Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 303 - 311

ALVEOLAR BONE REPAIR USING MESENCHYMAL STEM CELLS PLACED ON GRANULAR SCAFFOLDS IN A RAT MODEL

R. SAVA-ROSIANU, A. C. PODARIU, R. A. POPOVICI, G. TANASIE[a], L. ILIUTA[b], R. OANCEA[*]
Department I - Faculty of Dentistry, University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania
[a]Department of Physiology and Immunology, University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania
[b]Department of Marketing, Technology and Informatics, University of Pharmacy "Carol Davila", Bucharest

Recent progress in oral disease prevention has developed parallel to discovering new treatment protocols and materials which can replace damaged oral tissues. The purpose of this study was to asses alveolar bone repair by autologous mesenchymal stem cells and tri-calcium phosphate (TCP) granular scaffolds placed into surgically created bone defects in post-extractional sockets. Mesenchymal stem cells were obtained from femur bone marrow of 20 Sprague-Dawley rats without killing the animals, and characterized. Cells were suspended and placed onto a TCP scaffold into edentulous defects of the same rat's maxilla. As control, mandibular sockets from each animal were used. Tissue healing was histologically evaluated assessing bone formation at 5 and 21 days after implantation. Analysis of obtained data showed normal bone formation in the control sockets. Substantially more bone formation was observed in the sockets were the cell implants were placed. Cellular infiltrate was an indicator of normal granulation, new vascularization and osteoconduction processes. There were no signs of an immunological response from the host due to the autologous character of the implants. Mesenchymal stem cells placed on granular scaffolds offer the advantage of a direct delivery of cells that can be responsible for osteogenesis improving the physiologic bone repair.

(Received December 14, 2012; Accepted January 31, 2013)

Keywords: Mesenchymal Stem Cells, Tri-calcium phosphate Scaffolds, Bone Regeneration, Rat Model

1. Introduction

Recent progress in the field of oral disease prevention has developed parallel to discovering new treatment protocols and new materials which can replace damaged oral tissues. Traumatic lesions, periodontal disease, congenital defects or substance loss due to neoplastic lesions can lead to the decrease of alveolar bone volume and affect its properties. Patients suffering from these conditions need bone reconstruction and replacement of missing dental units. Novel bone repair and regeneration techniques involve osteogenesis, osteoinduction and osteoconduction processes[1]

One hypothesis of tissue engineering supposes regeneration with help of molecules, cells or a combination of those with biocompatible materials to assure their support and enhance physiological healing processes[2]. Thus, it involves the use of progenitor cells placed on biocompatible tridimensional scaffolds and stimulation by signaling molecules or growth factors. Implantation of in vitro expanded MSCs within the appropriate scaffold resulted in bone

[*]Corresponding author: roancea@umft.ro

regeneration in various animal models[1]. The specific aim of our study was to histologically assess the viability and the efficiency of autologous MSCs delivered within a TCP scaffold, in a rat alveolar defect having as control empty sockets and sockets with simple biomaterial implants.

Bone marrow contains a large population of pluripotent stem cells that are undifferentiated, also known as stromal cells[3] or mesenchymal stem cells[4-6]. They can be differentiated into several cell types including osteocytes, chondrocytes, adipocytes, cardiomyocytes, giving birth to a large number of tissues[7-9]. They can be isolated from the bone marrow, cultured in vitro and implanted into bone defects in order to repair bone loss[6]. MSCs have been previously shown to heal bony defects in an autologous setting. With this approach, in other studies, marrow was harvested; the MSCs were subsequently expanded and then administered to the subject. If feasible, allogeneic donor derived MSCs present an attractive alternative to using autologous cells. By using donor derived cells, the need for harvesting and expanding cells for each patient is eliminated. Because potentially billions of cells may be expanded from an individual donor, many devices may be created from rigorously tested and qualified cells. Therefore, an allogeneic MSC based bone regeneration construct for the augmentation and repair of alveolar bone was developed by other researchers. The constructs were also evaluated in the context of a critically sized mandibular defect in the dog[10].

The supporting scaffold plays a very important part because these cells need anchorage. The ideal scaffold has to have osteoconductive and osteoinductive properties, but at the same time to have a resorbtion rate that allows new bone formation and neovascularisation[1]. New polymeric scaffolds have yet to reach clinical investigation. Current hydroxyapatite/CaPO4 materials serve a space-maintaining function and are osteoconductive, but they are also slowly resorbed. Their behavior has been shown to vary with respect to patient, defect size and defect position[10]. MSCs adhered to hydroxyapatite/tricalcium phosphate (HA/TCP) matrices that were subsequently implanted into subdermal pouches of immunodeficient mice demonstrating bone formation as early as 3 weeks[11].

