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SHOCK WAVES INDUCE ACTIVITY OF HUMAN OSTEOBLAST-LIKE CELLS IN BIOACTIVE SCAFFOLDS

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ABSTRACT

Background: bone replacement is frequently needed in periodontal, orthopaedic and maxillofacial diseases. To avoid complications with autografts and allografts, artificial grafts (scaffolds) are candidates for stimulating bone regeneration after colonization with osteoblasts. Moreover, osteoblast activity can be induced by biological or physical stimulation. In this research extracorporeal shock waves were used to improve the ability of human osteoblasts to colonise scaffolds and to induce their osteogenic properties.

Methods: osteoblasts, treated with shock waves, were seeded on glass-ceramic macroporous scaffolds. Cells in scaffolds were counted after detachment, and examined for calcium nodule formation (Alizarin staining), for differentiation markers (real time PCR), and for scaffold colonization (scanning electron microscope).

Results: shock waves initially increased both the number and the activity of osteoblasts in the scaffold, but subsequently only increased osteoblast activity. Moreover, shock waves favoured scaffold colonization even in the deeper layers.

Conclusions: the calcium deposits and differentiation markers studied have demonstrated that shock waves increase osteoblast migration and penetration into scaffolds.

Clinical relevance: this study may provide an important starting point for the introduction of shock waves to boost bone formation through osteoblast stimulation in diseases characterized by bone defects.

KEYWORDS

Scaffold, osteoblasts, shock waves, BMP, SEM

INTRODUCTION

Bone replacements are needed in cases of trauma, neoplasia, and in many periodontal diseases and orthopaedic and in maxillofacial surgery (1-3).

At present, most injuries are not adequately treated because bone defects of critical size cannot be repaired by natural bone growth (4). Furthermore, due to the increase in mean population age and in surgery for removing tumors, bone regeneration is a clinical need of growing importance (5). Autografts, allografts or xenografts can be used as bone substitutes. The high degree of osteoinduction and osteogenesis obtained by autograft makes it the ideal choice. However, it presents some drawbacks, including scarce availability, the need for a second surgical operation and donor site morbidity (6-8). Allografts and xenografts, which can overcome these problems, are characterized by poorer bone induction, lower integration rate, a by no means negligible contamination risk, immune rejection and viral transmission (9,10). For these reasons, artificial grafts (scaffolds) are interesting and challenging candidates for stimulating bone regeneration and supporting newly formed bone (1-4,11).

In previous work (12,13), 3D bioactive glass-ceramic scaffolds were successfully obtained by the sponge impregnation method using a polymeric template. These scaffolds, showing pores in the 100-500 μm range and trabecular morphology analogous to spongy bone, were highly bioactive as they induced the precipitation of hydroxyapatite on their surfaces. They were also osteoinductive, as evinced by osteoblast proliferation within the scaffold and synthesis of calcium nodules. Since these scaffolds are interesting candidates for bone tissue engineering applications, in this research human osteoblast-like cells were exposed to shock waves before seeding on the scaffold to increase their osteogenic activity.

The adoption of shock waves to induce bone synthesis was prompted by several considerations. Extracorporeal shock waves were originally introduced in medical therapy to disintegrate calcific

deposits within renal, biliary and salivary tracts (14,15). More recently, shock waves have also been increasingly applied in various musculoskeletal disorders (16,17).

Extracorporeal shock wave treatment has also been shown to increase the expression of bone morphogenetic protein (BMP)-2, -3, -4 and -7 in rats with femoral defects (18).

In vitro studies on human osteoblast-like cells have shown that treatment with shock waves influence cell proliferation enhancing the transmembrane currents, as well as the voltage dependence of Ca-activated and K channels (19).

Since at the moment little is known about the parameters regarding osteoblast activity induced by shock waves, in this study we have evaluated alkaline phosphatase (ALP), osteocalcin, type I collagen, BMP-4 and -7, as well as calcium deposits.

MATERIALS AND METHODS

Scaffold preparation

Glass-ceramic macroporous scaffolds were obtained using an organic template (polyurethane sponge) and bioactive glass powders, as previously reported (12,13). The scaffolds (1 cm³) were soaked in Tris-buffered Simulated Body Fluid (SBF) before cell seeding to stimulate the precipitation of the hydroxyapatite layer, known to favour bone formation.

