DENTAL PULP STEM CELLS BIOADHESIVITY: EVALUATION ON MINERAL-TRIOXIDE-AGGREGATE

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Stem cells are undifferentiated cells that have the capacity to self-renew. They have been discovered in many adult tissues, including teeth. Dental Pulp Mesenchymal Stem Cells (DP-MSCs) are involved in dental repair by activation of growth factors, released after caries and have the ability to regenerate a dentin-pulp-like complex. The molecular/cellular research gives the possibility to grow new tissues and biological structures for clinical applications, providing cells for therapies including cell transplantation and tissue engineering. In this study DP-MSCs were derived from dental pulp of 10 donors. To evaluate material toxicity, after *in vitro* isolation, the cells were seeded on mineral trioxide aggregate (MTA). Initial light microscopy investigation of cells revealed no signs of cell death due to toxicity or infection, on the contrary the scaffolds supplied an excellent support for cell structures, the cells proliferated and adhered to substrate. Similar observation was seen in scanning electron microscopy, in particular the cells had proliferated and spread, covering a considerable part of the surface of the biomaterials investigated, with an elaborate form of attachment, in fact, the cells formed a continuous layer on the upper surface of the MTA. In conclusion, the aim of this study is to demonstrate that DP-MSCs combined with MTA could be a potential source for regenerative medicine, encouraging further study to evaluate the new-dentin formation.

Tooth development occurs through mutually inductive signalling between interacting oral epithelial and ectomesenchymal cells (1), both originating from migrating neural crest cells (2). These interactions result in the formation of an outer layer of enamel formed by the activity of cells derived from oral epithelium, known as ameloblasts, and an inner layer of mineralized dentin synthesized by odontoblasts, which are derived from the dental papilla. The central chamber of teeth is comprised of a soft fibrous pulp tissue also derived from the dental papilla, infiltrated by a network of blood vessels and nerve bundles, emanating from the apical foraman.. While the complex structural composition of teeth provides hardness and durability, these rigid structures are vulnerable to damage caused by mechanical trauma, chemicals, congenital defects, cancer, and bacterial infections. Unlike other tissues, such as bone, which have the capacity to repair and remodel throughout the post-natal period, the relatively static components of teeth do not readily undergo complete regeneration following insult. However, adult teeth demonstrate some limited reparative processes such as the formation of reparative (tertiary) dentin, a more poorly organized mineralized matrix to primary, and secondary dentin that serves as a protective barrier for dental pulp (3). Once the dentin/odontoblast layer has been breached it is thought that pre-odontoblasts are recruited from somewhere within the pulpal tissue (4) to the injured site, before developing into functional odontoblasts. Similarly, the periodontium (PDL, cementum, and alveolar bone) has a limited capacity for regeneration. Despite our extensive knowledge concerning the pathology of diseases of teeth, restoration of damaged or diseased dental tissues, to date, has relied primarily on the use of synthetic implants and structural substitutions comprised of inert compounds. For example, conventional protection of exposed pulp by capping, using calcium hydroxide, is widely used in clinical practice, which often leads to inflammation and necrosis of the pulp tissue (5). The consequences of this approach include tooth discoloring, increased susceptibility to dentin fracture and tooth loss. Generally, placement of a root canal filling is the only real alternative to save mature teeth with severe papal destruction. However, several reports have been testing a range of potential options for pulp capping, such as demineralized dentin particles, extracellular matrix components (collagen, fibronectin, and fibrin glue), and biocompatible calcium phosphate based cements (hydroxyapatite, a/b-tricalcium phosphate, tetracalcium, and octacalcium). More sophisticated tissue engineering approaches include the use of cytokines (BMPs, TGF-b), and artificial scaffolds (polyglycolic acid), to help regenerate viable endogenous pulp tissue in order to facilitate reparative dentin formation (6-7). More recently, several novel tissue-engineering approaches have started to emerge as prospective alternatives to conventional treatments, including gene therapy and

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the local administration of biocompatible scaffolds with, or without the presence of growth factors, such as BMPs, TGFb, bFGF, PDGF, and IGF-1 (7–10). While still largely experimental in nature, these developments are expected to offer new and improved alternatives to existing therapies to repair and maintain mature teeth. Most recently, the identification of putative dental stem cell populations capable of regenerating organized tooth structures has stimulated interest in the potential use of post-natal stem cell based therapies to treat the damage caused by trauma, cancer, caries, and periodontal disease (11-12).