The objective of this study was to create an animal model in order to test the viability and efficiency of autologous mesenchymal stem cells implants placed on tri-calcium phosphate (TCP) scaffolds. Scaffolds were placed into surgically created bone defects after the extraction of rat molars. The novelty of this study resides in the fact that bone marrow derived mesenchymal stem cells have been obtained from rats without having to kill the animals. After isolation, MSC have been expanded in vitro and placed on TCP scaffolds. The resulting structures were then implanted into bone defects of the same animals, surgically created after the extraction of first molars. Scaffolds and MSC implants have been placed directly in the bone defect where tooth extractions have been practiced allowing us to quantify real time results.

Knowing the fact that the adult regeneration potential is often limited, we tried to create new methods to replace damaged tissue through autologous cells using two of their most important properties: their proliferation capacity and their pluripotency.

2. Experimental

Animals - For the present study we used pluripotent mesenchymal stem cells obtained from femur bone marrow of 20 Sprague-Dawley rats. Cells were obtained according to a previously described working protocol[17], approved by the Ethics Committee of the University of Medicine and Pharmacy Timisoara, Romania. All experiments underwent full protection according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986) and to all european understandings signed by our country (EU Directive 93/35/EEC, Amendament 76/768/EEC, directive 86/609 CEE), as well as the romanian law (Law 471/2002, Ordonance 37/2002).

Animals were anesthetised after being weighed, using a Ketamine-Xilazine mixture. A needle was used to pierce the skin and muscles of the anterior thigh just above the knee (without opening the ankle) until the rough surface of the femur was felt. Keeping the needle perpendicular on the bone surface, an inlet was created at the epiphisis-diaphisis junction using a ferm rotative move. A syringe was attached to the needle and approximately 0,6-1 ml aspirate was obtained from each animal and placed onto 0.1 ml heparin to prevent coagulation, for further processing.

Each syringe received the label of the donor animal to make sure that cells will be received by the same animal, assuring the autologous source.

The wound was disinfected and bandaged and the animals were supervised until they recovered from the anesthesia.

Isolation of mesenchymal bone marrow derived stem cells - Mesenchymal stem cells were isolated from each animals aspirate 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.

Characterization of mesenchymal stem cells - MSC were charachterized by assesing quantitative parameters like cell density, cell viability, and qualitative parameters like population doubling time, clonogenity and plasticity ensuring their mesenchymal character.

Cell density was evaluated using the standard numbering method in the Neubauer chamber. For cell viability, the Trypan blue microscopy method was used which colors dead cells with lised cell membrane in blue while normal cells remain uncoloured and can be seen in the hemocytometer.

To study the clonogenic ability of the cells, they were placed onto 12,5 cm^2 plates at a density of 100.000/plate. Colonies have been assessed after 14 days.

For the plasticity assessment, mesenchymal stem cells from the second (day 42) or third (day 63**)** passage were used and their ability to differentiate into the adipogenic and osteogenic cell line was studied. Osteogenic differentiation was obtained on Miltenyi Biotec ready to use culture medium. Cells were cultured at a density of $10^5/cm^2$. Cell matrix mineralization was histochemically analyzed through the Von Kossa technique which evidentiates the presence of calcium salts that are coloured in black.

Adipogenic differentiation was determined by culturing the cells on DMEM with 60μm indomethacine and 10^{-6} rosiglitazone at a density of $10^5/cm^2$. Adipogenic differentiation has been assessed by the Oil Red staining.

Approximately 1×10^6 cells/well were incubated for 30 min at 4 °C with the following set of primary mouse anti-rat antibodies: anti-CD45, anti-CD90, anti-CD73 and anti-CD54 22,23 (BD Pharmingen, USA) at a dilution of 1:50.

After washing with phosphate buffered solution (PBS), the cells were incubated with secondary Alexa 488 anti-mouse antibody (Invitrogen, USA) for 30 min at 4 °C and then washed with PBS. The cells were fixed with 200 μL of 2% formaldehyde and analyzed using a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, USA). The data were analyzed with Cell Quest Software (BD Biosciences, USA). Approximately 30,000 event numbers were acquired.

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 as to cover a large surface at a density of $1{,}5x10^4 cells/cm^2$. They were allowed to adhere to the scaffolds for 20 minutes and then another 1,5 ml medium was added. 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.

Tooth extraction protocol and implants placement - Maxillary and mandibular first molars have been extracted under general anesthesia using a Gracey quirette for the sindesmotomy. Molars were sectioned with a tungsten carbide bur under constant cooling. The mesial half was removed with a small plier, the distal half being afterwards dislocated with the quirette the bone defects was enlarged using a special bur.