Cell culture conditions

Human osteoblast-like cell line, MG-63, (ATCC, Rockville, MD, USA) was grown in MEM medium containing 2 mM L-glutamine, 1% (v/v) antibiotic/antimycotic solution, 1 mM sodium pyruvate, and 10% (v/v) FBS (foetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37° C.

Treatment of cells with shock waves

The shock wave generator utilized was a piezoelectric device (Piezoson 100, Richard Wolf, Knittlingen, Germany) designed for clinical use in orthopaedics and traumatology. The instrument

generates focused underwater shock waves at various frequencies (1 to 4 impulses/sec) and intensities (0.05 to 1.48 mJ/mm²). For medical use, in orthopedics, shock waves of approximately 0.01 to 0.6 mJ/mm² are applied (20).

The experimental set-up has been previously reported (21). Briefly, each cell-containing tube was placed vertically. The shock wave unit was kept in contact with the cell containing tube by means of a water-filled cushion. Common ultrasound gel was used as a contact medium between cushion and tube.

MG-63 cells (10⁶/ml) were exposed to shock waves at different energy levels ranging from 0.08 to 0.32 mJ/mm². For each energy level, different numbers of impulses were tested (from 50 to 1000 at 4 impulses/sec). MG-63 cells, exposed or not to shock waves, were seeded (10,000 cells/cm²) in multiwells and used for counting cell numbers and analysing viability up to 10 days, to identify the shock wave exposure able to increase cell proliferation. After these preliminary experiments, only shock wave treatment corresponding to 0.22 mJ/mm² and 100 total impulses was used (named E6 100).

Cell growth within scaffolds

Sterilized scaffolds, pre-treated in SBF for 1 week, were preconditioned for 24 hours in multiwells containing culture medium. After removing preconditioning medium, MG-63 cells, treated with shock waves (0.22 mJ/mm² and 100 total impulses) or not (control cells), were seeded (10,000 cells/cm²) on the scaffolds.

6, 10 and 20 days after cell seeding, the medium was removed and the scaffolds were used to count the cells that had grown within them, and to evaluate cell viability, morphology, presence of calcium deposits, and osteoblast activity parameters. With this aim, the scaffolds were treated with trypsin/EDTA (0.25%/0.3%) to harvest the cells present within them.

Cell count and viability

Cells were counted in a Burker chamber by using a light microscope (Leitz, Wetzlar, HM-LUX, Germany). To determine viability, plasmamembrane integrity was checked microscopically by

trypan blue exclusion test (dye concentration 0.8 mg/ml); 400 cells were counted for each sample and results were expressed as percentages of trypan blue-positive cells.

Calcium deposit evaluation

The determination of calcium deposits was carried out on cells grown within the scaffolds. After trypsinization, fixation in 70% ethanol and washing with Tris-Buffered Saline (TBS), cells were stained with 1% Alizarin red S for 2 min, washed with TBS and observed under light microscope (22).

Morphology evaluation by scanning electron microscope (SEM)

At the different experimental times, scaffolds not treated with trypsin (containing cells), scaffolds not containing cells (negative control) and scaffolds after treatment with trypsin to remove cells were rinsed four times in PBS and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C. Dehydration was performed with water replacement by a series of graded ethanol solutions with final dehydration in absolute ethanol. Cross sections of the scaffolds were then gold sputtered for SEM observation.

Evaluation of osteoblast activity parameters by real-time PCR

After 6 and 20 days cells detached from scaffolds were examined for osteoblast-activity parameters: ALP, osteocalcin, type I collagen, bone morphogenetic protein-4 and -7 (BMP-7, BMP-4). Total RNA was extracted using RNeasy Mini Kit® (QIAGEN, GmbH, Germany).

Real-time PCR was performed using single-stranded cDNA prepared from total RNA (1 µg) with the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Forward (FW) and reverse (RV) primers were designed using Beacon Designer software (Bio-Rad, Hercules, CA, USA) (Table 1).