In this study the features and biocompatibility of expanded *ex vivo* human mesenchymal stem cells obtained from dental pulp tissues (DP-MSCs) seeded on MTA, were examined under light and scanning electron microscopy.

MATERIALS AND METHODS

Cell culture. The local Ethics Committee approved the experimental protocol and the patients participated after providing informed consent. For this study, ten (n=10) teeth were selected (VIII upper and lower, all protruding into the bucal cavity), which had to be extracted for orthodontic reasons, from patients of between 24 and 30 years old. After being informed on the applied therapy, all the patients signed the consent giving authorization use of the teeth. Extracted teeth were rinsed four times in phosphate – buffered saline (PBS) containing penicillin and streptomycin and subsequently the dental pulp was removed and cut into small pieces and cultured according to the manufacture's directions in MSCM medium provided by the company (Cambrex Company, Walkersville MD 21793-0127). After 20 days of cultures, numerous cells forming colonies (CFU-F) migrated from the explants. At day 7 adherent cells, which were 80-90% confluent by phase contrast microscopy, were isolated using 0.1% trypsin solution and plated in tissue culture polystyrene flasks at 5x103 cells/cm2. Primary cultures of DP-MSCs mainly consisted of colonies of bipolar fibroblastoid cells which, after subcultivation, proliferated with a population doubling time of 48h, reaching a confluent growth-arrested condition. Cells in the developing adherent layer were used for the experiments described after removal with 0.1% trypsin solution and cell growth was evaluated by trypan blue exclusion test.

Surfaces/scaffold. MTA biomaterials were prepared on the sterile surface, mixing the powder and liquid (sterile solutions). MTA was delicately applied directly into the pellet. The materials were treated with 1% penicillin/streptomycin for 1h, washed with PBS and seeded with cells. The cells were incubated with MSCM medium and cultured for 24, 48, 72 h. Subsequently, they were processed for light and scanning microscopy analysis.

Flow cytometry analysis. Mesenchymal stem cells were washed in PBS and subsequently suspended in PBS with saturating concentrations (1:100 dilution) of the following conjugated mouse- anti human antibodies fluorescein isothiocyanate (FITC) conjugated: HLADr, HLAB27, CD2, CD3,CD7, CD34,CD14, CD45, CD117, CD44; CD29, STRO1,

