Investigation of a Hydroxyapatite/tri-calcium-phosphate Scaffold as Carrier for Mesenchymal Stem Cells in Alveolar Bone Regeneration

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The aim of this study was to asses alveolar bone repair by allogeneic mesenchymal stem cells using synthetic hydroxyapatite (HA)/tricalcium phosphate (TCP) granular scaffolds as a carrier for alveolar bone tissue engineering. Rat mesenchymal stem cells were obtained from iliac crest bone marrow of Sprague-Dawley rats and expanded without differentiation. Cells were resuspended at a final concentration of 5×10^6 cells/ mL, half in simple osteogenic differentiation medium and the other half in BMP4 – enriched medium on a synthetic HA/TCP scaffold. After another 7 days they were characterized in vitro to prove the viability of the implanted cells. The structures were further implanted in the surgically created bone defects. After 4 weeks, cell viability and differentiation was evaluated by histological analysis. After 4 weeks, cells presented an obvious change in their aspect, having a round shape, polygonal, having a tendency to conflict in concentric circles. Cell cultures with or without BMP contained 58.25 ±18.43% or 43.35 ± 17.68% bone area in vitro ($p = 0.049$), respectively. There was no statistically significant difference in percent bone area when cells in simple osteogenic differentiation medium or BMP4 enriched medium placed on HA/TCP scaffolds were compared at either time point. The HA/TCP scaffolds allowed for the delivery of allogeneic mesenchymal stem cells in a clinically manageable form that enhanced bone formation at early stages of alveolar repair.

Keywords:synthetic hydroxyapatite (HA), tricalcium phosphate (TCP), stem cells

Advancements in the prevention of dental disease have been paralleled by innovation in treatment techniques and materials to replace missing teeth. Trauma, periodontal disease, congenital and acquired defects, or surgical resection of cancer also result in reduced volume of bone with inferior physical properties. Predictable, simple, economical bone grafting procedures are desired.

Stem cells are present in most tissues and play a very important role in tissue repair processes and homeostasis. Adult stem cells have the capacity to regenerate but also to generate different types of tissues. Understanding the mechanism of stem cell regeneration, can unveil the origin of multicellular organisms and the regeneration of several tissues.

Mesenchymal stem cells can differentiate into osteoblasts and undergo mineralization when they are cultured in the presence of ascorbic acid, b-glycerophosphate and dexamethasone (Dex) [1, 2]. From physicochemical and biochemical analyses, such mineralization is not a simple precipitation of calcium and phosphorous but is a biological accumulation of apatite, which exists in natural bone [3]. Cultured MSCs can differentiate into osteoblasts not only on tissue culture polystyrene (TCPS) dishes but also on the surfaces of biomaterials such as bioactive calcium phosphate ceramics [4, 5] and bioinert alumina ceramics [6] or titanium alloys [7–9]. The cultured osteoblast/biomaterial constructs show in vivo osteogenic capability as evidenced by new bone formation after implantation [10–13].

The purpose of this study was to asses alveolar bone repair by using synthetic hydroxyapatite (HA)/tricalcium phosphate (TCP) granular scaffolds as a carrier for allogeneic mesenchymal stem cells as an alveolar bone tissue engineering technology.

Experimental part

Materials and methods

Scaffold preparation

As support scaffolds for the mesenchymal stem cells, highly purified, biocompatible β tri-calcium phosphate cylinders (RTR Septodont, France) consisting of micro- and macroporouse granules, was used. The particle dimension varies between 500μm – 1 mm, having macro porosities of 100μm – 400μm and microposities smaller than 10μm which gradually deliver calcium and phosphate ions, supporting the colonisation of osteoprogenitor cells. Scaffolds were adapted to the dimension of the alveolar sockets at a size of a 4 mm radius and a height of 2 mm. After sterilizing them, they were put on culture plates. Cells from the second passage were resuspended in 15-20 μL culture medium and placed onto the scaffolds so that to cover a large surface at a density of 1.5×10^4 cells/ cm^2. They were allowed to adhere to the scaffolds for 20 min and then another 1.5 mL medium was added. Cells were resuspended at a final concentration of 5×10^6 cells/mL, half in simple osteogenic differentiation medium and the other half in BMP4 – enriched medium on a synthetic HA/ TCP scaffold. After another 7 days they were characterized in vitro to prove the viability of the implanted cells. The structures were further implanted in the surgically created bone defects. Results were evaluated by histo-morphometric analysis 4 weeks after implantation.

