osteoblasts, of which cell function can be electromagnetically modulated [6].

Hydroxyapatite is widely used in reconstructive bone surgery owing to its biocompatibility. The *in vitro* modification of porous hydroxyapatite, with osteogenic signals of the transforming growth factor-β superfamily and with bone morphogenetic proteins, enhances the tissue regeneration *in vivo* [13], suggesting that the modification of hydroxyapatite could play an important role in tissue engineering.

As consequence, aiming, in a future work, at accelerated and enhanced bone regeneration *in vivo*, in the present study of tissue engineering, we show a particular "biomimetic strategy" that consists in the *in vitro* modification of porous hydroxyapatite with proliferated osteoblasts and their extracellular matrix produced *in situ*. In other words, applying an electromagnetic wave [6], our aim was to enhance a bone cell culture inside porous hydroxyapatite, that is, to coat the hydroxyapatite internal surface with physiological and biocompatible cell-matrix layers. Using this approach, the *in vitro* cultured material could be theoretically used as an osteointegrable implant in clinical applications.

2. *Materials and Methods*

2.1 *Hydroxyapatite disks*

Porous Orthoss® bovine hydroxyapatite disks (diameter, 8 mm; height, 4 mm) were kindly provided by Geistlich Pharma AG (Wolhusen, Switzerland) [10-12]. The biomaterial had the following characteristics: internal surface density of 97 m^2/g, average porosity equal to 60%, crystal dimensions of 10÷60 nm, and Ca/P ratio equal to 2.03, as in normal human cancellous bone.

2.2 *Cells*

The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (HTB85, ATCC, Rockville, MD). The cells were cultured in McCoy's 5A modified medium with L-glutamine and HEPES (Cambrex Bio Science Baltimore, Inc., Baltimore, MD), supplemented with 15% fetal bovine serum, 2% sodium pyruvate, 1% antibiotics, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich, Inc., Milwaukee, WI). Ascorbic acid, another osteogenic supplement, is a component of McCoy's 5A modified medium. The cells were cultured at 37°C with 5%

CO_2, routinely trypsinized after confluency, counted, and seeded onto the hydroxyapatite disks.

2.3 *Cell seeding*

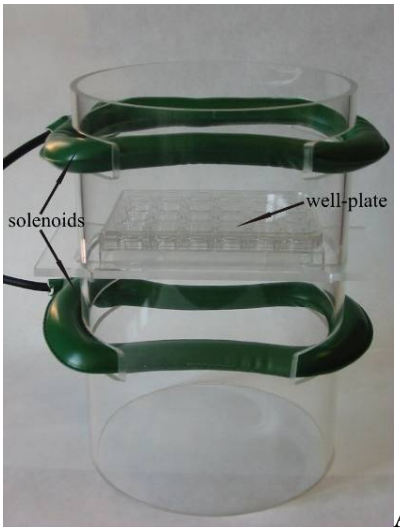
The disks were sterilized by ethylene oxide at 38°C for 8 h at 65% relative humidity. After 24 h of aeration to remove the residual ethylene oxide, the disks were placed inside the two culture systems: the "static" one, a standard well-plate system, far from the electromagnetic bioreactor, and the "electromagnetic" one, where the well-plate system is positioned inside the electromagnetic bioreactor.

A cell suspension of 10×10^6 cells in 400 µl was added onto the top of each disk and, after 0.5 h, 600 µl of culture medium was added to cover the disks. Cells were allowed to attach overnight, then the static culture continued in the standard well-plate and the electromagnetic bioreactor was turned on.

2.4 *Electromagnetic bioreactor*

The electromagnetic bioreactor [6] consisted of a carrying structure custom-machined in a tube of polymethylmethacrylate: the windowed tube carried a well-plate and two solenoids, the planes of which were parallel (Fig. 1A). In this experimental setup the magnetic field and the induced electric field were perpendicular and parallel to the disks top, respectively. The top of the disks were 5 cm distant from each solenoid plane, and the solenoids were powered by a Biostim SPT pulse generator (Igea, Carpi, Italy).