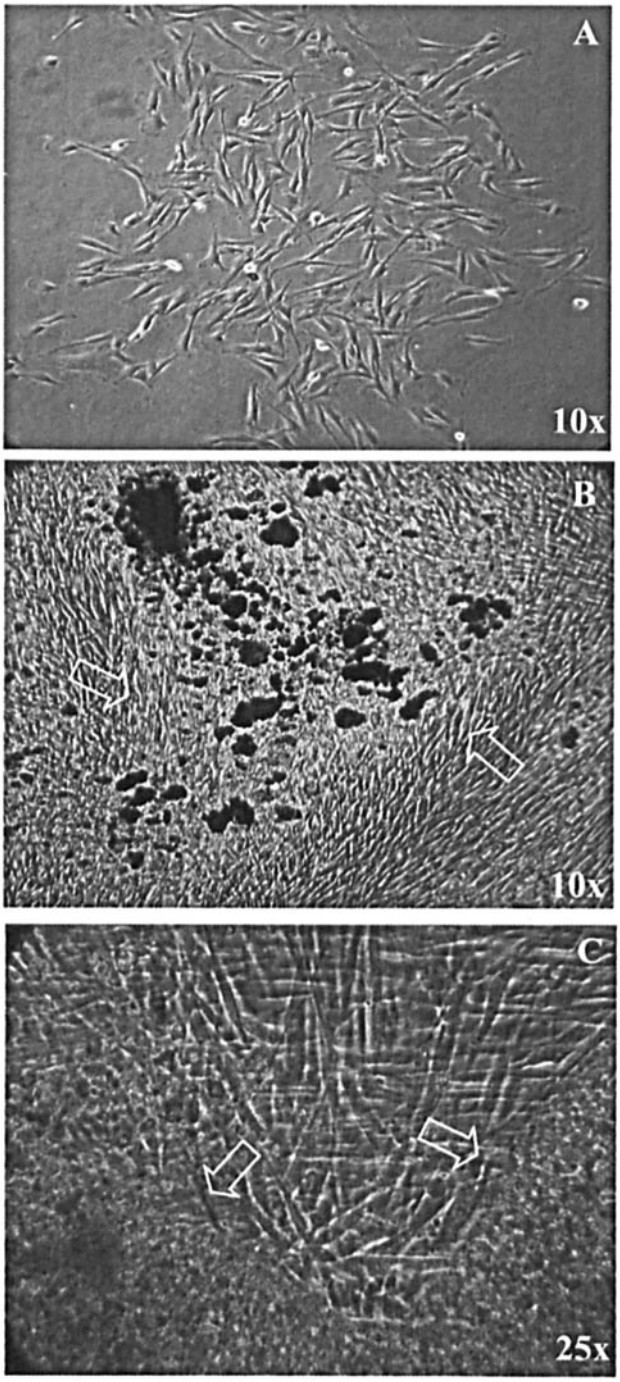

Fig. 1. *Section A: Micrograph of DP- MSCs expanded ex vivo, forming colonies, observed under inverted microscopy, shows the characteristic fibroblastoid-like morphology. Section B and C: DP-MSCs seeded on MTA biomaterials. It is possible to observe the high performance and biocompatibility of the three dimensional scaffold. No signs of infection are evident in culture cell medium. The cells revealed adhesion and continuous contact to substrate (arrows). Confluent living cells were observed on uppermost biomaterials surface. Cellular margins were indiscernible due to intimate contact between neighbouring cells. Original magnification: A and B:10x; C:25x.*

