Safety and efficacy of *in vitro* generated bone-like material for in vivo bone regeneration – a feasibility study

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**ABSTRACT**

Bone-like viable tissue can be generated *in vitro* by utilizing a combination of inorganic matrix, osteoblasts, osteogenic media and application of adequate mechanical stimulation of the cells. To pursue the proof that the *in vitro* generated bone-like tissue (BLT) is capable of bridging a critical bone gap in *vivo* without adverse effects, the *in vitro* cytotoxicity method (MTT) and murine *in vivo* model were implemented, by implanting the BLT into calvaria critical bone gap in rats. The endpoints for the evaluation of this concept were histological and radiographic data which should show the effectiveness of this method. We found that there was no cytotoxic effect of the BLT according to the MTT assay and no carcinogenic or other morbid effects of the BLT in *vivo* (mice experiment, n = 10). The critical gaps in BLT-implanted animals (experimental model with rats) demonstrated full bridging of the calvaria critical bone gap with vascularized woven bone (n = 3) as opposed to animals treated with vehicle material (n = 3), which maintained an open gap without any visible closure, according to gross examination, X-ray imaging and histological analysis. The newly formed bone tissue was characterized by pronounced presence of bone marrow regions and newly formed host blood vessels, a strong indication for functional osseointegration. Therefore, the *in vitro* generated BLT, which causes bone regeneration in critical gaps, has the translational potential to bridge bone non-union defects, without harmful systemic or cytotoxic effects. These initial feasibility results indicate a high safety profile following *in vivo* implantation of BLT and its potential clinical ability to be used as autologous bone graft.

1. Introduction

Bone has a vital potential to regenerate after damage; however, the efficacious repair of large defects resulting from resection, trauma with extended fractures or inadequately vascularized bone gaps will not achieve a satisfactory healing and will be considered as nonunion causing symptomatic skeletal instability. These clinical conditions are treated by stabilization techniques utilizing metal hardware augmented by bone grafting.

Bone grafts materials should demonstrate three major interrelated properties for optimal bone regeneration [1]: i) Osteoconduction - supporting the attachment of new osteoblasts and osteoprogenitor cells; ii) Osteoinduction - induction of osteoprogenitor cells (or other non-differentiated cells) to differentiate towards osteoblast lineage. iii) Osteogenesis – formation of new bone via osteoblasts-derived osteoid that subsequently mineralizes. In a clinical setting most bone grafting options rely on bone graft replacement materials, e.g., calcium phosphate matrices, collagen sponges and natural bone grafts (autografts, allografts and xenografts) [2]. The best clinical results are achieved by autologous bone grafting, but this method requires additional surgical procedures with considerable morbidity and commonly doesn’t provide enough amount of tissue for bridging of large gap in bone. Therefore, the ability for generation of autologous bone tissue *in vitro*, in the required amount and without additional surgical morbidity, is highly desired.

Previously we reported on an *in vitro* strategy to generate human bone-like tissue [3]. Upon culturing bone marrow-derived autologous human osteoblasts in 2-D osteogenic conditions with porous β-TCP granules, accompanied with precisely timed mechanical stimulation. The end-product had the characteristics of bone-like tissue (BLT) with expression of bone-specific markers as well as human bone-like spatial organization.
This method utilizes the combination of bone inductive media and mechanical stimulation to mimic the biomechanical conditions for bone formation in vivo [4]. While the in vitro BLT generation, the applied optimal mechanical stimuli parameters, in terms of amplitude, wave of movement shape and frequency, are similar to physiological stimulation of bone by bone-surrounding contracting muscles in vivo [5]. The in vitro activation of mechano-sensing mechanisms in osteoblasts promotes the preservation of bone-like properties, such as deposition of bone matrix components [6]. Thus, mechanical stimulation during BLT generation led to an abundance of collagen type I and osteocalcin proteins, the main and essential components of human bone matrix and typical bone-like microscopic appearance, which is similar to normal bone. Moreover, transient cartilage generation, as evident in the first week of 3-D culture, suggests that the end-product bone-like tissue follows an endochondral-like developmental path [3]. Therefore, generation of the three-dimensional human BLT by the means of mechanical activation, in parallel with sequential culture in 2-D, followed by 3-D on inorganic porous matrix, is a suitable candidate for further in vivo proof-of-concept investigation, as a candidate for the clinical use as autologous bone graft. We hypothesize that such in vitro generated material is safe when implanted in vivo and is able to bridge critical bone defects. The proof of this concept might be clinically important for in vitro generation of autologous bone grafting material.

