Objectives
The need for bone tissue supplementation exists in a wide range of clinical conditions involving surgical reconstruction, including in limbs, the spine, and skull. The bone supplementation materials currently used include autografts, allografts, and inorganic matrix components; but these pose potentially serious side-effects. In particular, the availability of the autograft is usually limited, and their harvesting causes surgical morbidity. Therefore, for the purpose of autologous bone graft supplementation, we have developed a method for extracorporeal human bone-like tissue generation.

Methods
Human osteoblast-like cells were seeded on porous granules of tricalcium phosphate and incubated in osteogenic media while exposed to mechanical stimulation by vibration in the infrasonic range of frequencies. The generated tissue was examined microscopically following haematoxylin eosin, trichrome, and immunohistochemical staining.

Results
Following 14 days of incubation, the generated tissue showed histological characteristics of bone-like material due to the characteristic eosinophilic staining, a positive staining for collagen trichrome and a positive specific staining for osteocalcin and collagen 1. Macroscopically, this tissue appeared in aggregates of between 0.5 cm and 2 cm.

Conclusions
We present evidence that the interaction of cellular, inorganic, and mechanical components in vitro can rapidly generate three-dimensional bone-like tissue that might be used as an autologous bone graft.

Keywords: Bone graft, Bone regeneration, Osteoblast, Bone, Osteogenesis, Extracorporeal
complications and have different levels of efficiency. The availability of autografts, which have the highest osteoinductive ability, is usually limited to their anatomical site and their harvesting can cause considerable surgical morbidity. The successful use of allografts is also limited because of the high risk of ‘docking site’ nonunion (or rejection of whole graft) and infection. The osteoconductive properties of inorganic material such as tricalcium phosphate, calcium phosphate and calcium sulphate are effective, predominantly in the filling of small gaps in bone. Therefore, the possibility for in vitro generation of a sufficient amount of autologous bone for inductive and conductive purposes might resolve these difficulties and complications.

For the purpose of supplementation of autologous bone graft sufficient for the requirements of an individual patient, we developed a method for the generation of extracorporeal autologous bone. By this method, bone matrix generating cells – osteoblasts – were seeded on an inorganic supporting matrix of porous tricalcium phosphate, in a specially designed bioreactor, allowing exposure of cells to osteogenic medium and their stimulation by biomechanical activation by mechanical vibration in an infrasonic range of frequencies. We hypothesised that by using this method we could generate live tissue with biochemical characteristics identical or similar to human bone.

Figure 1a – a schematic representation of mechanical stimulation of the generated tissue, adherent to a plastic surface of culture flask, by a horizontal vibration in the infrasonic range of frequencies. Figure 1b – profile of the mechanical vibration pattern for the generated tissue stimulation. Sine shaped vibration at 20 Hz frequency, (25 to 30) × 10^{-6} m of displacement amplitude and peak-to-peak acceleration of 0.5 m/sec^2 (± 0.1 m/sec^2) was applied to the well plates.
Materials and Methods

Cells. The source of the osteoblasts were mesenchymal precursor cells that originated from disposable human cancellous bone samples, each between 2 g and 3 g in total, collected during elective hip replacements in four patients. These patients were two men and two women who had no systemic illnesses and who were aged between 60 and 65 years. The patients gave signed informed consent and the use of these cells was approved by the Institutional Ethical Committee. The site of the collection of bone samples was at least 5 cm distant from the subchondral bone area.

Culture technique. The osteoblasts were initially grown as explant primary cultures in a special bone inductive medium containing Dulbecco’s Modified Eagles Medium (DMEM) with heat-inactivated fetal calf serum (10%), 20 mM HEPES buffer, 2 mM L-Glutamine, 100 μM ascorbate-2-phosphate, 10 nM dexamethasone, 50 U/ml penicillin, and 150 μg/ml streptomycin at 37°C in a humidified atmospheric environment of 95% air with 5% CO₂ (v:v), for between 20 and 30 days. The human bone cell cultures obtained by this standard method have been shown to express osteoblast-like characteristics⁵,⁶ such as polygonal multipolar morphology, expression of the enzyme alkaline phosphatase, synthesis of a collagen-rich extracellular matrix.
matrix with predominantly type I collagen and small amounts of collagen types III and V, and non-collagenous proteins, such as sialoprotein (BSP) and osteocalcin. Additionally, these cells demonstrate matrix mineralisation in vitro and bone formation in vivo. We have previously shown that these cells have osteoblastic characteristics such as multipolar morphology, adhesion to plastic surfaces, cellular alkaline phosphatase activity, positive Von Kossa staining, and osteopontin and osteocalcin expression.7

Following monolayer confluency, 10^6 cells were passaged onto three-dimensional granules of tricalcium phosphate (diameter 0.5 mm, pores 300 μ to 500 μ, total volume 5 cc) and cultured in the same type of osteogenic media in the same environmental conditions. Mechanical stimulation. These cultures were also exposed to mechanical stimulation by horizontal vibration at an infrasonic range of frequencies. The reason for application of mechanical stimulation to the osteoblasts is the ability of these cells to increase bone matrix elaboration according to the vector (direction and magnitude of the mechanical forces to which they are exposed).8