25 µl of a PCR mixture, containing cDNA template equivalent to 80 ng of total RNA, 5 pmoles each of the forward and reverse primers and 2× iQ™ SYBR® Green SuperMix (Bio-Rad, Hercules, CA, USA), were amplified using an iCycler PCR (Bio-Rad, Hercules, CA). Each sample of the 3

different experiments was tested 6 times and the threshold cycle (Ct) values were the corresponding mean. The fold change was defined as the relative expression compared to that at time 0 (time of seeding cells), calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{time 0}}$. Data are reported as variation percentages, calculated taking the values of control cells as 100.

Statistical analysis

All data were expressed as means \pm S.D. of 3 different experiments. The significance of differences between group means was assessed by variance analysis, followed by the Newman-Keuls test ($p < 0.05$).

RESULTS

Human osteoblast-like cells MG-63 were treated with shock waves at different energy levels. For each energy level, the effect on cell proliferation of different numbers of impulses (ranging from 50 to 1000, 4 impulses/sec) was tested (data not shown). On the basis of these preliminary experiments the energy level, as well as the number of impulses required to stimulate cell proliferation were identified (0.22 mJ/mm² per 100 impulses, named E6 100), and adopted in subsequent experiments. Immediately after shock wave exposure, this experimental condition caused a decrease in cell viability (-23%), then a significant increase in cell number, as shown in Figure 1 (panel A), and finally a tendential though not significant increase in the number of cell divisions (panel B). Increase in cell number reached the highest value 10 days after shock wave treatment. The variation percentage calculated for treated cells was 125%, taking the values of control cells as 100. Viability during all experimental times was the same for cells treated with shock waves and control cells, and remained about 100% (data not shown). The tendential increase in the numbers of new cells generated after treatment with shock waves, compared with those of the control cells, demonstrates the entity of increased colonization of the scaffold by osteoblasts as well

as their stimulating effect. Figure 1 also shows the total number of cells present within the scaffold at 6, 10 and 20 days after cell seeding (panel C). In shock wave-treated cells an increase in cell number was observed at 6 and 10 days after treatment, whereas a decrease was observed at 20 days.

MG-63 cell spreading and migration within the scaffolds were evaluated by SEM analysis. Different cross-sections of the scaffolds were obtained (up to 5 mm from the surface) in order to compare the depth of colonization of the shock wave-treated cells with that of the control cells. In both cases MG-63 cells were observed to attach, spread, and proliferate to a greater degree at 10 and 20 days than at 6 days. Moreover, SEM analysis showed that at 10 and 20 days, shock wave-treated cells that penetrated to layers up to 5 mm deeper than control cells, which were only found on the surface. Figure 2 and Figure 3 report SEM micrographs of the surface and of the deepest layer (5 mm) of the scaffolds at 10 and 20 days, respectively. The cells colonising the scaffold strongly adhered to its porous structure and appeared to be closely attached to the surface.

To determine the mineralization process, calcium deposits were shown up by staining the cells harvested from the scaffolds with Alizarin red S. Histochemical analysis (Figure 4) showed that after 10 days, a few Alizarin-positive areas had developed only in scaffolds colonised by shock wave-treated cells; at 20 days calcium deposits were observed in both control and shock wave-treated cells, but they were more frequent and larger in scaffolds containing shock wave-treated cells.

Markers of osteoblast activity were also examined. ALP, type I collagen, BMP-7, BMP-4 and osteocalcin mRNA was evaluated by real-time PCR at 6 and 20 days after shock wave exposure. Figure 5 shows that all the parameters examined were higher in shock wave-treated cells than in control cells, except for type I collagen at 6 days, BMP-7 and BMP-2 at 20 days, when the values were 120%, 90% and 53%, respectively, with control cell values taken as 100. The percentage values of increased gene expressions in treated cells were: at 6 days, 200% for ALP, 173% for osteocalcin, 283% for BMP-4, 152% for BMP-7 and 141% for BMP-2; at 20 days, 422% for ALP, 312% for type I collagen, 207% for osteocalcin and 230% for BMP-4.