Accordingly, we performed studies in vitro and murine in vivo models aiming to assess:

(i) The BLT general safety and potential toxic or carcinogenic effects. (ii) Bone critical gap bridging following BLT implantation.

2. Methods

The BLT was engineered in vitro by synergistic combination of human derived osteoblast-like cells, three-dimensional biocompatible inorganic bone matrix and defined mechanical stimulation applied during the process [3]. Briefly, the source of the osteoblasts was mesenchymal precursor cells that originated from disposable human cancellous bone samples containing bone marrow, each 2–3 g in total, collected during elective hip arthroplasty. The samples were obtained from Bone Bank where they harvested according the Institutional Ethical Committee requirements (Rambam Med Center No A1240). The site of the collection of bone samples was distant from the subchondral bone area.

The osteoblasts were initially grown as explant primary cultures in a bone inductive medium containing DMEM (Dulbecco’s Modified Eagles Medium, ThermoFisher Scientific, Life Technologies, Paisley, UK, Cat No. 41965) with heat-inactivated fetal calf serum 10% (HyClone USA, Cat. No SH30071), 20mM HEPES buffer (Life Technologies, Cat. No. 15630), 2mM L-Glutamine (ThermoFisher Scientific, USA, Cat. No. A12860), 100μM ascorbate-2-phosphate (Sigma-Aldrich, USA Cat. No. 49752), 10mM dexamethasone (Kern Pharma Barcelona), 50 U/ml penicillin, and 150 μg/ml streptomycin (ThermoFisher Scientific, USA, Cat. No. 15140) at 37 °C in a humidified atmospheric environment of 95% air with 5% CO2 (v:v), for 20–30 days. The human bone cell cultures obtained by this method have been shown previously to express osteoblast-like characteristics [7, 8], i.e. polygonal multipolar morphology, expression of the enzyme alkaline phosphatase, synthesis of a collagen-rich extracellular 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 mineralization in vitro and bone formation in vivo. It has been shown previously that these cells have osteoblastic characteristics, e.g., multipolar morphology, adhesion to plastic surfaces, cellular alkaline phosphatase activity, positive Von Kossa staining, and osteopontin and osteocalcin expression [9].

Following monolayer confluence, 10⁶ cells were passaged onto three dimensional granules of β-tricalcium phosphate (βTCP, diameter 0.5mm, pores 300-500μ, 5cc total volume) and cultured in the same type of osteogenic media in the same environmental conditions as described above for three weeks.

During the two dimensional and three-dimensional culture condition the samples were exposed to mechanical stimulation by horizontal vibration at an infrasonic range of frequencies, i.e. well plates containing the cultured tissue were connected to a horizontally oriented 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. Sine shaped vibration at 20Hz frequency, (25–30) x10^-16 m of displacement amplitude and peak-to-peak acceleration of 0.5 +/- 0.1 m/s^2 was applied to the well plates [10]. These vibration parameters have been found previously to be optimal for the induction of human osteoblast-like cells proliferation and bone generation [3, 10]. The samples were exposed to the vibration protocol for 4 min once in 24 h [3].

The eventually generated tissue comprised autologous human osteoblast-like cells in 3-D βTCP construct with microscopic characteristics of BLT (Figure 1). We have shown previously that the generated tissue has the characteristic bone-like features, i.e. abundant with collagen type 1, contains osteocalcin and has the characteristic microscopic appearance (Figure 1) [3].

2.1. Safety studies

2.1.1. In vitro safety evaluation

The general cytocompatibility and safety of the tested BLT was evaluated by concomitant culture of mouse fibroblast cells L929 using MTT cell viability assay, according to the ISO Standard 10993-5 (Cell Proliferation Kit – MTT, No 11465007001 Merck) [11]. Briefly, 1929 cells were cultured in medium containing 5% serum, 50 μ/l penicillin and 150 μg/ml streptomycin at 37 °C and 1% glutamine to MEM. Cells were harvested at 80% confluence, counted and re-suspended to final concentration of 1 x 105/ml and 3 x 105 in growth medium supplemented with 1% fetal calf serum. Cell suspension of L929 was seeded in the 3 concentrations; no cells, 1 x 104 and 3 x 104 cells/well) in a 96-well microtiter plate. Cell cultures were incubated (37 ± 1°C, 5% CO2) for 24 h. Growth medium was removed from the 96-microtiter plate and the cells were washed twice with 1–2 ml of PBS-supplemented MEM. Medium was replaced on all plates with fresh medium and samples of BLT were added into the relevant wells as well as the growth medium. Positive (2% Triton), negative controls (cells only) and pure scaffold (by MTT) of more than 30% was considered a cytotoxic effect (compared to the average OD was subtracted from each reading. Reduction of cell viability product was 550 nm. The reference wavelength was 620nm (and subtracted by the software for each well). Blank wells were read, and the average OD was subtracted from each reading. Reduction of cell viability (by MTT) of more than 30% was considered a cytotoxic effect (compared to control). The results were recorded in OD units. Five replicates for experiment conditions were used.