Isolation and characterization of mesenchymal stem cells

Rat mesenchymal stem cells were obtained from iliac crest bone marrow of Sprague-Dawley rats, by fine needle aspiration technique, approved by the Ethics Committee of the University of Medicine and Pharmacy "Victor Babes" Timisoara. Cells were isolated from each animals aspirate

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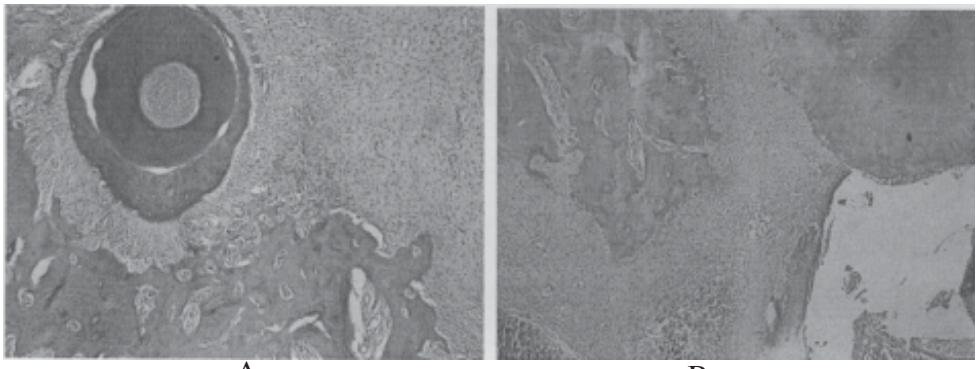

A B

Fig. 1 A, B: high volume of "de novo" formed alveolar bone, positive response from the host shown by osteoconduction at the bone-implant interface, intermembranous osteogenesis on the intern wall in proximity of the neighboring tooth.

Table 1

GROUP STATISTICS – BONE FORMATION AFTER IMPLANTATION OF TRI-CALCIUM-PHOSPHATE SCAFFOLDS LOADED WITH STEM CELLS IN OSTEOGENIC DIFFERENTIATION MEDIUM WITH OR WITHOUT BMP

	Medium	N	Mean	Std. Deviation	Std. Error Mean
x	tepstem	10	56.6220	10.35390	3.27419
	tepstembmp	10	60.7850	12.07596	3.81876

by simple plastic adherence on MEM Alpha culture medium (MEM - Alpha; Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented by 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 10 ng/mL fibroblastic growth factor (FGF; Sigma, St. Louis, MO, USA) and 2% mixture of Penicillin/Streptomicin (Pen/Strep, 10,000 IU/mL; PromoCell) and further expanded on DMEM. MSC were characterized by assessing quantitative parameters like cell density, cell viability, and qualitative parameters like population doubling time, clonogenity and plasticity ensuring their mesenchymal character.

Statistical analisys

Statistical analysis of bone formation was performed by t-test analysis using SPSS 17. Significance level was set at $p<0.05$.

Results and discussions

Microscopy culture scanning

After 7 days of culture, cells observed using the Nikon Eclipse E600 showed small conglomerates. Adherent conglomerates had fibroblastoid aspect, characteristic for mesenchymal stem cells.

Scaffold analysis

Before implantation, the maintenance of cell viability on the scaffold was assesed by observing the extra cellular matrix deposits using the Jakob von Kossa mineralization technique, showing clear presence of mineralization areas.