DISCUSSION

Current opinion holds that significant improvements in bone regeneration will be obtained only by using new technologies based on tissue engineering supported by biochemical or biophysical stimulation. This science requires 3D-scaffolds able to mimic bone and to be colonised by osteoblast-like cells and their products. Among possible forms of biophysical stimulation, shock waves have recently been applied in a broad range of musculoskeletal pathologies (16,17), even though some aspects of the mechanisms involved are still unclear. In this research it has been found that treating human osteoblast-like cells MG-63 with shock waves generated by a piezoelectric apparatus produces an increase in the number of osteoblasts and their degree of penetration into the scaffold. Regarding the increase in osteoblast number, the effect was different at various experimental times: at the earlier times (6 and 10 days after shock wave exposure) numbers of shock wave-treated cells present within the scaffold were higher than those of control cells, whereas at the last experimental time (20 days) the numbers were lower than in the controls. On the other hand, it has been shown that there were more and larger calcium deposits present in scaffolds colonized by shock wave-treated cells than those produced by control cells. In the light of these observations, we supposed that shock wave-treated cells at 20 days might proliferate less, but function more actively. To measure the parameters induced by shock waves resulting in an increased osteogenic activity of MG-63 cells, some markers have been studied. It has emerged that MG-63 cells exposed to shock waves expressed more ALP, osteocalcin, type I collagen, and all BMP-4, BMP-7 and BMP-2. ALP, type I collagen and osteocalcin showed the highest increase after 20 days, whereas BMP-4, -7 and -2 after 6 days with a decrease after 20 days. It is known that ALP expression, which is an early marker of osteoblast differentiation, could be increased by BMP-4 (23,24).

BMPs enable skeletal tissue formation during embryogenesis, growth, adulthood, and healing. BMPs (BMP-2, -4, and -7) are the only growth and differentiation factors which can singly induce

de novo bone formation both in vitro and at heterotopic sites in vivo (25). Following a fracture, BMPs apparently diffuse from reabsorbing bone matrix and activate osteoprogenitor cells which, in turn, produce more BMPs. The temporal and spatial distribution of the BMPs during fracture healing has been moderately well characterized as a complex, interactive and site specific process (26,27,28).

The observations described above suggest that shock waves initially induce an increase in cell number and osteogenic activity, whereas induction of osteogenic activity prevails later.

This research has shown that the use of physical stimulus, such as shock waves, induces osteoblast activity producing the same effect as using biological molecules, such as BMP-2, an osteoinductive growth factor able to determine osteoblast differentiation by increasing calcium deposits and accelerating the healing process when implanted in a bone defect. Our previous paper (12) described how in scaffolds colonised with human osteoblast-like cells treated with BMP-2, more and larger calcium deposits were produced than in the controls, suggesting that the higher degree of mineralization ability of the cells, though there was only a small number of them in the scaffold, was attributable to the BMP-2. In the same way, in SEM analysis, osteoblast-like cells were observed to attach, spread, proliferate and form mineralized nodules when cultured on bioactive scaffolds to a greater extent in BMP-2-treated cells than in controls (12). Therefore, shock waves treatment may be assumed to provide a good opportunity to stimulate osteoblast activity, preferable to the use of chemical substances.

It should be noted that the importance of the use of shock waves lies in their ability to stimulate scaffold colonization and migration: since shock wave-treated cells have been found to penetrate further into scaffolds, whereas untreated cells remain on the surface.

These encouraging results have led us to conclude that this study may well provide an important point of departure in the introduction of shock waves to enhance bone healing through osteoblast activity in bioactive glass-ceramic scaffolds.

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Table 1

Gene Access Number	Sequence FW: forward primer; RV: reverse primer	Annealing (°C)	Cycles
GAPDH NM_002046	FW-5'-GTC GGA GTC AAC GGA TTT GG-3' RV-5'-GGG TGG AAT CAT ATT GGA ACA TG-3'	52	30
ALP NM_000478	FW-5'-CTC CCA GTC TCA TCT CCT-3' RV-5'-AAG ACC TCA ACT CCC CTG AA-3'	58	40
Osteocalcin NM_199173	FW-5'-GTG ACG AGT TGG CTG ACC-3' RV-5'-CAA GGG GAA GAG GAA AGA AGG-3'	59	35
Type I Collagen NM_000089	FW-5'-ACA GCC GTC TCA CCT ACA GC-3' RV-5'-GTT TTG TAT TCA ATC ACT GTC TTG CC-3'	60	45
BMP-7 NM_001719	FW-5'-GTG GAA CAT GAC AAG GAA T-3' RV-5'-GAA AGA TCA AAC CGG AAC-3'	58	40
BMP-4 D30751	FW-5'-CTC GCT CTA TGT GGA CTT C-3' RV-5'-ATG GTT GGT TGA GTT GAG G-3'	58	45

Figure

Figure 1

Osteoblast proliferation after treatment with shock waves.