2.1.2. In vivo safety evaluation

Testing the biosafety profile and the tumorgenicity potential of the in vitro generated BLT was done in mouse model. The model was approved by the Ethical Committee for animal experiments (Technion – IIT, Haifa No IL No 1008346) and were conducted according to established animal welfare guidelines and complies with all regulations for animal studies. The test was based on subcutaneous inoculation of BLT in NOD-SCID mice and examining the biosafety and tumorigenicity of the tested material. A total of 10 mice were tested. Each tested group included five animals (n = 5 for vehicle βTCP implanted and n = 5 for BLT implanted in the doses of 0.1 ml/kg). Duration of the experimental period was 62 days. We used a standard surgical procedure for subcutaneous implantation of the testing material. Briefly: Animals were anesthetized by isofouran 4%. The right flank area was sanitized with alcohol. Using microsurgery scissors, 1–1.5 cm vertical incision on right flank was cut. The incision was spread into 2 cm to create a pocket for the bone-like tissue. Using straight forceps, the bone-like tissue was inserted into the incision. The incision site was elevated with forceps and staple. The animal was placed back in cage on its left side. The animals woke up within a minute.

The vehicle and BLT-implanted animals were monitored for general well-being in terms of change in body weight and neoplastic changes in histologic slides at the site of implantation.

Observations for signs of morbidity and mortality were made twice daily. Body weight was measured once a week starting on day 1. At termination individual body weight changes was calculated. Blood was collected on day 1 and at the termination of the experiment, briefly - animals were subjected to light anesthesia with isoflurane and using glass capillary, the membrane of the retro-orbital sinus was punctured. Gentle pressure on the collateral jugular vein was applied; blood flowing out the eye was collected. On day of termination (day 62) animals were euthanized by Pental 30%, rib cage was opened, maximum blood was collected via the heart using a 27- gage needle. Blood was collected into EDTA-coated tubes for whole blood and into serum tubes for serum. The serum tubes were kept at room temperature for at least half an hour for clotting. The blood for serum was centrifuged at room temperature for 10 min at 1790 g (4000 RPM). Following centrifugation, the serum (supernatant) was separated from the blood cells (pellet). Blood samples were tested by the standard laboratory procedures for blood hematology and biochemistry analyses (including blood electrolytes levels, liver functions and kidney functions). At study termination (day 62) animals were euthanized by Pental 30%.

All animals were subjected to an excision of injection site for histological preserved in 10% Neutral buffered formalin (4%) and kept at 4 °C until they were transferred for further histopathological evaluation. Histopathological analysis was performed on the implantation area. Tissues were trimmed, embedded in paraffin, sectioned at approximately 5 μm thickness and stained with Hematoxylin & Eosin (H&E).

2.2. Efficacy in vivo studies

To test the osseointegrative capacity of the BLT in vivo, i.e. the ability of the implanted tissue to interact with the host bone at orthotopic site, we implanted the in vitro generated BLT into rat calvarial critical size defect model. The studies were conducted according to established animal welfare guidelines and complies with all regulations for animal studies (approved by the Ethics Committee of Technion – IIT, No II. No 1008346). The ability of the BLT to promote critical size defect healing was monitored during the experiment using X-ray imaging and at the experimental endpoint using histological evaluation.

2.2.1. Animal model

The critical size calvarial defect is a commonly used an in vivo model for investigation of cancellous bone repair by alternative bone graft materials, tissue engineered constructs and regenerative therapies [12]. In this study the BLT was applied directly into the 6 mm in diameter calvarial critical bone defect in rat experimental model (Sprague Dawley males 10–11 weeks of age at study initiation, the average (±SD) body weight at study initiation was 372.9 ± 8.0 g). The model was approved by the Ethical Committee for animal experiments.

The human extrapolation factor in the rat model is 19.9, providing sufficient exposure to the human derived BLT with adequate estimation of the safety profile. Thus, the calvarial defect in rat model provides the opportunity to implant higher doses of the test materials with an adequate estimation of safety and biodistribution parameters and without the need for the bone gap stabilization.