Autologous MSC on TCP scaffolds were placed in the resulting maxillary defects of each animal, immediately after the extraction, under general anesthesia. As control one mandibular sockets was kept empty and the other one received only TCP implants, for each animal. Sockets were sutured.

A group of 5 animals have been sacrificed 5 days after implantation to observe early signs of bone formation, the other 15 being kept alive for 21 days after implantation to observe the final phase of bone formation. The bone fragments were collected, preserved in 10% formaldehyde and transported to the Pathology laboratory for further processing. Fragments have been decalcified, sectioned, put on slides, stained with Hematoxylin-Eosin and examined at the optical microscope.

Statistical analisys - Statistical analysis of bone formation was performed by t-test analysis using SPSS 17. Significance level was set at $p<0.05$. The t-test analysis generates three sets of data. The first set indicates mean bone formation standard deviation and mean standard error for both groups. The second set shows how the values are correlated and the third data set provides the statistical significance of the obtained results. By dividing the mean difference between the two groups to the mean standard error the value of „t" can be seen. It also indicates the two-tailed significance level that has to be smaller than 0.05/0.01 in order for the results to be statistically significant.

3. Results

Microscopy culture scanning - Cultures were observed using the Nikon Eclipse E600 every 48 h. After the first week small conglomerates of adherent cells were seen. Even though there weren't many cells, they had fibroblastoid aspect, characteristic for mesenchymal stem cells (Fig.1 A).

Cell growth analysis - Cell growth analysis and viability staining with trypan blue showed that viability was over 90% at the first passage and 80-85% at the second passage.

After Giemsa coloration a number of $5,3\pm0,8/\ 10^5$ cells have been observed during clonogenity tests (Fig1 B).

Following the calculation of the population doubling time we obtained the results of 5.9 (±0.7) days for the first passage and 6.8 (± 1.2) days for the second passage (Fig 1 B).

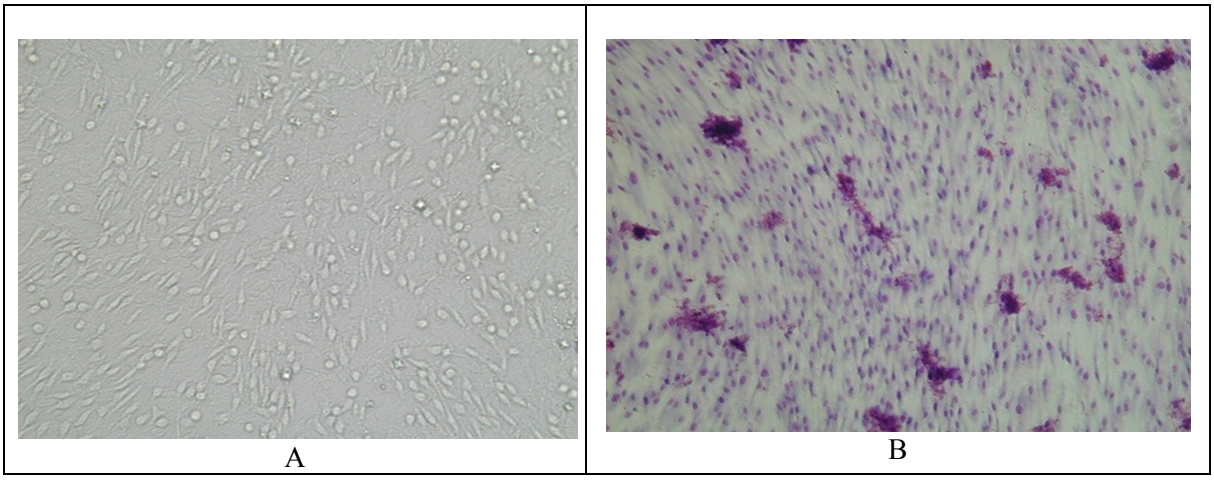

Fig. 1. Characterization of mesenchymal stem cells; A. Characteristic aspect of mesenchymal stem cells with fibroblastic shape, disposed in small conglomerates, magnification x 10; B. Giemsa coloration used for the clonogenity testing.

Plasticity assessment - Osteogenic differentiation was assessed 15 days after placing the cells on osteogenic differentiation medium. Approximately 20% of the cultured cells changed their shape becoming polygonal and having a characteristic osteoblast aspect.

Further evaluation of the culture showed a clear change of the cellular shape. They became rounded, polygonal with a tendency to gather in concentric circles (Fig 2A, B).