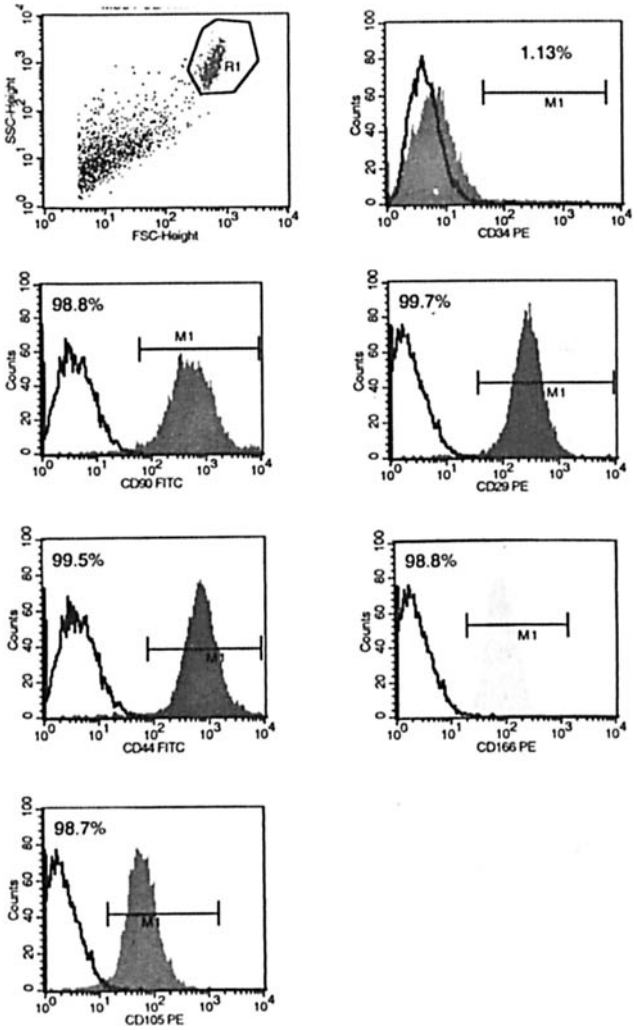

Fig. 2. *Flow cytometry of the mesenchymal-related antigens in DP-MSCs. Section A shows a representative flow cytometry plot of DP-MSCs reflecting cytoplasmic granularity and large cell size. Sections B, C, D, E and F show the positivity expression of mesenchymal related antigens.*

and CD105, CD33, CD90, CD166 phycoerythrin (PE) labelled, for 30 minutes at 4°C. The cell suspension was washed twice with PBS and analysed on a FACStar plus flow cytometer (Becton–Dickinson, Mountain View, CA, USA). All antibodies were from Becton Dickinson. Experiments were repeated at least three times, with at least four different donors, with similar results.

Scanning electron microscopy. The cells were washed in 0.1M phosphate buffer with 0.15M of sucrose to ensure the osmolarity remained at about 360 mOsm, prefixed for 20 h at 4° C in a 5 ml of glutaraldehyde at 2% in 0.05M phosphate buffer (pH 7.4) and then washed again with buffer solution. Following prefixation the specimens were treated with OTOTO method of post fixation as MALIK-WILSON (13), involving repeated exposure to osmium tetroxide and thiocarbohydrazide. All specimens were than dehydrated using graded alcohol series and Critical Point Dried (CPD) from liquid CO2 in an Emitech K 850 (Emitech Ltd. Ashford, Kent,UK). All samples were mounted onto aluminium specimens holders using carbon adhesive discs, and very lightly coated with gold in an Emitech K 550 (Emitech Ltd. Ashford, Kent,UK) sputter coater before imaging by means of a SEM (LEO 435 Vp Cambridge, UK) (13).

RESULTS

The present study is limited to the investigation of the cellular morphology of dental pulp mesenchymal stem cells, in response to MTA incubation.

Initial light microscopy investigation of primary cultures of DP-MSCs showed a morphologically homogeneous fibroblast- like appearance with a stellate shape and long cytoplasmic processes. In general, the cells were adhered to each other, subsequently forming colonies (Fig.1A). DP-MSCs seeded on MTA showed a high affinity to the surface of biomaterial. The cells growing in the direction of scaffolds and with contact and anchorage on the surface of the biomaterials, was evident. The cells had no signs of cell death or infection. (Fig. 1B and C).

The immunophenotype studied by flow cytometric analysis, disclosed the homogeneous expression of the mesenchymal-related antigens CD90, CD29, CD166, CD105, CD 44 (Fig 2) and did not express HLADr, HLAB27, CD15, CD33, CD34, CD45, CD14, CD2, CD7, CD3. A representative flow cytometry plot of mesenchymal stem cells visualized according to light scatter reflect cytoplasmic granularity and large cell size (Fig 2). The immunephenotype and morphological properties showed that DP-MSCs were similar to periodontal ligament, bone marrow mesenchymal cells and human umbilical cord perivascular cells. Scanning electron microscopy confirmed characteristic fibroblastoid-mesenchymal stem cells, with many cytoplasmic processes. The nuclei are round or oval-shaped and the secretory apparatus is very well represented. At higher magnification, the cytoplasmic elongation shows several vesicles of different size and very long filopodia that make contact with neighbour cells. Moreover, newly synthesized and secreted vesicles are present, probably the enhanced functioning of the secretory apparatus of the mesenchymal stem cells may be associated with the secretion of molecules required to survive and proliferate, probably involved in the paracrine and autocrine mechanisms (Fig. 3A).