During the in vivo studies the highest possible dose of BLT was used, which provided full depth filling of the experimentally generated calvarial defect. In order to extrapolate the dose from rat to human subjects, the burr hole is operationally considered as having a cylinder shape. We calculated the cylinder volume according to the radius and the thickness of the burr hole in calvarial bone. In rat the thickness of calvarial bone is 1 mm [13], therefore, the volume of a burr hole with 6 mm in diameter is 28 mm³. The human skull has a variable thickness in the range between 5.3 mm and 8.4 mm, 6.62 ± 0.08 mm [14, 15]. Thus, using the cylinder volume calculation, the average volume of a
standard human burr hole with 16 mm diameter is 1,331 mm³ and ranging from 1,066 to 1,685 mm³. The average rat’s 6 mm defect (with 28 mm³ volume) provides an extrapolation ratio of 47.5-folds. Moreover, in order to avoid possible local pressure on the underlining dura, in the clinical trial synopsis we applied less than maximal possible volume and limited the highest dose to 1,000 mm³ volume of BLT (30 mg). Since BLT contains osteoblast-like cells from human origin, all rats were immunosuppressed using 5 mg/kg/day per-oral cyclosporine A (Novartis, H5148), starting two weeks prior to surgery and thereafter until the end of the experiment. The bone healing effects of BLT were evaluated in rats bearing 6 mm calvarial defects. The study was done according to the following: upon performing the calvarial defect surgery one group was implanted with ~20–30 mg BLT (n = 3), additional group (vehicle controls) implanted with Normal Saline (sodium chloride 0.9 % TEVA®, vehicle control, n = 3) and in the negative control group left untreated (n = 2). The BLT and vehicle material were placed inside the defect orifice, covered with periosteum and all surrounding soft tissue was sutured. The control group underwent similar surgical procedure. All rats were subjected to X-ray imaging at the 6th week of experiment. At the end of the 6th week, rats were euthanized and the calvarial defect sites were subjected to histological analysis (Figure 2).

2.2.2. Histological evaluation of critical gap closure

Upon termination of the experiment at 6th postoperative week, the calvarial bones of both BLT-treated and vehicle control groups were stained using H&E and subjected to histological evaluation. The histological evaluation included a gap closure with woven bone score, as a marker for newly in-situ developed bone tissue, based on the following gradation: Grade 0 = 0% closure; Grade 1 = up to 25% closure; Grade 2 = up to 50% closure; Grade 3 = up to 75% closure; Grade 4 = up to 100% closure. Since non-healing bone wounds generally result in a thin collagenous fibrous tissue within the defect with no visible bone ingrowth [16], the amount of fibrous tissue was scored based on the following gradation: (-) no fibrosis; (+) marginal; (++-) minor; (++++) pronounced. The length of remaining gap, if any, was measured under the microscope using calibration scale in millimeters.

3. Results

3.1. In vitro safety evaluation

The cytotoxicity MTT assay revealed that the L929 fibroblasts’ mean viability increased following exposure to the BLT (mean 0.937 OD +/- 0.054 SD vs. 0.719 OD +/- 0.028 SD in unexposed controls) while examining 1 x 10⁴ cells, mean 1.477 OD +/- 0.171 SD vs. 1.041 OD +/- 0.042 SD in unexposed controls. The results are presented in Figure 3.

![Figure 2](image1.png)

Figure 2. Graphical representation of the in vivo experiment of critical bone gap bridging design. (A) Schematic presentation of the anatomical location and size of rat calvarial defect model. (B) Timeline and major steps of in vivo experiment design to evaluate the BLT implantation.

![Figure 3](image2.png)

Figure 3. Viability (%) comparison of L929 cells after exposure to BLT for 72 h and to control conditions (mean values are presented).
0.109SD in unexposed controls while examining $3 \times 10^4$ cells, $n = 5$ experimental condition).

The exposure of L929 fibroblasts to porous βTCP porous granules alone had some toxic effect, however, the viability was not reduced by more than 65% and therefore is not considered toxic [17] (mean 0.383 OD +/- 0.210 SD vs. 0.719 OD +/- 0.028 SD in unexposed controls while examining $1 \times 10^4$ cells, mean 0.814 OD +/- 0.060 SD vs. 1.041 OD +/- 0.109SD in unexposed controls while examining $3 \times 10^4$ cells, $n = 5$ each experimental condition, Figure 3).

The test with BLT treatment had higher percent of viability as osteoblasts in the BLT (with unknown number) were added on-top of tested L929 fibroblasts that were seeded in each of culture wells, but the results were consistent in both cell densities indicating on an overall lack of cytotoxic effect.