Adipogenic differentiation was visible 10 days after placing the cells on adipogenic differentiation medium when approximately 25-30% of the cultured cells changed their shape, staring to accumulate cytoplasmic globular deposits, typically for adipose cells (Fig. 2 C**).**

This aspect could be observed with the contrast phase microscope as well as using OIL red O staining that evidences cellular lipoid inclusions.

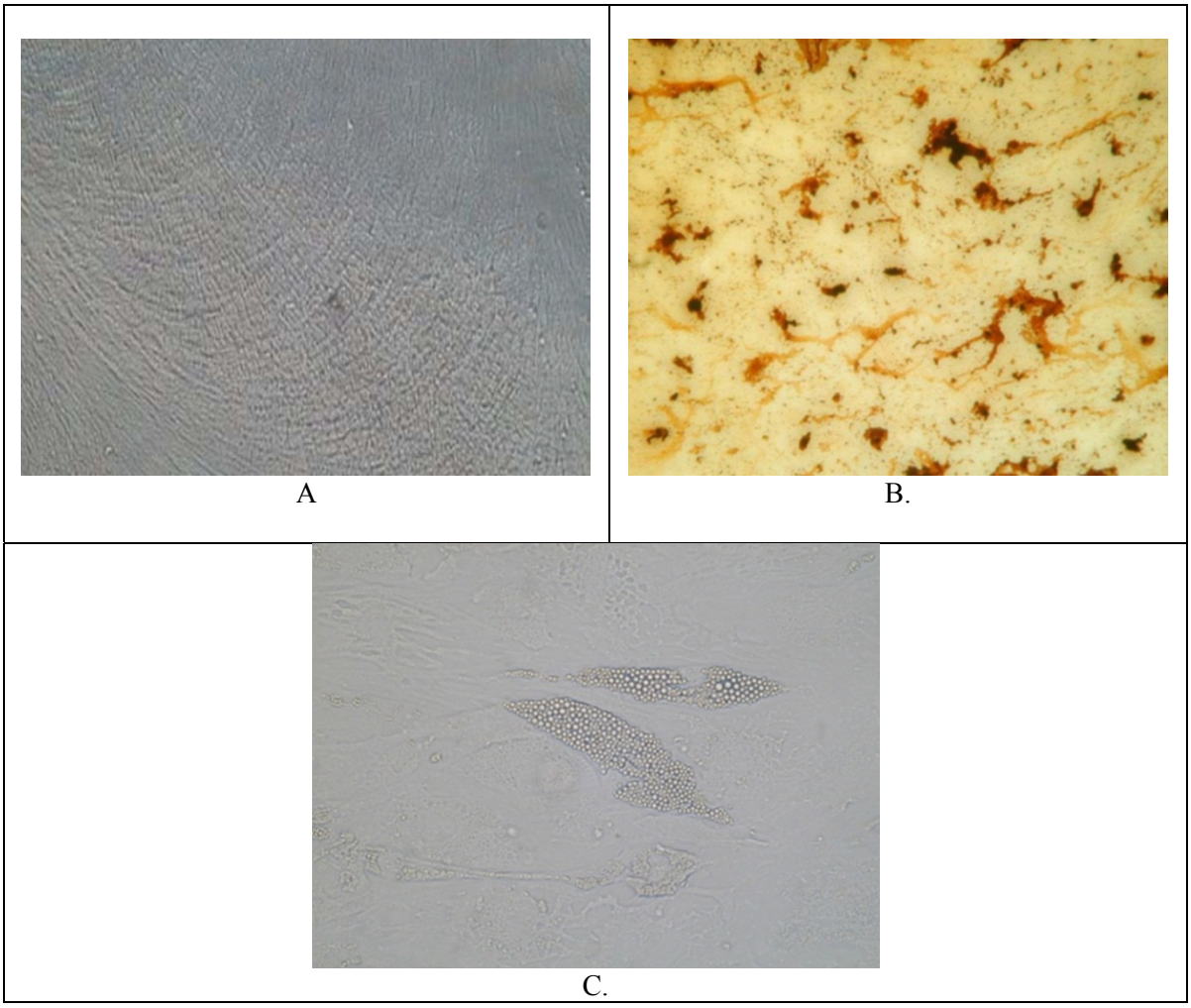

Fig. 2. A. Plastic potential of mesenchymal stem cells; Osteogenic cell line differentiation, mesenchymal stem cells changing their shape and starting to deposit extracellular matrix, magnification x 10; B. Osteogenic differentiation; Mineralization areas on mesenchymal stem cell implants placed on tri-calcium-phosphate scaffolds, Jakob von Kossa coloration. C. Adipogenic cell line differentiation, cells changing their shape and starting to accumulate cytoplasmatic globular adipose deposits, magnification x 10.

