In vivo evaluation of chitosan-glycerol gel scaffolds seeded with stem cells for full-thickness mandibular bone regeneration

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Abstract: The aim of this study was to evaluate in vivo bone regeneration, mediated by adipose-derived stem cells (ADSCs), induced to differentiate into osteoblasts and carried by a scaffold gel. In the test group, bone regeneration was mediated by ADSCs, induced to differentiate into osteoblasts, and carried by a scaffold gel. In the control group a scaffold without cells was used. The scaffold, consisting of chitosan and glycerol phosphate, was maintained in situ by a cross-linked resorbable membrane. The osteogenic potential of ADSCs was confirmed by osteocalcin assay and Von Kossa staining performed before implantation. Histological assays detected an initial increase in bone formation in the test group compared with the control group. Microcomputed tomography analysis did not show significant differences between the two groups. Both histological and microcomputed tomography analysis were performed on the ex vivo specimens after a follow-up period of 8 weeks. We observed that differentiated ADSCs could increase bone regeneration and that the scaffold used here can be a suitable carrier to entrap and maintain the cells in situ. On the contrary, the membrane used was not functional in isolating the site of the defect from surrounding soft tissues and caused a significant inflammatory reaction.

Keywords: bone regeneration; chitosan; regenerative medicine; stem cells; tissue engineering.

Introduction

In extreme situations of bone loss that result in a critical-sized bone defect, complete spontaneous regeneration cannot occur and warrants artificial regenerative treatments (1,2). Autologous bone graft remains the gold standard for the reconstruction of bone defects owing to its advantages in terms of osteogenic potential, mechanical properties, and the absence of any adverse immune responses (1-3). However, autologous bone graft presents certain disadvantages, including a limited amount of available bone and donor site morbidity (4). On the other hand, allografts, xenografts, or artificial bone substitutes may expose the patient to severe risks of foreign body reactions and infections (5). Stem cells represent the new frontier in the field of regenerative medicine and are seen as a promising and suitable means to overcome the aforementioned drawbacks (6-8). Numerous studies report that adult stem cells can be isolated from several organs and tissues. In particular, adipose tissue contains cells that have the ability to proliferate and differentiate into multiple cell lines (9-11). Various studies have widely demonstrated the ability of adipose-derived stem cells (ADSCs) to differentiate into mesenchymal-derived cell lines (adipocytes, osteoblasts, chondroblasts, and endothelial cells) and not mesenchymal cell lines (skeletal muscle cells, cardiac cells, and neuronal cells) (12-15). Numerous papers have been published in recent years on bone regeneration for skeletal and mandibular repair using ADSCs (16-18).
Tissue engineering is a very promising approach for repair and regeneration of defective or damaged tissues and organs (19-21). However, a prerequisite when using tissue engineering approach is a suitable scaffold with an appropriate architectural design. Several preassembled three-dimensional scaffolds from synthetic or natural biomaterials have been prepared, but filling irregularly shaped bone defects using a preassembled scaffold is often difficult (22,23). A valid alternative is represented by injectable in situ-forming scaffold that can fill irregularly shaped bone defects and can be introduced using minimally invasive procedures (24-26).

Chitosan (CTS) is a natural polymer obtained by alkaline de-acetylation of chitin and CTS-based scaffolds have been shown to be biodegradable, nonimmunogenic, and biocompatible (27). Recent studies have shown that the addition of glycerol phosphate disodium salt (GP) to CTS makes it possible to obtain a liquid solution at room temperature (RT). By means of the body temperature, this injectable solution undergoes a phase transition and becomes a gel (28,29). This behavior was observed to be related to electrostatic interactions between positively charged amine groups of CTS and phosphate anions of GP (29).

The aim of the present study was to evaluate bone regeneration, mediated by ADSCs, induced to differentiate into osteoblasts and carried by a CTS/GP gel.