Given the position of the solenoids and the characteristics of the pulse generator, the electromagnetic stimulus had the following parameters: intensity of the magnetic field equal to 2 ± 0.2 mT, amplitude of the induced electric tension equal to 5 ± 1 mV, frequency of 75 ± 2 Hz, and pulse duration of 1.3 ms (Fig. 1B). *In vivo* experiments demonstrated that a continuous exposure to a pulsed electromagnetic field, similar to that used in this study, stimulates the bone repair in the healing process of transcortical holes in adult horses [14].



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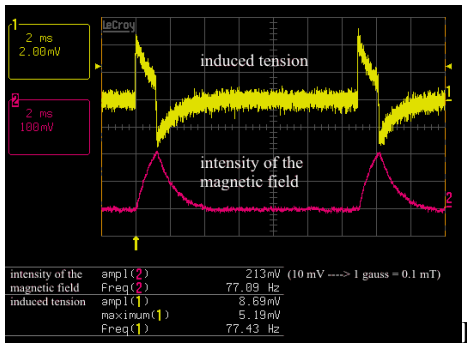

B

Fig. 1. Electromagnetic bioreactor (A) and signal (B) [6].

The electromagnetic bioreactor was placed into a standard cell culture incubator with an environment of 37°C and 5% CO_2. The electromagnetic culture was stimulated 24 h per day for a total of 22 days. The culture medium was changed on days 4, 7, 10, 13, 16, and 19.

2.5 *Standard well-plate culture*
The static culture was placed into an incubator, where the electromagnetic stimulation was not detectable. The duration of the static culture was 22 days and the culture medium was changed on days 4, 7, 10, 13, 16, and 19.

2.6 *Scanning electron microscopy (SEM) analysis*
At the end of the culture period, the disks were fixed with 2.5% (v/v) glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH=7.2) for 1 h at 4°C, washed with Na-cacodylate buffer, and then dehydrated at room temperature in a gradient ethanol series up to 100%. The samples were kept in 100% ethanol for 15 min, and then critical point-dried with CO_2. The specimens were mounted on aluminum stubs, sputter coated with gold (degree of purity equal to 99%), and then observed with a Leica Cambridge Stereoscan microscope (Leica Microsystems, Bensheim, Germany).

2.7 *DNA content*
At the end of the culture period, the cells were lysed by a freeze-thaw method in sterile deionized distilled water and the released DNA content was evaluated with a fluorometric method (PicoGreen, Molecular Probes, Eugene, OR). A DNA standard curve [6], obtained from a known amount of osteoblasts, was used to express the results as cell number per disk.

2.8 *Set of rabbit polyclonal antisera*
L.W. Fisher (http://csdb.nidcr.nih.gov/csdb/antisera.htm, National Institutes of Health, National Institute of Dental and Craniofacial Research, Craniofacial and Skeletal Diseases Branch, Matrix Biochemistry Unit, Bethesda, MD) kindly provided us with the following rabbit polyclonal antibody immunoglobulins G: anti-osteocalcin, anti-type-I collagen, anti-type-III collagen, anti-decorin, and anti-osteopontin (antiserum LF-32, LF-67, LF-71, LF-136, and LF-166, respectively) [15].

2.9 *Set of purified proteins*
Decorin, type-I collagen (purification processes described in [16] and [17], respectively), osteocalcin (immunoenzymatic assay kit, BT-480, Biomedical Technologies, Inc., Stoughton, MA), osteopontin (immunoenzymatic assay kit, 900-27, Assay Designs, Inc., Ann Arbor, MI), and type-III collagen (Sigma-Aldrich).