Scaffold analysis - The maintenance of cell viability on the scaffold was assesed by observing the extra cellular matrix deposits using the Jakob von Kossa mineralization technique. The structures were fixed for 15 minutes in buffered neutral formalin and placed for 20 minutes in the dark chamber in a 2% silver nitrate solution. After washing them in distilled water they were

treated with 0.5% hydroquinone. The last step consisted in the fixation of the cells in sodium-thio-sulphate for 5 minutes (colouring the reaction product brown) and cell nuclei were coloured with Meyer Haematoxilin.

Results were analyzed using the Nikon Eclipse E600 microscope showing clear presence of mineralization areas.

Hystological evaluation of bone formation - Bone fragments analysed 5 days after implant placement revealed an intense inflammatory process as indicated by typical polymorphonuclear (PMN) leukocyte infiltration. These cells were especially abundant in the superficial alveolar area in contact with the oral cavity (Fig. 3 A, B, C, D).

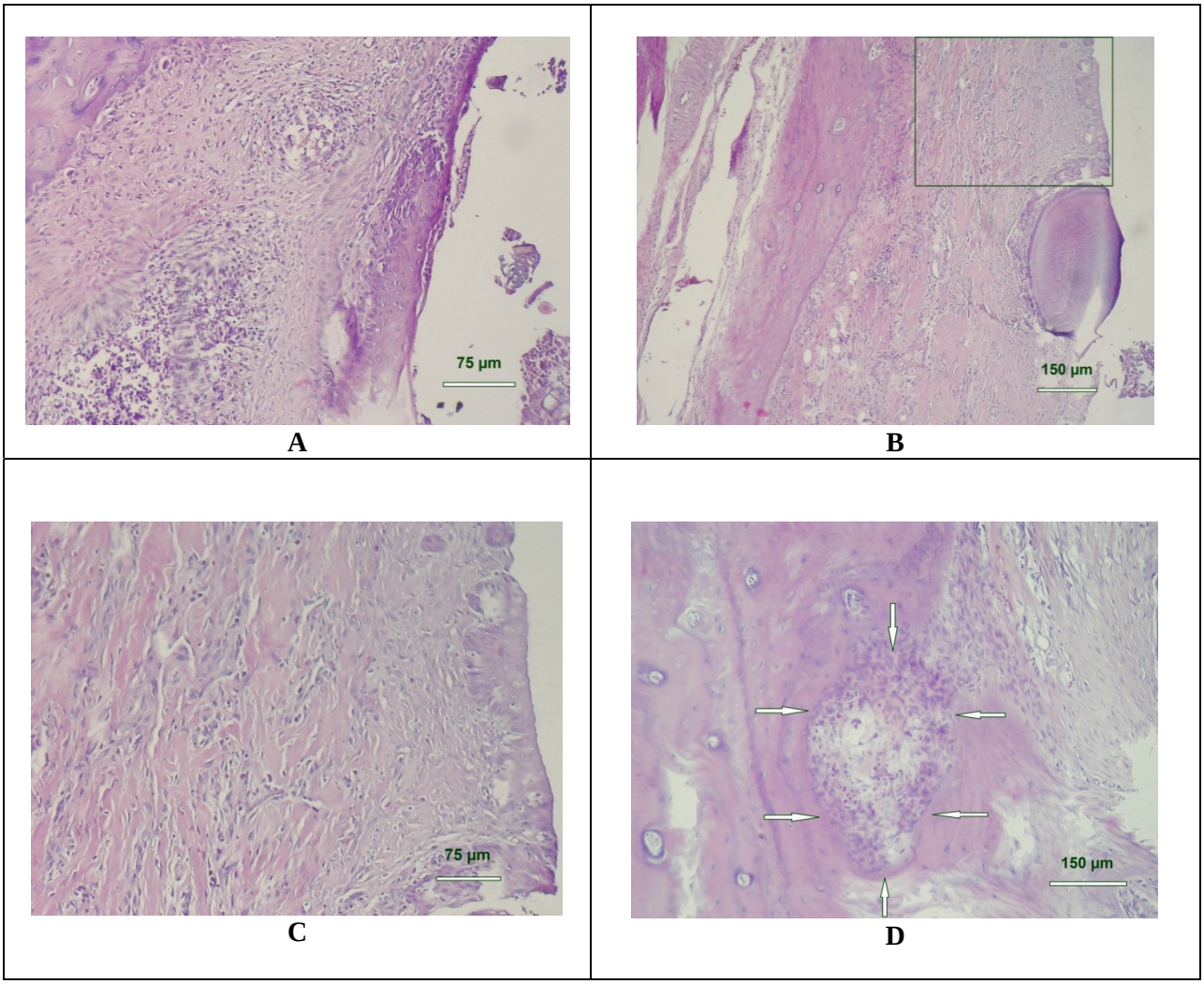

Fig. 3. A. Bone formation after MSC implantation; area where implant has been placed, intense inflammatory process as indicated by typical polymorphonuclear (PMN) leukocyte infiltration, 75 µm; B. detail of implant area, 150 µm; C. inflammatory cells abundant in the superficial alveolar area, 75 µm; D. inflammatory cells abundant in the superficial alveolar area in contact with the oral cavity, 150 µm.

The defects that were kept empty and those where only granular TCP or was implanted showed signs of bone formation and part of the granular material could be observed. In the alveolar bone, immature bone areas, slightly basolphilic matrix and nutritive blood vessels can be seen (Fig.4 A, B).

In the sockets where the stem cells on TCP scaffolds have been placed massive bone formation could be observed. The rich cellular infiltrate is an indicator of normal granulation, new vascularization and osteoconduction processes. Sections through the central part of the scaffold show the existence of a large amount of bone formed *„de novo”*. In the area where the scaffold was implanted, distant from the surgical lesion margins bone formation can be seen. The positive response of the host to the implantation of stem cells loaded scaffolds is indicated by the osteoconduction processes at the bone-implant interface (Fig. 4 C).

At the limit of the bone cementant lines with incremental matrix deposits and transitional aspects towards adult lamellar bone can be seen. The surrounding areas show signs of fibrous tissue proliferation, mesenchymal osteoprogenitor sites, with the aspect of intramembraneous osteogenesis of the alveolar bone. Also the enlargement of bone tissue and the numerical growth of blood vessels show the important stimulation of osteogenesis (Fig. 4 D). No immunological response from the hosts has been seen. Two cases showed signs of inflammation due to the existence of food debries on the gingival surface and the sulcus of surrounding teeth.