Hystomorphometric analysis of bone formation

In the areas where TCP scaffolds have been implanted, bone formation could be observed at an extended degree. Some differences could be observed between implants with or without BMP but they were not statistically significant. Sections through the central part of the scaffold show the existence of a large amount of bone formed *„de novo"*. Osteoconduction processes at the bone-implant interface indicate a positive response of the host. (fig. 1).

Statistical analysis

Cell cultures with and without BMP contained 60.78 ±12.07% and 56.62 ± 10.35% bone area (p = 0.049), respectively. There was no statistically significant difference in percent bone area when cells in simple osteogenic differentiation medium or BMP4 enriched medium placed on HA/TCP scaffolds were compared at either time point (table 1).

A large number of materials have been used as support for mesenchymal stem cells in other studies. Titanium alloys including pure titanium are widely utilized as substitutes for dental implants [14] and the fixing of fractures (as fixation plates and screws) [15]. Titanium is bioinert and biocompatible, and shows osteoconductive property when implanted in bone defects. In previous study, Ohgushi and Caplan [3] have reported that osteoinductivity was given by making the marrow cell/titanium composites [3]. However, mineralization through the osteogenic differentiation of osteogenic cells on titanium has not been fully examined. In contrast, TCP dishes are recognized as the gold standard of substrates for cultured cells because their surfaces are chemically modified to be suitable for cell adhesion. During recent research we concluded that tri-calcium-phosphate scaffolds can be successfully used as carriers for stem cell implants used for bone regeneration.

The direct implantation of a large number of osteoprogenitor cells (MSCs) within the appropriate matrix accelerates the process of bone formation and reduces the need for massive proliferation of the osteoblast progenitor cells into the defect. Only a single concentration of cells was used in this study ($5x10^6$/mL). The 4-week time point for analysis was selected as an opportunity to view bone formation at an early time point. This was based on earlier studies using canine MSCs in a critical size defect. In other preliminary studies, using less than $0.5–1.0\times10^6$ cells/ml to load HA/TCP scaffolds did not support reproducible bone formation. While higher cell numbers may be effective, 1.0×10^6/mL was successful in regenerating bone in 8 weeks by use of a polyglycolic acid scaffold [4,5].

Tissue engineering supposes the use of progenitor cells on biocompatible tridimensional scaffolds and their stimulation with different signaling molecules or growth factors. In case of tissue engineering, the support scaffold is very important because cells need anchorage. The ideal

matrix has to be biocompatible, easy to handle, have osteoconductive and osteoinductive properties and at the same time have a resorbtion rate that allows the formation of new tissue [5,6].

Another hypothesis implicates growth factors that modulate the innate cell activity. They have pleiotropic effects, being involved in the healing processes of all tissues, including alveolar bone, periodontal ligaments and cement. Bone morphogenic proteins (BMP) represent osteoinductive key molecules, being involved in the complex regulation of bone physiologic processes. BMPs are abundant in bone tissues, being also expressed by many cell types, having the role to commit pluripotent undifferentiated cells to differentiate into bony tissues or other tissues such as dental structures [3]. This is why is used the applying BMPs on to support matrices in order to regenerate dental tissues.

Our studies have indicated that implant constructs can be transplanted into different sites with no adverse immunologic or inflammatory consequences. For a successful regeneration, it will be necessary to utilize and recruit progenitor cells that can differentiate into specialized cells with a regenerative capacity, followed by proliferation of these cells and synthesis of the specialized connective tissues which they are attempting to repair. Clearly, a tissue-engineering approach will need to utilize the regenerative capacity of cells and will involve the isolation of such cells and their subsequent proliferation within a three-dimensional (3D) framework. The use of a prefabricated 3D scaffold, with the appropriate cells or instructive messages (e.g. growth factors and matrix-attachment factors) incorporated into it, may overcome many of the limitations associated with current regenerative technologies.

Conclusions

Our study shows that the HA/TCP scaffolds allowed for the delivery of allogeneic mesenchymal stem cells in a clinically manageable form that enhanced bone formation at early stages of alveolar repair.

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