2.10 *Confocal microscopy*
At the end of the culture period, the disks were fixed with 4% (w/v) paraformaldehyde solution in 0.1 M phosphate buffer (pH=7.4) for 8 h at room tem-

perature and washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH=7.4) three times for 15 min. The disks were then blocked by incubating with PAT (PBS containing 1% [w/v] bovine serum albumin and 0.02% [v/v] Tween 20) for 2 h at room temperature and washed.
L. Fisher's anti-decorin, anti-osteocalcin, anti-osteopontin, anti-type-I collagen, and anti-type-III collagen rabbit polyclonal antisera were used as primary antibody with a dilution equal to 1:1000 in PAT. The incubation with the primary antibodies was performed overnight at 4°C, whereas the negative controls were obtained by the incubation, overnight at 4°C, with PAT instead of the primary antibodies. The disks and the negative controls were washed and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes) with a dilution of 1:500 in PAT for 1 h at room temperature.
At the end of the incubation, the disks were washed in PBS, counterstained with Hoechst solution (2 μg/ml) to stain the cellular nuclei, and then washed. The images were taken by blue excitation with a confocal microscope (TCS SPII, Leica Microsystems) equipped with a digital image capture system at 100× magnification.

2.11 *Extraction of the extracellular matrix proteins from the cultured disks and enzyme-linked immunosorbent assay (ELISA)*
At the end of the culture period, to evaluate the amount of the extracellular matrix constituents over the internal and external hydroxyapatite surface, the disks were washed extensively with sterile PBS to remove the culture medium, and then incubated for 24 h at 37°C with 1 ml of sterile sample buffer (1.5 M Tris-HCl, 60% [w/v] sucrose, 0.8% [w/v] sodium dodecyl sulphate, pH=8.0). At the end of the incubation period, the sample buffer aliquots were removed, and then the disks were centrifuged at 4000 rpm for 15 min to collect the sample buffer entrapped into the pores.
The total protein concentration in the two culture systems was evaluated by the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). The total protein concentration was 862 ± 135 μg/ml in the static culture and 1640 ± 251 μg/ml in the electromagnetic culture ($p<0.05$).
After matrix extraction, the disks were incubated, once again, for 24 h at 37°C with 1 ml of sterile sample buffer, and no protein content was detected.
Calibration curves to measure decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen were performed. Microtiter wells were coated with increasing concentrations of each purified protein, from 1 ng to 2 μg, in coating buffer (50 mM Na_2CO_3, pH=9.5) overnight at 4°C. Some of the wells were coated with bovine serum albumin (BSA) as a negative control. To measure the extracellular matrix amount of each protein by an ELISA, microtiter wells were coated, overnight at 4°C, with 100 μl of the extracted extracellular matrix (20 μg/ml in coating buffer). After three washes with PBST (PBS containing 0.1% [v/v] Tween 20), the wells were blocked by incubating with 200 μl of PBS containing 2% (w/v) BSA for 2 h at 22°C. The wells were subsequently incubated for 1.5 h at 22°C with 100 μl of the L. Fisher's anti-

decorin, anti-osteocalcin, anti-osteopontin, anti-type-I collagen, and anti-type-III collagen rabbit polyclonal antisera (1:500 dilution in 1% BSA). After washing, the wells were incubated for 1 h at 22°C with 100 µl of HRP-conjugated goat anti-rabbit IgG (1:1000 dilution in 1% BSA).
The wells were finally incubated with 100 µl of development solution (phosphate-citrate buffer with *o*-phenylenediamine dihydrochloride substrate). The color reaction was stopped with 100 µl of 0.5 M H_2SO_4 and the absorbance values were measured at 490 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). The amount of extracellular matrix constituents inside the disks is expressed as fg/(cell×disk).

2.12 *Statistics*
The disks number was 24 in each repeated experiment (12 disks in the control culture and 12 disks in the electromagnetic culture). The experiment was repeated 4 times. Results are expressed as mean ± standard deviation. To compare the results between the two culture systems, one-way analysis of variance (ANOVA) with *post hoc* Bonferroni test was applied, electing a significance level of 0.05.