Figure 3B shows DP-MSCs seeded on biomaterials. The cells revealed adhesion and continuous contact to substrate (arrows). Confluent cells layers were observed on uppermost biomaterials surface. In some cases cellular boundaries were not discernible due to the intimate contact between neighbouring cells. (Fig. 3B and C). At higher magnification cellular proliferation and subsequent colonization of MTA was evident after 72

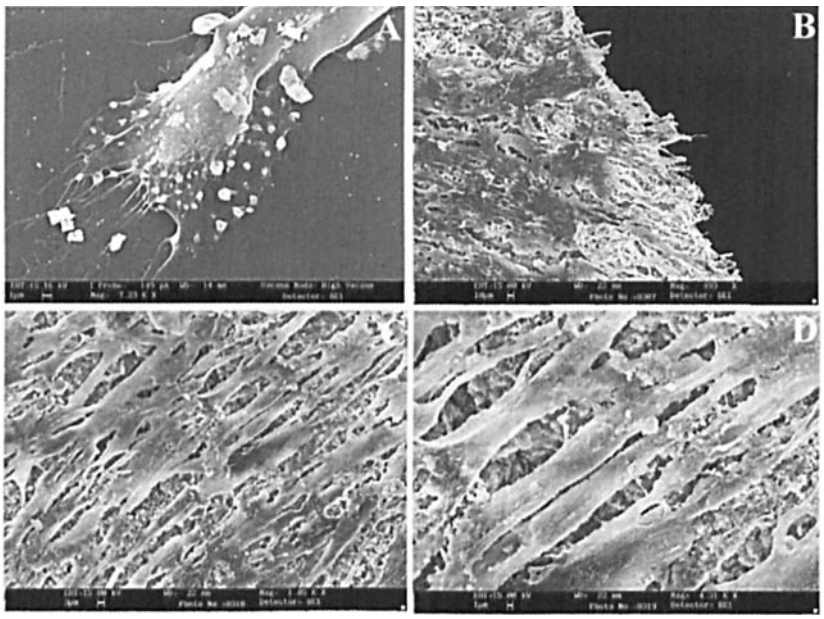

Fig. 3. *Section A: Scanning electron microscopy picture of mesenchymal stem cells from dental pulp with characteristic fibroblastoid morphology. A well marked vesicular secretory pathway , cytoplasmic processes and filopodia were evident. Section B, C, and D show the DP-MSCs grown on MTA. The cells are present and adhere tightly, anchoring themselves onto the biomaterial surfaces by cytoplasmic processes. Original magnification: Bar, A and D: 1μm, C: 3 μm and B:10 μm.*

h of incubation starting from 24 h. DP-MSCs changed their morphology, in fact no philopodia and cytoplasmic processes are evident, on the contrary a lateral attachment between cells is clearly visible (Fig. 4D).

DISCUSSION

Using an *in vitro* study, many authors have demonstrated that the regulation of proliferation rate in anchorage –dependent cells was determined by cell morphology, which, in turn, was determined by the ability of cells to attach to the substratum and also by surface chemistry of the biomaterials (14). An essential component in the regeneration of tissue is the use of a proper scaffold material. The function of the scaffold is to provide sites for cell adhesion, to support cell proliferation and differentiation and, thus, to promote tissue regeneration. Scaffolds for tissue engineering are usually 3-D, and are often designed to mimic all characteristics of natural structure. In previous research on dental pulp stem cells, a HA/TCP ceramic powder was used for the regeneration of dentin-pulp complex (15). After several weeks, implanted human dental pulp cells produced a structure quite similar to a dentin–pulp complex (15). Recently, Gronthos et al. have shown ex vivo that expanded ovine DP-MSCs mixed with hydroxyapatite/tricalcium phosphate formed a DSPP-positive dentin-like matrix surrounded by a distinct odontoblast layer and fibrous pulp tissue when implanted into immunocompromised mice (16).

The aim of the this study is to evaluate the relationship between DP-MSCs and MTA. The immunephenotype of DP- MSCs disclosed the homogeneous presence of mesenchymal –related antigens as: CD29, CD44, CD105, CD 90, CD166 and it has been demonstrated that they show multipotent differentiative capacity. Dental pulp is an easily accessible and efficient source of MSCs, with different kinetics and differentiation potentialities from MSCs as isolated from the bone marrow. The rapid proliferative capacity together with the immunoregulatory characteristics of DP-MSCs may prompt future studies aimed at using these cells in the treatment or prevention of T-cell alloreactivity in hematopoietic or in solid organ allogeneic transplantation.