Materials and Methods

Adipose tissue harvesting

In this study, 10 male Sprague-Dawley rats weighing approximately 500 g were used. Upon arrival at the University of Trieste animal house, the animals were quarantined for 1 week. In vivo experiments were conducted in accordance with the National Institute of Health guidelines for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), in accordance with local laws and regulations (d.l.v. 116/92). All efforts were taken to minimize pain or discomfort to the animals used in this project: rats were anesthetized with intraperitoneal injection of tiletamine-zolazepam 5% w/v in sterile water (Zoletil, Virbac Laboratories, Carros, France; dosage: 30 mg/kg) and xylazine 2% w/v in sterile water (Virbaxil, Virbac Laboratories; dosage: 10 mg/kg). Before incision, a subcutaneous injection of lidocaine hydrochloride (10 mg/kg) was administered to achieve local anesthesia. After shaving and cleaning the skin with betadine, a vertical incision of 2 cm was performed, the adipose tissue was removed, and discontinuity was sutured using Vicryl 3-0 sutures (Ethicon Inc., Somerville, NJ, USA). Both before and after surgery, animals were treated with the antibiotic and analgesic enrofloxacin (Zobuxa, Novartis Animal Health Inc., Greensboro, NC, USA; dosage: 10 mg/kg) and carprofen (Quellin, Bayer HealthCare, LLC, Shawnee Mission, KS, USA; dosage: 5 mg/kg), respectively. Both test (n = 5) and control animals (n = 5) were identified using a microchip implanted into the dorsal region. Each rat in the test group was subjected to explant of adipose tissue in the interscapular region.

Isolation of adipose-derived stem cells

First, the adipose tissue was washed twice with phosphate buffer solution (PBS) and then cut into small pieces with a blade. Subsequently, it was transferred in a 50 mL centrifuge tube and digested with 0.1% collagenase type II at 37°C for 60 min. Collagenase was neutralized with equal amount of control medium (CM)—Dulbecco’s modified Eagle medium containing 10% fetal calf serum and 1% penicillin-streptomycin. The suspension was centrifuged at 1,500 rpm, the cellular pellet was re-suspended in the CM, distributed, and plated on 25 cm² cell culture petri dishes. Nonadherent cells were removed by replacing the CM. The medium was changed with a fresh one every 3 days. After 5-6 days, the cells reached confluence and were detached using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) and diluted to a 1:3 ratio in fresh culture dishes. After three passages, the adherent nondividing adipocytes were diluted, tested for proliferation, and then cultured in an osteogenic medium (OM). All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation

Immediately after isolation, ADSCs culture was saturated with 0.1 M glycine, permeabilized with 0.1% triton X-100, and saturated with 0.5% BSA in PBS. For bromodesoxyuridine (BrdU) staining, a passage of denaturation of DNA with 20 mM NaOH was performed for 20 s. After three washing steps in PBS, the cells were incubated for 1 h in a humid chamber at RT with primary antibody: mouse monoclonal anti-BrdU cell proliferation kit (Cell Biolabs Inc., San Diego, CA, USA) and OCG3 mouse monoclonal anti-osteocalcin (Abcam, Cambridge, MA, USA). The secondary antibody was goat anti-Mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). Total nuclei were stained with 2 µg/mL in PBS Hoechst 33342. When not specified, reagents were purchased from Sigma-Aldrich.
Osteogenic differentiation
ADSCs were cultured in an OM prepared by mixing CM with 10 mM dexamethasone, 10 mM β-glycerophosphate, and 82 mg/mL ascorbate-2-phosphate (30). For the transplant, cells were kept in OM for 14 days, changing the medium every 3 days. Subsequently, the cells were detached using 0.05% trypsin-EDTA for 5 min. After trypsin neutralization with OM, the medium cells were centrifuged and the pellet re-suspended in fluid scaffold at a concentration of 10⁶ cells/mL. Reagents were purchased from Sigma-Aldrich.

Chitosan scaffolds preparation
CTS scaffolds were prepared as described elsewhere (28,29). In brief, a CTS solution was obtained by dissolving the polymer in 0.1 M acetic acid solution prepared in deionized water (DW) at a final concentration of 2.2% w/v (polymer/solvent) and sterilized by autoclave. Glycerol phosphate disodium salt was dissolved in DW at a concentration of 44.4% w/v, sterilized by filtration (0.22 μm; Millex Syringe Filters, Merck-Millipore, Darmstadt, Germany), and added dropwise to the CTS solution. Reagents were purchased from Sigma-Aldrich.