3. *Results*
Human SAOS-2 osteoblasts were seeded onto porous hydroxyapatite disks, and then cultured without or with an electromagnetic stimulus for 22 days. These culture methods permitted the study of the SAOS-2 cells as they modified the biomaterial through the proliferation and the coating with extracellular matrix. The cell-matrix distribution was compared between the two culture systems.

3.1 *Microscope analysis*
In comparison to static condition, SEM images revealed that, due to the electromagnetic stimulus, the osteoblasts proliferated and built their extracellular matrix over the available internal hydroxyapatite surface (Figs. 2 and 3). At the end of the culture period, statically cultured cells were few and, essentially, not surrounded by extracellular matrix, therefore wide biomaterial regions remained devoid of cell-matrix complexes (Fig. 2). In contrast, the physical stimulus caused a wide-ranging coat of the internal surface of the biomaterial: several osteoblasts proliferated and the biomaterial was tending to be hidden by cell-matrix layers (Fig. 3).
The immunolocalization of type-I collagen and the counterstaining of the cellular nuclei showed the stimulation effects in terms of higher cell proliferation and more intense building of the extracellular matrix (Figs. 4 and 5). The immunolocalization of decorin, osteocalcin, osteopontin, and type-III collagen revealed similar results (data not shown).
These observations were confirmed by the measure of the DNA content at the end of the culture period: in the static culture the cell number per disk grew to $45.5\times10^6 \pm 4.1\times10^4$ and in the electromagnetic culture to $73.0\times10^6 \pm 3.6\times10^4$ with $p<0.05$.

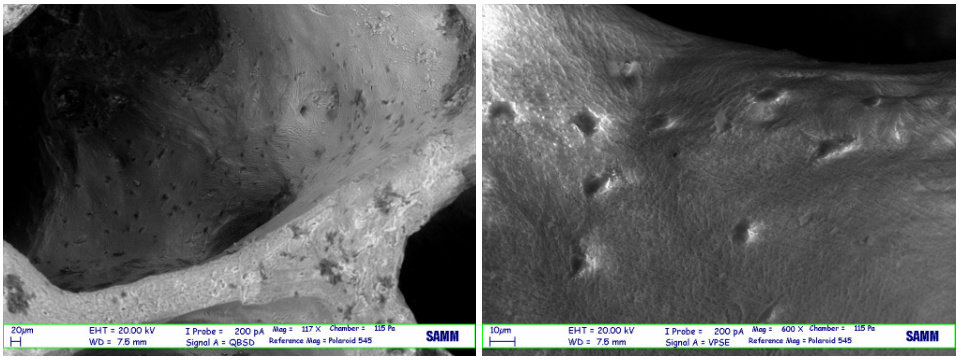

Fig. 2. SEM images of the static or control culture, bar equal to 20 µm (left) and to 10 µm (right). The osteoblasts are in the "backscattered depressions".

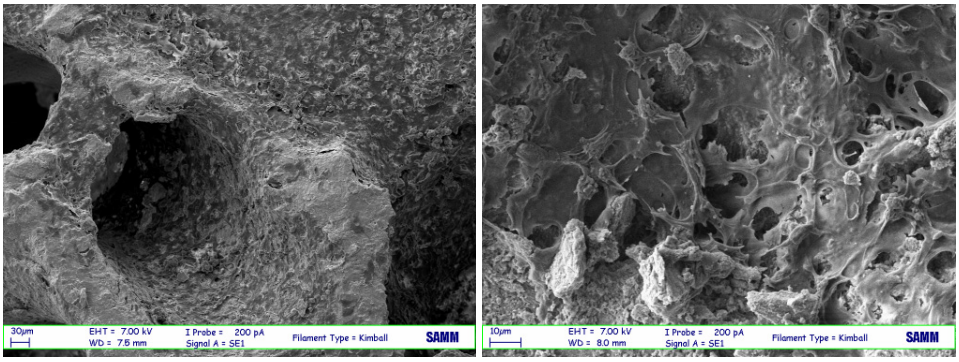

Fig. 3. SEM images of the electromagnetic culture, bar equal to 30 µm (left) and to 10 µm (right). The osteoblasts deposited wide matrix layers.