Moreover, different authors have also shown that dental pulp, periodontal ligament and human umbilical cord perivascular cells represent a population of normal, rapidly expandable cells with intrinsic capacity of self-renewal and can furthermore generate multiple cells that are able to differentiate in different tissues (17-19). Recently, comparing the gene expression profiles by microarrays analysis of human dental pulp cells and bone marrow stromal cells, it has been demonstrated that they possess similar levels of gene expression in more than 4000 known human genes (20). Moreover, dental pulp is a viable source of easily attainable cells with a possible potential for cell transplantation therapies, indicating the presence of a cell population in the dental pulp with neuronal differentiation capacity (21), and also having the ability to differentiate in mesenchymal tissue, after induction, offering a novel approach for

regenerative medicine (22-24). These results are in accordance with the existence in the adult organism of a biological niche of the mesenchymal stem cells that are not only restricted to bone marrow but are present in different tissues and, under defined inductive conditions, are able to acquire characteristics of cells derived from embryonic mesoderm, as well as possessing ectodermal and neuronal properties (25-27). In this paper, apart from cytofluorimetric determination, we report the isolation and characterization, by morphological analysis, of a homogeneous mesenchymal stem cells population expanded *ex vivo* from dental pulp and their ability to grow in the presence of biomaterials. In fact, we have successfully demonstrated the cellular proliferation and subsequent colonization of MTA from human DP-MSCs.

In conclusion, these cells could represent an ideal carrier for delivering genes into the tissues for gene therapy applications and tissue engineering, and, in particular, human DP-MSCs obtained from individual patients can eliminate all complications associated with immune rejection of allogenic tissue and do not induce any immunoreactivity in the host upon local transplantation or systemic administration. In our laboratory, several studies are being carried out to enhance their clinical usefulness, to evaluate the maintenance and differentiation of this cell population and the signal transduction regulating their mechanism *in vivo* and *in vitro* and to estimate the ability of MTA to provide a mechanical and inductive support for the time needed to obtain dentin/pulp regeneration.