Von Kossa staining
Cells cultured on coverslip were fixed with 4% paraformaldehyde for 20 min at RT after 0, 7, and 14 days of culturing in OM. After several washes in DW, samples were incubated with 1% silver nitrate in water for 20 min at RT under ultraviolet (UV) light. Un-reacted silver was removed by dipping the samples in 5% sodium thiosulfate for 5 min. Subsequently, samples were washed with DW, and cells were counterstained with 0.1% nuclear fast red solution (0.1% fast red + 5% aluminum sulfate). After a final washing step in DW, coverslips were mounted on microscope slides and observed under light microscope. Reagents were purchased from Sigma-Aldrich.

Creation of the mandibular defect
The surgical strategy used in this phase followed previous published protocols (28). In detail, 2 weeks after the removal of adipose tissue, surgery was performed in both groups of animals. Rats were anesthetized with tiletamine-zolazepam 5% w/v in sterile water (Zoletil, Virbac Laboratories; dosage: 40 mg/kg) and xylazine 2% w/v in sterile water (Virbaxil, Virbac Laboratories; dosage: 10 mg/kg). After local infiltration of lidocaine hydrochloride (4 mg/kg), a horizontal incision was made approximately 1 cm below the lower border of the jaw. After performing dissection of the subcutaneous tissue, the masseter muscle was disengaged to expose the underlying bone, taking up the mandibular angle as a surgical landmark. A bone defect of approximately 3 × 3 mm was created from the bottom edge of the jaw mesially to the gonion and distally up to the molars by using a diamond burr (1.5 mm of diameter) under constant saline irrigation. Subsequently, a resorbable cross-linked collagen membrane (Cytoplast, Osteogenics Biomedical Inc., Lubbock, TX, USA; dosage: 10 mg/kg) was applied on the bone defect and sutured with Vicryl 3-0 (Ethicon Inc.) to the bone by means of two holes (one mesial and one distal to the defect) obtained by a diamond burr under irrigation. In the test group, osteoblasts derived from stem cells of the same rat and suspended in the CTS/GP gel scaffold were injected using a syringe into the defect covered by the membrane. In the control group, only the scaffold without cells was injected into the defect covered by the membrane.

The surgical wounds were sutured with Vicryl 5-0 (muscle) and Vicryl 3-0 (Ethicon Inc.). In order to prevent the possible development of infections or painful inflammatory complications, enrofloxacin (Zobuxa, Novartis Animal Health Inc.; dosage: 10 mg/kg), carprofen (Quellin, Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS, USA; dosage: 10 mg/kg), and tramadol hydrochloride (Zydol, Grünenthal Ltd, Stokenchurch, UK; dosage: 10 mg/kg) were administered immediately before and for 3-5 days after surgery.

Sacrifice and sampling
All animals were sacrificed after 8 weeks from the date of the surgery (31). The sacrifice was performed by carbon dioxide inhalation. A portion of the mandible of approximately 1.5 cm in length that included the defect was sectioned using a diamond burr. Immediately after the explant, all samples were immersed in a 4% formaldehyde-buffered fixative solution (Sigma-Aldrich).

X-ray microcomputed tomography (μCT)
Samples treated with 4% formaldehyde-buffered solution were placed in 1.5 mL sealed centrifuge tubes filled with fixative solution before μCT scanning. This procedure made it possible to perform μCT under wet conditions without evaporation of the solvent. The μCT of the samples was obtained by means of a cone-beam system called TOMOLAB (www.elettra.trieste.it/Labs/TOMOLAB). The device is equipped with a sealed microfocus X-ray tube, which guaranteed a focal spot size of 5 μm in an energy ranging 40-130 kV, and a maximum current of 300 μA. As a detector, a CCD digital camera was used with a field of view of 49.9 × 33.2 mm² and a pixel size of 12.5 × 12.5 μm². The samples were positioned onto
the turn-table of the instrument, and acquisitions were performed using the following parameters: distance source-sample (FOD), 100 mm; distance source-detector (FDD), 200 mm; magnification, 2×; binning, 2 × 2; resolution, 12.5 μm; tomographic dimensions (pixels), 1,984 × 1,024; slice dimensions (pixels), 1,984 × 1,984; number of tomographies, 1,440; number of slices, 864; E = 40 kV, I = 200 μA; and exposure time, 2.5 s. The slice reconstruction process, achieved by means of commercial software (Cobra Exxim, Pleasanton, CA, USA), was initiated once the tomographic scan was completed and all projections were transferred to the workstation. Input projections and output slices were represented by files (one file per projection and one file per slice) by using arrays of 16-bit integers.