Plates containing the cultured tissue were connected to a horizontally orientated shaker. The amplitude, wave of movement shape, and frequency of the vibration provided by the shaker were controlled by an amplifier and pulse generator. Vibration peak-to-peak acceleration was measured with a piezoelectric accelerometer and displayed on a vibration measuring amplifier. The displacement of vibration movement was calculated from the acceleration values. A sine-shaped vibration at 20 Hz frequency, (25 to 30) × 10^-6 m of displacement amplitude and peak-to-peak acceleration of 0.5 m/sec^2 (± 0.1 m/sec^2) was applied to the well plates (Fig. 1). These vibration parameters have been found to be optimal for the induction of human osteoblast-like cells proliferation and metabolic activity.4 The rationale to use these parameters of vibration, i.e. frequency of 20 Hz, is based on the normal vibromiogram pattern, which reflects the basic skeletal muscle contraction at rest with subsequent mechanical effect on the adjacent bone and essentially osteoblast stimulation in vivo.9

The samples were exposed to the vibration protocol for four minutes once every 24 hours, a protocol previously found to be effective in stimulating the metabolic activity of human osteoblasts.4,10

Staining methods. On three, seven and 14 days following the start of the experiment, samples of the generated material were decalcified, embedded in paraffin, sectioned and stained by haematoxylin and eosin (HE) according to standard protocols, and inspected microscopically in order.
to evaluate the tissue morphology. After 14 days the samples were stained by the trichrome method for the general detection of collagen and by immunohistochemical assays for collagen 1 (mouse anti IgG collagen type 1, cat. SC-59772), and by osteocalcin (rabbit anti osteocalcin, cat. SC-30044) in order to determine the characteristics of the bone in the generated material. The microscopic HE-stained images of generated tissue were compared with microscopic images of stained samples of normal control bone tissue. All samples used as controls were from biopsies taken for non-related clinical reasons from patients who were not involved in the study. Similarly, the microscopic images of the generated tissue stained immunohistochemically for collagen 1 and osteocalcin were compared with the immunohistochemically stained normal bone samples as a positive control. They were also compared with cartilage samples as a negative control for collagen 1 staining, and to a sample of kidney tissue as a negative control for osteocalcin staining. Images of the generated and normal bone tissue stained without the addition of the antibodies to collagen 1 and osteocalcin were inspected as double negative controls.

**Results**

Three days after treatment of the cells in the bioreactor, there was histological evidence by HE-staining of islets of bone-like matrices with strong eosinophilic staining (Fig. 2) which became abundant after two weeks of culture (Fig. 3). There was also evidence of collagen deposition by the osteoblasts after two weeks of treatment in the bioreactor (trichrome staining; Fig. 4). Immunohistochemical staining showed that the deposed collagen was mostly of type 1 (Fig. 5) and that the tissue contained osteocalcin (Fig. 6). On examination of the generated tissue following seven days of incubation, islets of cartilage were seen in HE-stained samples (Fig. 7). These cartilage islets disappeared after 14 days of incubation. Macroscopically, the generated tissue had a three-dimensional granular shape, between 5 mm and 20 mm in diameter (Fig. 8).

**Discussion**

We used human osteoblasts in osteogenic media seeded on an inorganic scaffold and exposed to infrasonic mechanical stimulation in order to generate bone tissue in vitro. All of these components were planned to mimic the optimal biomechanical conditions for bone formation in vivo. The unique use of mechanical stimulation in the infrasonic range of movements should be similar the physiological mechanical stimulation of bone by resting muscles according to a normal resting vibromiogram, which contract in this range of mechanical parameters.

There are other methods for generating a stock of osteoblast-like cells from the progenitor cells without using the
and poly-caprolactone (PCL) nanotubules. Currently it is suggested that metaphysically active osteoblasts that generate calcified bodies can be considered as an efficient source for inducing the enhancement of bone generation following their implantation. Obviously, in these circumstances, the intended clinical use would be an uncontrolled cell implantation, rather than the use of biologically active bone graft material. The clinical efficiency of these methods has not been proven. The biologically active bone-like tissue presented here might be more effective in its osteoinductive and osteoconductive characteristics. This hypothesis should be investigated in future clinical studies investigating fracture union. In this report we show that such viable bone-like material can be generated in vitro.

There is evidence that cultured osteoblasts may generate calcified bodies in a monolayer culture in in vitro perfusion chambers on different scaffolds. In these studies, it was suggested that metabolically active osteoblasts that generate calcified bodies can be considered as an efficient source for inducing the enhancement of bone generation following their implantation. Obviously, in these circumstances, the intended clinical use would be an uncontrolled cell implantation, rather than the use of biologically active bone graft material. The clinical efficiency of these methods has not been proven. The biologically active bone-like tissue presented here might be more effective in its osteoinductive and osteoconductive characteristics. This hypothesis should be investigated in future clinical studies investigating fracture union. In this report we show that such viable bone-like material can be generated in vitro.

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- N. Rosenberg: Writing the paper, Design of the experiments
- O. Rosenberg: Design of the experiments, Performing the experiments, Writing the paper

**ICMJE Conflict of Interest:**

- None declared

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