Panel A: Numbers of osteoblast were counted after treatment with shock waves at the energy level of 0.22 mJ/mm^2 and 100 impulses (E6 100) at the indicated experimental times. Data are means \pm S.D. of 3 different experiments.

Panel B: Numbers of cell divisions were counted as above described.

Panel C: Numbers of osteoblasts, treated with shock waves (E6 100) and seeded on scaffolds, were counted at the indicated experimental times after detachment from scaffold with trypsin. Data are means \pm S.D. of 3 different experiments.

For each panel, means with different letters are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls test.

Figure 1

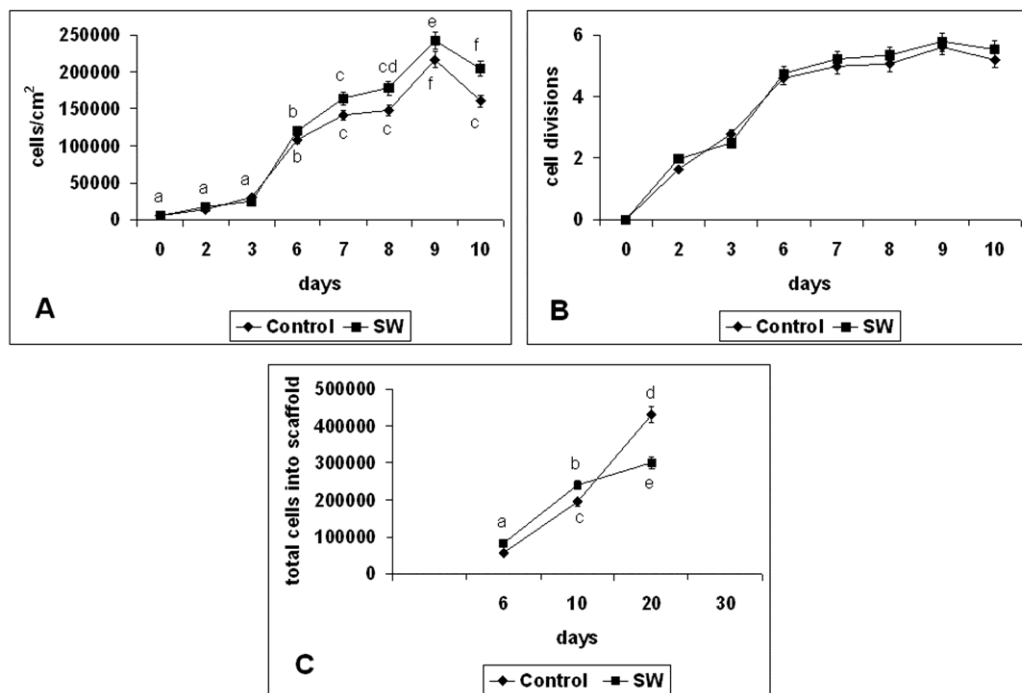
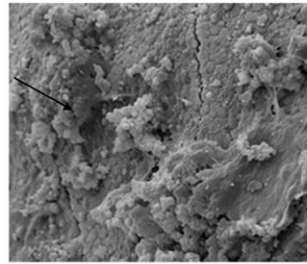


Figure 2

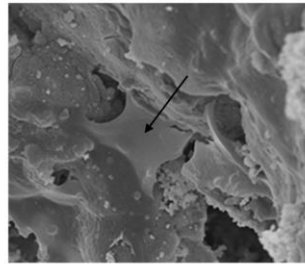
Osteoblast spreading and migration onto the scaffolds evaluated by SEM analysis at 10 days.

SEM micrographs of the surface and of deepest layer (5 mm) of the scaffolds show the osteoblasts treated (E6 100) or not treated with shock waves at 10 days after seeding on the scaffolds.