3.2 *Extracellular matrix extraction*

To evaluate the amount of bone extracellular matrix inside the hydroxyapatite disks, an ELISA of the extracted matrix was performed.

At the end of the culture period, in comparison with the static culture, the electromagnetic stimulation significantly increased the internal surface coating with decorin, osteocalcin, osteopontin, type-I collagen, and type-III collagen ($p<0.05$) (Table 1).

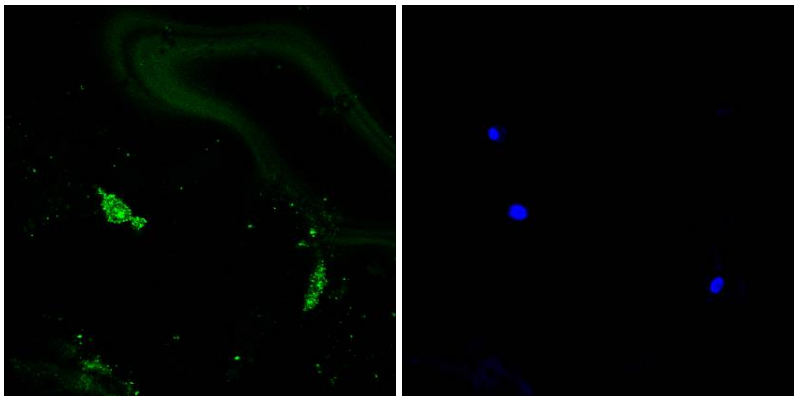

Fig. 4. Localization of type-I collagen (green) and cellular nuclei (blue) in the static or control culture, 100× magnification.

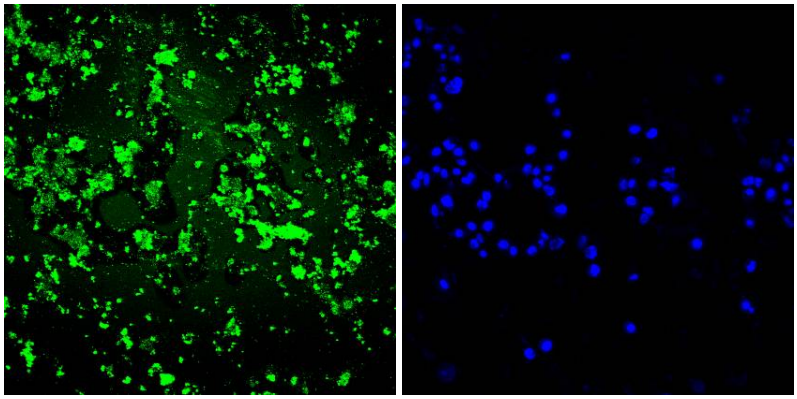

Fig. 5. Localization of type-I collagen (green) and cellular nuclei (blue) in the electromagnetic culture, 100× magnification.

Table. 1. Amount of extracellular matrix constituents inside hydroxyapatite.

	Matrix protein total coating after 22 days of culture in fg/(cell×disk)		
	Static culture	Electromagnetic culture	Electromagnetic/Static
Decorin	6.38 ± 0.12	13.28 ± 0.22	2.08-fold
Osteocalcin	1.47 ± 0.35	4.26 ± 0.37	2.89-fold
Osteopontin	1.23 ± 0.43	2.95 ± 0.53	2.39-fold
Type-I coll.	2.22 ± 0.54	14.76 ± 0.71	6.64-fold
Type-III coll.	3.47 ± 0.17	10.99 ± 0.65	3.16-fold

Table note: $p<0.05$ in all "Static" vs. "Electromagnetic" comparisons.