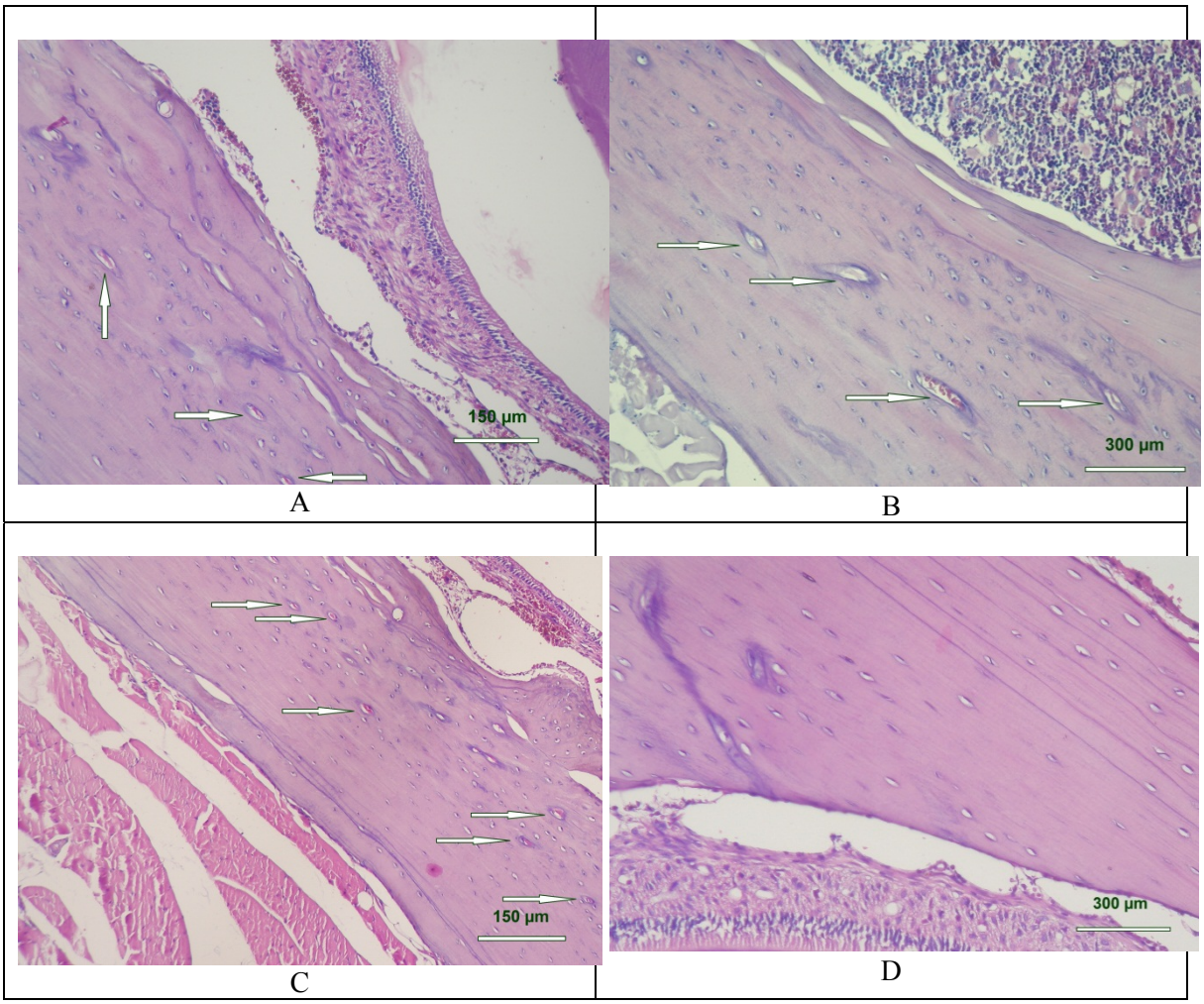

Fig. 4. Alveolar bone formation; A. alveolar bone, immature bone areas, slightly basolphilic matrix and nutritive blood vessels, pointed by the arrows, 150 µm; B. Implant area – „de novo” bone formation, pointed by the arrows, 300 µm; C. osteoconduction processes at the bone-implant interface, pointed by the arrows 150 µm; D. important stimulation of osteogenesis, numerical growth of blood vessels 300 µm.

Statistical analysis - Statistical analysis revealed that the procentual average of bone formation in the empty control sockets was 41,76 (SD =9,62), in sockets where only granular TCP was implanted 41,79 (SD = 9.68) and in the sockets where mesenchymal stem cells were implanted 56,62 (SD =10,35). The difference between the obtained values is statistically significant for the two-tailed significance level of 0.01.

4. Discussion

Our study clearly showed massive bone formation when autologous mesenchymal stem cells placed on TCP scaffolds were implanted in alveolar sockets. The newly formed tissue was purely intramembraneous showing that osteogenesis that was stimulated by stem cells occurred through direct transformation of mesenchymal stem cells into osteoblasts and not through endochondral sequency. Similar studies identified the presence of labelled mesenchymal stem cells in the bone tissue lacunae and in the calcification base[11]. Previous studies[12,1] showed that after 4 weeks bone tissue formation within sites with MSC loaded scaffolds implants was much more enhanced than in sites with simple scaffold implants (HA/TCP) and physiological repair processes were much slower.