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REFERENCES

1. **Thesleff I. and P. Sharpe.** 1997. Signalling networks regulating dental development. *Mech. Dev. 67:111.*
2. **Lumsden A.G.** 1988. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development 103(S):155.*
3. **Tziafas D.** 2004. The future role of a molecular approach to pulp-dentinal regeneration. *Caries Res. 38:314.*
4. **Carlile M.J., M.G. Sturrock, D.M. Chisholm, G.R. Ogden and A.M. Schor.** 2000. The presence of pericytes and transitional cells in the vasculature of the human dental pulp: an ultrastructural study. *Histochem. J. 32:239.*
5. **Schuurs A.H., R.J. Gruythuysen and P.R. Wesselink.** 2000. Pulp capping with adhesive resin-based composite vs. calcium hydroxide: a review. *Endod. Dent. Traumatol. 16:240.*
6. **Mooney D.J., C. Powell, J. Piana and B. Rutherford.** 1996. Engineering dental pulp-like tissue in vitro. *Biotechnol. Prog. 12:865.*
7. **Rutherford B., L. Spangberg, M. Tucker and M. Charette.** 1995. Transdentinal stimulation of reparative dentine formation by osteogenic protein-1 in monkeys. *Arch. Oral Biol. 40:681.*
8. **Jin Q.M., O. Anusaksathien, S.A. Webb, R.B. Rutherford and W.V. Giannobile.** 2003. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *J. Periodontol. 74:202.*
9. **Giannobile W.V., C.S. Lee, M.P. Tomala, K.M. Tejeda and Z. Zhu.** 2001. Platelet derived growth factor (PDGF) gene delivery for application in periodontal tissue engineering. *J. Periodontol. 72:815.*
10. **Howell T.H., J.P. Fiorellini, D.W. Paquette, S. Offenbacher, W.V. Giannobile and S.E. Lynch.** 1997. A phase I/II clinical trial to evaluate a combination of recombinant human platelet-derived growth factor-BB and recombinant human insulin-like growth factor-I in patients with periodontal disease. *J. Periodontol. 68:1186.*
11. **Seo B.M., M. Miura, S. Gronthos, P.M. Bartold, S. Batouli and J. Brahim.** 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet 364:149.*
12. **Gronthos S., M. Mankani, J. Brahim, P.G. Robey and S. Shi.** 2000 Postnatal human dental pulp stem cells (DPSCs) in vitro and invivo. *Proc. Natl. Acad. Sci. USA 97:13625.*
13. **Malik L.E. and R.B. Wilson.** 1975. Evaluation of modified technique for SEM examination of vertebrate specimens without evaporated metal layer. *Scan. Micr. 1: 259.*
14. **Annaz B., K.A. Hing, M.Kayser, T. Buckland and L. Di Silvio.** 2004 Porosity variation in hydroxyapatite and osteoblast morphology: a scanning electron microscopy study. *J. Microscopi. 215:100.*
15. **Weibo Z. , X.F. Walboomers, T.H. Van Kuppevelt, F.D. Willeke, Z. Bianc and J.A. Jansen.** 2006 The performance of human dental pulp stem cells on different three-dimensional scaffold materials. *Biomaterials 27: 5658.*
16. **Gronthos S., K. Mrozik, S. Shi and P.M. Bartold.** 2006. Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. Calcif. Tissue Int. 79:310.
17. **Sarugaser R., D. Lickorish, D. Baksh, M.M. Hosseini and J.E. Davies.** 2005. Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. *Stem Cells 23:220.*
18. **Erices A., P. Conget and J.J. Minguell.** 2000. Mesenchymal progenitor cells in human umbilical cord

blood. *Br. J. Haematol. 109:235.*

19. **Lee O.K., T.K. Kuo, W.M. Chen, K. D. Lee, S. Hsieh and T.H. Chen**. 2004. Isolation of multipotent mesenchymal stem cells umbilical cord blood. *Blood 103:1669.*
20. **Shi S., P.G. Robey and S. Gronthos**. 2001. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone 29:532.*
21. **Nosrat I.V., C.A. Smith, P. Mullally, L. Olson and C.A. Nosrat**. 2004. Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair un the nervous system. *Eur. J. Neurosci. 19:2388.*
22. **Iohara K., M. Nakashima, M. Ito, M. Ishikawa, A. Nakasima and A. Akamine.** 2004. Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. *J. Dent. Res. 83:590.*
23. **Gronthos S., M. Mankani, J. Brahim, P.G. Robey and S. Shi**. 2000. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo. Proc. Natl. Acad. Sci. US A. 97:13625.*
24. **Batouli S., M. Miura, J. Brahim, T.W. Tsutsui, L.W. Fisher, S. Gronthos, P.G. Robey and S. Shi**. 2003. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. *J. Dent. Res. 82:976*
25. **Trubiani O., D. Tripodi, T. Delle Fratte, S. Caputi and R. Di Primio**. 2003. Human dental pulp vasculogenesis evaluated by CD34 antigen expression and morphological arrangement. *J. Dent.Res. 82:742.*
26. **Mu L.J., P. Lazarova, G. Gaudernack, S. Sæbøe-Larssen and G. Kvalheim**. 2004. Development of a clinical grade procedure for generation of mRNA transfected dendritic cells from purified frozen CD34+ blood progenitor cells. *Int. J. Immunopathol. Pharmacol. 17:255.*
27. **Kaneider N.C., A. Kaser, H. Tilg, G. Ricevuti and C.J. Wiedermann**. 2003. CD40 ligand-dependent maturation of human monocyte-derived dendritic cells by activated platelets. *Int. J. Immunopathol. Pharmacol. 16:225.*