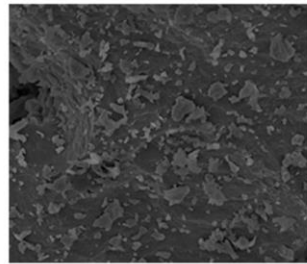
→ cells

Figure 2**10 days**

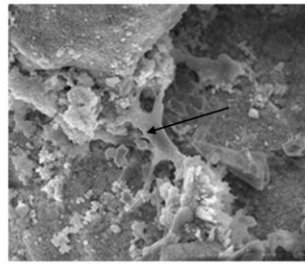
control surface



SW surface



control 5 mm



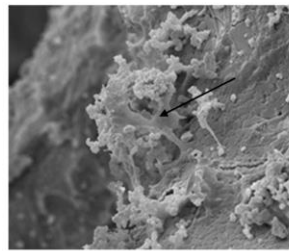
SW 5 mm

Figure 3

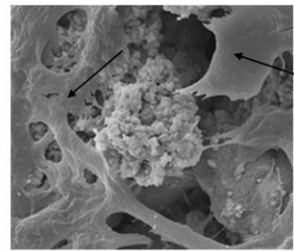
Osteoblast spreading and migration onto the scaffolds evaluated by SEM analysis at 20 days.

SEM micrographs of the surface and of deepest layer (5 mm) of the scaffolds show the osteoblasts treated (E6 100) or not treated with shock waves at 20 days after seeding on the scaffolds.

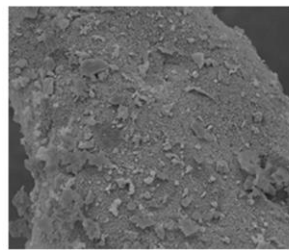
→ cells

Figure 3**20 days**

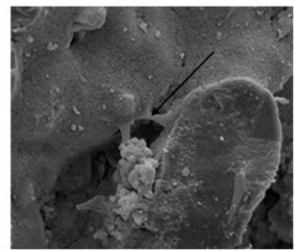
control surface



SW surface



control 5 mm



SW 5 mm

Figure 4

Calcium deposits evidenced with Alizarin S staining

Osteoblasts harvested from the scaffolds were stained with Alizarin red S and observed at light microscope. The cells treated (E6 100) or not treated with shock waves were harvested at 10 and 20 days after seeding on the scaffolds.

→ calcium deposits

Figure 4

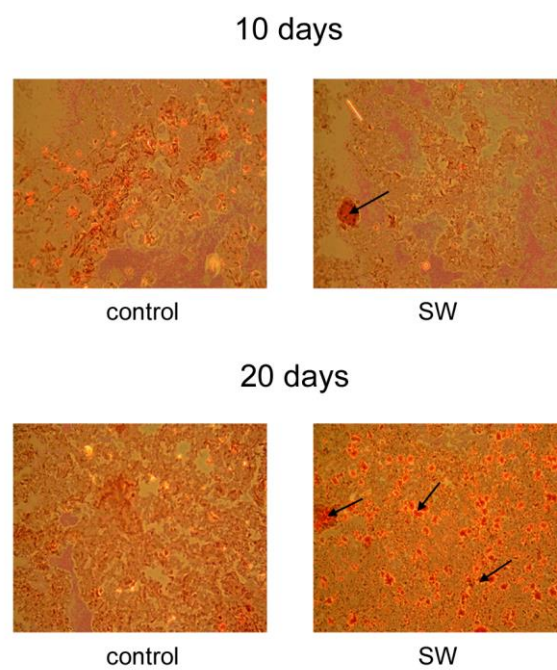


Figure 5

Alkaline phosphatase (ALP), osteocalcin, type I collagen, bone morphogenetic protein-4, -7 and -2 (BMP-4, BMP-7, BMP-2) mRNA content.

mRNA content, at 6 and 20 days after seeding cells on scaffolds, was evaluated by real-time PCR and the values of osteoblasts treated with shock waves (E6 100) were referred to those of control cells taken as 100% (black line).

Data are means \pm S.D. of 3 different experiments. For each panel, means with different letters are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls test. The control values are indicated as a.