Another important observation regarding the autogenous stem cell implants was the absence of significant mononuclear cellular infiltrate. When other isogenic or xenogenic grafts were used, it was shown by other research studies that antiinflammatory treatment was needed to upgrade bone formation and tissue repair. The role of T lymphocytes has been suggested to mediate the degree of survivor for xenogenic cells on these experimental models[12]. In contrast, experiments that use autologous mesenchymal stem cells prove a high degree of tissue repair without the need of an immunomodulating treatment. Local inflammation of the implant sites has been absent in most cases and regional lymph nodules showed normal consistency.

Occasionally, a low degree of inflammation was seen at the surgical site, associated with bacterial contamination due to food debree present on the oral mucosa, having as a result minimal bone regeneration, as shown in other studies[13]. These studies attributed the dehiscency of the sutures to the fact that animals chewed the cages where they were kept[14].

One of the advantages of tissue regeneration based on autologous cell implants, and the high level of bone formation is represented by the fact that barrier membranes and their association to infection can be excluded from the bone regeneration protocol. The direct implantation of a large number of osteoprogenitor cells on a scaffold has proven to accelerate the osteogenesis process and to reduce the need for chemotaxy and excessive proliferation of progenitor osteoblasts. Other studies showed that a concentration of 5 x 10^4 cells/ml can induce bone formation whereas a concentration of 0,5-1 x 10^6 cells/ml don't[1,10].