Three-dimensional visualizations of the reconstructed slices were performed by means of OsiriX v.3.9.4. 64 bit Imaging Software (open source). This software made it possible to identify accurate angulation and segmentation of the samples from which the planar three-dimensional reconstruction was obtained (Fig. 1). From the three-dimensional reconstruction, a segmentation based on gray levels was used to identify both newly formed bone and native bone. The gray-level threshold between newly formed bone and native bone was selected with the help of the surgeon and an expert radiologist (32,33). The percentage of newly formed bone was calculated by dividing the volume of the newly formed bone by the volume of the original defect. Calculation was performed by means of Image ProPlus 6.2 software (Media Cybernetics, Rockville, MD, USA). To evaluate the newly formed bone, an internal control was used. To this end, the site of the holes used by the surgeon to fix the membrane around the defect was exploited as a virtual reference. These circular defects were initially 1.5 mm in diameter, and by means of the image analysis procedure the value of the newly formed bone in the original defect was obtained.

Histological analysis
Immediately after µCT scans, the samples were sent to the Department of Anatomy and Histology of the University of Trieste for histological analysis.

Samples were immersed in a solution of EDTA disodium in acid buffer for 5 h. Subsequently, the samples were placed in histology cassettes, and the biocassettes were included in the histo-processor, where with a predetermined sequence and timing, the samples were postfixed in 10% buffered neutral formalin, dehydrated through an ascending scale of ethanol (from 50 to 100%), clarified with xylene, and permeated by liquid paraffin at 60°C. Thereafter, the material was embedded in paraffin and allowed to solidify on chilled plates. The obtained block was then sectioned using a microtome, and 8 μm thick histological sections were spread on a glass slide and placed in an oven at 60°C for 1 h to ensure good adhesion of the sections to the glass slides and, at the same time, to dissolve the paraffin excess. Subsequently, the sections were stained with hematoxylin and eosin. After staining, the preparations were dehydrated and mounted with resin, which was placed on the coverslip.

Statistical analysis
Statistical analysis was performed using SPSS 21.0 software for Macintosh (SPSS Inc., Chicago, IL, USA). Because the data for the newly formed bone was not normally distributed in both test and control groups according to Levene’s test, a Mann-Whitney nonparametric test was used. Values of $P < 0.05$ were considered statistically significant.

Results

Proliferation of ADSCs
The ability of isolated cells to proliferate in culture was assessed using BrdU incorporation assay. This test is related to the synthesis of new DNA and could thus be used to identify cells in active proliferation. ADSCs were treated as described in the Materials and Methods section. BrdU incorporation indicated substantial proliferation activity during the incubation period (Fig. 2).

Differentiation of ADSCs
Differentiation of ADSCs into osteoblasts was assessed by means of a Von Kossa assay performed at 0, 7, and 14 days from culturing in OM. The dark calcium salt deposits were barely visible at time zero (Fig. 3A), fairly represented after 1 week (Fig. 3B), and abundant after 2 weeks (Fig. 3C) of culturing in OM. The differentiation level was detected by means of an immunofluorescence assay against osteocalcin, a protein abundant in mature
bone tissue but weakly expressed in developing tissue. Osteocalcin was absent at time zero (Fig. 4A), barely represented after 1 week (Fig. 4B), and abundant after 2 weeks (Fig. 4C) of culturing in osteogenic medium.

**X-ray µCT**

Figure 5 presents the quantitative results obtained by means of image analysis. In the internal controls, i.e., the site of the holes used by the surgeon to fix the membrane around the defect (Fig. 1A, black arrows), the percentage of the newly formed bone was obtained by dividing the volume of the newly formed bone by the volume of the cylindrical hole generated by the burr. In the defects, the percentage of newly formed bone was obtained by dividing the volume of the newly formed bone (Fig. 1B) by the volume of the original defect (Fig. 1C). Mann-Whitney U test revealed no significant difference between the two groups for the following subgroups: internal control ($P = 0.275$) and newly formed bone ($P = 0.827$).