### 3.2. In vivo safety evaluation (mice model)

None of the animals were found in a moribund condition and overall presented a normal behaviour pattern. The animals were steadily gaining weight in both vehicle control and BLT treated groups, indicating a general compliance with the treatment, there were no differences in body weight between the groups (P > 0.05, ANOVA, $n = 10$, Figure 4).

Blood analysis revealed no pathological alterations of kidney function, liver function and blood electrolytes in the two tested groups on the day 62.

Histological analysis of the implantation sites revealed no neoplastic alterations. Neoplastic cells were not found in all tissue samples that were analyzed.

### 3.3. Photographic and X-ray-assisted monitoring of critical gap closure (rat model)

Upon termination of the experiment at the end of 6th week, photographs of wounds were taken and confirmed the X-ray imaging observations, which included complete closure of BLT treated wounds ($n = 3$) and minimal change in untreated wound area ($n = 3$, Figures 5 and 6).

### 3.4. Histological analysis (rat model)

Histological analysis indicated that BLT-implanted animals demonstrated significantly improved results in all evaluated parameters. BLT-filled gaps were completely closed (grade 4 of bone formation score, $n = 3$) as compared to untreated vehicle controls ($n = 3$), which had 0 mm bone regeneration (grade 0 of bone formation score, $n = 3$), with generated fibrous tissue in the critical gap (+++ of fibrosis score, $n = 3$).

The newly formed bone tissue was characterized by woven bone morphology with only minor fibrotic appearance (marginal fibrosis +) as opposed to control groups (Figure 7). In the treated by BLT animals there
was evident osseointegrative capacity as inferred from formation of bone marrow regions and pronounced vascularization (Figure 7).

4. Discussion

In this report, we aimed to assess the safety and potential efficiency of the in vitro generated BLT to be used for the bridging of critical bone defects. The in vitro generated BLT has the potential to be used as autologous bone graft and as such should resolve the current problems of harvesting morbidity and the lack of autologous bone availability for the use in the skeletal surgery.

The in vitro cytotoxicity MTT assay revealed that the BLT is not cytotoxic. The in vivo murine study showed no adverse effect of the implanted BLT.

To evaluate the proof of the BLT capability to serve as bone graft tissue in critical bone gap, the rat critical size calvarial defect model was adapted. The experiment design in terms of animal model, size of the defect and the end-point for evaluation are well suitable to provide pathophysiologically relevant data as opposed to bone formation assays in ectopic sites [18]. The critical gaps in the BLT implanted animals demonstrated complete closure of the bone critical gap by vascularized bone, as opposed to the treated by vehicle material animals that

Figure 6. Representing photographs of the BLT-treated, vehicle control with critical size calvarial defects and untreated animals. The photographs were taken at the 6th postoperative week. The circles indicate the original circular shaped gap in the calvarial bone. Normal skull photograph was taken from unoperated and untreated rat for the visual comparison.

Figure 7. Representing histological photomicrographs of calvarial defects at the 6th postoperative week. A: H&E stained preparations of controls and BLT treated rats upon completing 6 weeks of experiment. Black triangles indicate the borders of original gap prior to implantation. Long arrows point towards newly formed blood vessels inside the BLT. Normal H&E-stained skull was prepared from unoperated and untreated rat for comparison purpose. B: Enlarged micrograph image of the in situ regenerated bone – blood vessels filled with erythrocytes are presented by red arrows.
maintained an open gap without any visible closure, according to gross examination, X-ray imaging and histological analysis. The newly formed bone tissue at the sites of implantation included a characteristic woven bone morphology, which is indicative for the ongoing active and normal healing process [19]. The newly formed bone tissue was also characterized by pronounced presence of bone marrow regions and newly formed host blood vessels, a strong indication for functional osseointegration [1].

Materials or strategies, which cause bone regeneration in critical gaps are considered to have the translational potential to bridge non-union defects, or capable of generating bone at a site and time when bone would otherwise not be present [20]. Therefore, the ability of the BLT to induce significant coverage of the calvarial defects demonstrates its osteoinductive potential at pathologically relevant orthotopic sites. Furthermore, the fact that the tested BLT showed no adverse systemic or cytotoxic effects supports our hypothesis of its high safety profile following in vivo implantation.

This preliminary report indicates the potential feasibility of generating bone graft via tissue engineering for clinical use as an autologous bone graft. Following these promising results, additional future large scale in vivo studies are required for the substantial proof of concept of the presented method. In this report we show that implantation of viable human bone material, generated in vitro, for bridging critical bone defects in vivo is feasible and safe. Accordingly, there is a promise for the future development of the method for the tissue engineered autologous bone graft material availability for clinical use.

Declarations

Author contribution statement

N. Rosenberg and O. Rosenberg: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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