We decided to use solid granular scaffolds for this experiment. Some other studies previously showed some advantages when using granular scaffolds[15]. Mankani et al.[16] observed a maximum of bone formation when using particles of 0,1-0,25 mm. Based on these studies we determined that the use of scaffolds having different types of particles offers the advantage of perfectly adapting to the shape of the defect without interfering with the vascularization processes. Biological, granular scaffolds can stimulate vascularization and tissue integration because of appropriate spaces between the particles of the anorganic material. Using granular materials also accelerates the scaffold's resorbtion process and the replacement of the anorganic material with new formed bone because of stimulating the osteoclastic activity at it's surface. TCP is a synthetic biocompatible material of high purity. Scaffolds contain crystalline β-tri-calcium phosphate with a purity of 99%. It's qualities have been studied and it has proven that it lacks every local or systemic toxicity. It's granular consistency with a porosity ranging from <10μm up to 400 μm provide an optimal osteoconductive environment for the development of bony tissues. Tri-calcium phosphate is extremely hidrophyl, making it easy to apply inside the defect. The microcavities provided by the macro- and microporosities determine the deep colonisation of osteogenic cells providing therefore the fixing of the biological process by gradual resorbtion. Tri-calcium phosphate scaffolds ensure rapid resorbtion, which is an advantage for small or medium size

defects and they also gradually release calcium and phosphate ions assuring rapid mineralization of the newly formed tissue.

Contrary to hidroxiapatite, TCP gradually releases calcium and phosphate ions to maintain bone repair. Hidroxyapatite also has a slower resorbtion rate blocking neovascularization processes and slowing down the repair processes.

We can conclude that the implantation of autologous mesenchymal stem cells offer the advantage of direct osteoprogenitor cell delivery, cancelling the steps needed for physiological bone repaire which is a slow process. By incorporating living cells on specific scaffolds, one can overcome the disadvantages of osteoinductive single factors that can affect bone formation.

Acknowledgements

The authors express their thanks to the staff of the University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania. The Pathology Department of the University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania, especially Alis Dema, deserve thanks for their continued excellence in tissue processing. Finally, we thank Liliana Vasile for her assistance in the histomorphometric analysis.

References

[1] D. Mylonas, D. M. Vidal, I. J. De Kok, J. D. Moriarity, L. F. Cooper, J. Prosthodont. **16**(6), 421 (2007).
[2] J. O. Hollinger, S. Winn, J. Bonadio, Tissue Eng., **6**, 341 (2000).
[3] P. Bianco, M. Riminucci, S. Kuznetsov, P. G. Robey, Crit. Rev. Eukar. Gene., **9**, 159 (1999).
[4] A. I. Caplan, J. Orthopaed. Res., **9**, 641 (1991).
[5] S. P. Bruder, N. Jaiswal, S. E. Haynesworth, J. Cell. Biochem., **64**, 278 (1997).
[6] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, et al. Multilineage potential of adult human mesenchymal stem cells, *Sci.*, **284**, 143 (1999).
[7] S. P. Bruder, A. A. Kurth, M. Shea, W. C. Haynes, N. Jaiswal, S. Kadiyala, J. Orthopaed. Res., **16,** 155 (1998a).
[8] S. P. Bruder, K. H. Kraus, V. M. Goldberg, S. Kadiyala, J. Bone Joint Surg. Am. **80**, 985 (1998b).
[9] E. Kon, A. Muraglia, A. Corsi, P. Bianco, M. Marcacci, I. Martin, et al., J. Biomed. Mater. Res., **49**, 328 (2000).
[10] I. J. De Kok, S. J. Peter, M. Archambault, C. Van den Bos, S. Kadiyala, I. Aukhil, L. F. Cooper, Clin. Oral Impl. Res., **14**, 481 (2003).
[11] L. F. Cooper, C. T. Harris, S. P. Bruder, R. Kowalski, S. Kadiyala, J. Dent. Res., **80,** 314 (2001).
[12] G. Voggnereiter, S. Assenmacher, E. Kreuzfelder, M. Wolf, M. R. Kim, D. Nast-Kolb, F. U. Schade, J. Bone Miner. Res**.**, **15,** 1825 (2000).
[13] M. Nevins, S. A. Jovanovic, Curr. Opin. Periodont., **4,** 109 (1997).
[14] H. Schliephake, M. Dard, H. Planck, H. Hierlemann, U. Stern, , Int. J. Oral Max. Impl., **15,** 364 (2000).
[15] P. H. Krebsbach, S. A. Kuznetsov, K. Satomura et al., Transplantation, **63,** 1059 (1997).
[16] M. H. Mankani, S. A. Kuznetsov, B. Fowler et al., Biotechnol. Bioeng., **72,** 96 (2001).
[17] V. Ordodi, F. A. Mic, A. A. Mic, G. Tanasie, M. Ionac, D. Sandesc, V. Paunescu, Lab. Anim. **35(5)**, 41 (2006).