**Histology**

Histological analyses revealed that the presence of initial bone regeneration (Fig. 6A) with elements arranged to form an edge close to the surface of the vital bone (Fig. 6B) was higher in the test cases than in the control ones.
(Fig. 7). In addition, an increased cellularity inside the membrane fibers (Fig. 6C) was observed in the test group samples. In both test and control samples, foci of necrosis and a marked fibroblastic cell component were observed, but the inflammatory aspect was more prominent in the control group. Moreover, in the latter group, the regenerative aspect was less represented (Fig. 7A), abundant necrotic areas could be detected, and nevertheless, membrane fibers were poorly colonized by cells (Fig. 7B). In all control samples and in certain test samples, a well-capsulated abscess-type lesion, surrounding the bone defect, was detectable (Fig. 7C).

**Discussion**

Two groups of five rats each were used for this study. After surgery and until the date of sacrifice, recovery of all animals involved in this study was considered normal in terms of eating behavior, weight, and quality of life. The cue for the morphology of mandibular defect used here was derived from the study conducted by Kostopoulos and Karring (31). They performed a 2 × 3 mm split-mouth defect at the level of the inferior edge of the mandibular branch. The defect was covered only on one side with a resorbable membrane, leaving the contralateral side exposed. In our experiment, which was performed unilaterally to reduce postsurgical morbidity, we used stem cells for bone regeneration in the test group alone, whereas a scaffold gel and a cross-linked collagen membrane were used in both groups to fill the defect. To the best of our knowledge, this is the first study wherein a CTS scaffold was used to regenerate mandibular defects in an animal model by means of ADSCs, where the cells were differentiated before implantation. Histologically, we observed better outcomes in the test group owing to the presence of initial bone regeneration demonstrated by the presence of osteoblastic cells forming a parallel edge at the level of healthy bone. This observation allowed to conclude that the scaffolds used were able to maintain cells in situ, concordant with the results presented by Kim et al. (29).

On the contrary, the membrane used here, besides not being particularly easy to manipulate, exhibited a marked inflammatory reaction in all samples with the presence of necrotic areas (occasionally extremely wide and up to 10% over the total area analyzed), foreign body cells, and fibroblasts. Kozlovsky et al. (34) reported that by treating the membrane with cross-linking agents, such as UV radiation, glutaraldehyde, diphenyl-phosphoryl-azide, or hexamethylenediisocyanate, to slow down re-adsorption, the membrane itself could become partially cytotoxic and thus not be compatible with attachment and proliferation of human osteoblasts.

The results obtained by means of μCT did not show significant differences in bone regeneration between the test and control samples; this could be attributed to the limited resolution of this technique (i.e., 12.5 μm). μCT is extremely efficient in detecting three-dimensional features of solid samples, but this ability is also limited by device resolution (32,33,35-38). Improving this aspect would highlight the detailed differences between the two groups tested in the present study. Unfortunately, the efforts of the authors to obtain the best setup for the μCT device were not sufficient to detect if any substantial differences were present between both groups. For the variable “percentage of newly formed bone” detected in the defect (Fig. 5), the standard deviation of the test group was larger than that of the control group. We speculated that this behavior could be attributed to the presence of ADSCs in the scaffold. These cells could have contributed to enhance the differences between a regenerative site supported by just a scaffold and a site wherein a system (scaffold and cells) is implanted (39).

Following Frost’s theory (40), the process of bone healing initiates with an inflammatory response and recruitment of several cell types, including osteoprogenitor mesenchymal stem cells (MSCs) at the site of injury. Any condition that alters this mechanism can disrupt the normal bone healing process (41). We think that the presence of the scaffold seeded with ADSCs could have interfered with the healing mechanism not in a way of hampering it, as indicated by the lack of statistical differences in the percentage of newly formed bone between the test and the control groups, but enhancing the differences among the samples of the test group. Histological examination revealed the presence of early bone changes in the test group. We assessed the presence of initial bone regeneration when the scaffold with osteoblasts, derived by adipose stem cells, was placed in the bone defect, even if the inflammatory reaction slowed the healing and regeneration processes. Additional studies will be
conducted to quantify the role of stem cells associated with a scaffold in bone regeneration, including that on defects with critical dimensions and irregular geometry. Moreover, further research will overcome the limitations of restricted population and the absence of a positive control (i.e., a commercial gold standard for bone regeneration) that arose in the present preliminary study.

Conflict of interest
None of the authors have any commercial or proprietary interest in the product used in this study.

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