4. *Discussion*

The aim of this study was the *in vitro* modification of a porous hydroxyapatite with extracellular matrix and osteoblasts to make the biomaterial more biocompatible for the bone repair *in vivo*.

A discussion about the concept of "biocompatibility" is necessary. When a biomaterial is implanted in a biological environment, a non-physiologic layer of adsorbed proteins mediates the interaction of the surrounding host cells with the material surface. The body interprets this protein layer as a foreign invader that must be walled off in an avascular and tough collagen sac. Therefore, the biomedical surfaces must be developed so that the host tissue can recognize them as "self". Castner and Ratner think the "biocompatible surfaces" of the "biomaterials that heal" as the surfaces with the characters of a "clean, fresh wound" [18]: these "self-surfaces" could obtain a physiological inflammatory reaction leading to normal healing. In this study we have followed a biomimetic strategy where the seeded osteoblasts built a biocompatible surface made of bone matrix [19].

To obtain a biomimetic modification of the internal porous surface of the hydroxyapatite scaffold, an electromagnetic wave was applied to the seeded biomaterial [6,19]. The electromagnetic stimulus increased the cell proliferation around 1.6-fold; a similar result has been obtained with the same physical stimulation applied to SAOS-2 osteoblasts seeded onto a polymeric biomaterial [6]. Furthermore, the electromagnetic field significantly enhanced the syn-

thesis of type-I collagen, decorin, osteopontin, osteocalcin, and type-III collagen, which are fundamental constituents of the physiological bone matrix. In particular [6], type-I collagen is the most important and abundant structural protein of the bone matrix; decorin is a proteoglycan considered a key regulator for the assembly and the function of many extracellular matrix proteins with a major role in the lateral growth of the collagen fibrils, delaying the lateral assembly on the surface of the fibrils; osteopontin is an extracellular glycosylated bone phosphoprotein secreted at the early stages of the osteogenesis before the onset of the mineralization, it binds calcium, it is likely to be involved in the regulation of the hydroxyapatite crystal growth, and, through specific interaction with the vitronectin receptor, it promotes the attachment of the cells to the matrix; osteocalcin is secreted after the onset of mineralization and it binds to bone minerals.

The preceding results could be explained with a signaling model. The electromagnetic stimulation raises the net Ca^{2+} flux in the osteoblast cytosol and the release of the intracellular Ca^{2+} [20-22]. According to Pavalko's signaling model, the increase of the cytosolic Ca^{2+} concentration is the starting point of signaling pathways, which cause the secretion of prostaglandins enhancing the osteoblast proliferation, and which target specific bone matrix genes [20].

In this study the electromagnetic stimulus was a physical method to obtain the biomimetic modification of the material, whose internal surface was coated by osteoblasts and by a layer of bone matrix. The use of a cell line showed the potential of the electromagnetic stimulation; nevertheless, appropriately tuning the parameters of the electromagnetic wave and the culture time, a better result could be obtained with autologous bone marrow stromal cells instead of SAOS-2 osteoblasts for total immunocompatibility with the patient.

In conclusion, we theorize that the cultured "self-surface" could be used fresh, that is, rich in autologous cells and matrix, or after sterilization with ethylene oxide, that is, rich only in autologous matrix. In future work, we intend to use our constructs, which are rich in autologous matrix, as a simple, storable, tissue-engineering product for the bone repair [19].

Acknowledgements

The authors thank L.W. Fisher, S. Setti, R. Cadossi, A. Mortara, D. Picenoni, M.G. Cusella De Angelis, G. Mazzini, and P. Vaghi. The Biostim SPT pulse generator was a gift from Igea (Carpi, Italy). This work was supported by Fondazione Cariplo Grants (2004.1424/10.8485 and 2006.0581/10.8485) to F. B., by PRIN Grant (2006) from Italian Ministry of Education, University and Research to L. V., and by FIRB Grant (RBIP06FH7J) from Italian Ministry of Education, University and Research to M.G. C. De A..

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