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Regeneration of dentin-pulp complex by using dental pulp stem cells in dog

Fatemeh Dehghani Nazhvani[1], Setareh Kazempour[2], Seyed-Mojtaba Hosseini[3], Ali Dehghani Nazhvani[4], Pardis Haddadi[5]

[1]Bone and Joint Diseases Research Center, Shiraz University of Medical Sciences, [2]Research Committee, School of Dentistry, Shiraz University of Medical Sciences, [3]Research Committee, School of Medicine, Shiraz University of Medical Sciences, [4]Department of Oral and Maxillofacial Pathology, Biomaterials Research Center, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, [5]Department of Periodontology, School of Dentistry, Lorestan University of Medical Sciences, Khorramabad, Iran

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Address for correspondence:
Dr. Ali Dehghani Nazhvani, Department of Oral and Maxillofacial Pathology, Biomaterials Research Center, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail: alidehghaninazhvani@yahoo.com

ABSTRACT

Introduction: Although missing tooth is not life-threatening, it affects the quality of daily life. Stem cells have emerged as an important player in the generation and maintenance of many tissues. The role of scaffolds has changed from a passive carrier to a bioactive matrix, which can be used to induce cellular behavior. The aim of this study was to determine the possibility of regeneration of dentin-pulp complex with dental pulp stem cells (DPSCs) in an animal model.

Materials and Methods: In this animal study after extraction of DPSCs and cultivation, 10 types of scaffolds were made by using platelet-rich plasma (PRP), cancellous bone, and collagen pad. They were inserted in different parts of the dog's mouth. After the 4th month, the area was operated, and the scaffolds were removed.

Results: Microscopic examination revealed no sign of cell differentiation and formation of new structures in those models which used collagen scaffolds. However, the dentin-pulp complex emerged in models that the combination of bone scaffolds and PRP or stem cells was used.

Conclusion: Using bone scaffolds in combination with PRP or DPSCs to regenerate dentin-pulp complex in dog helped odontoblastic and pulpal differentiation as well as the formation of predentin and tubular dentin.

Key Words: Complex, dental pulp stem cell, dentin, differentiation, platelet-rich plasma, pulp

INTRODUCTION

Although damaged or missing tooth is not life-threatening, it clearly influences the quality of an individual's daily life. Stem cells have emerged as significant players in the generation and maintenance of several tissues of the body.[1,2] However, it is difficult to precisely stimulate the native stem cells niche since there is no comprehensive definition of essential factors for the stem-cell niche based on *in vivo* models.

Recent advancements in stem cells biology reported the presence of these undifferentiated precursor cells in dental pulp, as well.[3,4] In addition to possessing the general properties of stem cells, namely the ability of self-renewal and differentiation into several types of cells, accessibility, and high proliferation ability have made the dental pulp stem cells (DPSCs) more prominent than the other sources of stem cells.[1]

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This cell population is not only found in an adult's permanent teeth but also in primary teeth. Hence, both wisdom teeth and deciduous teeth, which are mostly extracted due to orthodontic reasons or impaction, are among the most accessible sources of these cells.[5] They easily provide the chance of preserving these cells to be transplanted to the individual himself through tissue engineering. It promises the possibility of regenerative treatment of dentin-pulp complex in future in contrast to the current routine endodontic treatments.[6]

Yu's studies proved that the soluble factors produced by the germ cells of rat incisor can induce differentiation of DPSCs and resulted in regeneration of regular-shaped dentin-pulp complex.[7] Yamada *et al*. used a sort of gelatin, which was a combination of platelet-rich plasma (PRP) and mesenchymal stem cells extracted from the pelvic crestal bone marrow. Regeneration of interdental papilla and reduction of depth of bone lesion as observed in radiology indicated that the employed gel helped regeneration of periodontal tissue.[8]

Three-dimensional (3D) cell culture systems have resulted in the development of useful models for physiological assessment of stem cells in response to the surrounding environment. The role of scaffolds has changed from a passive carrier towards a bioactive matrix which can induce the desired cell behavior.[9] The recent advancements revealed that placing the stem cells within a 3D substrate scaffold causes them to function more effectively in the regeneration process and also better maintain their physiological strength.[10,11] Hence, the first and most significant step in tissue engineering is to choose the proper scaffold.

Several biomaterials are available including synthetic or natural polymers, extracellular matrix, hydrogels, bioceramics, and self-assembling systems. Each material has its specific structure, chemical composition, formability, and analytical profile.[12] Some bioactive scaffolds can regenerate dental tissues such as dentin-pulp complex. Calcium-phosphate ceramics are among the most prevalent materials whose inductive effects have been proven on differentiating osteoblastic stem cells *in vitro* and on bone formation *in vivo*.[13,14] Nam *et al*. investigated the influence of 3D scaffolds of calcium-phosphate granules on the growth and odontogenic differentiation of human DPSCs (HDPSCs) in dental tissue engineering. They detected that employing this scaffold in combination with HDPSCs provides the appropriate substrate for growth and odontogenic development; thus it can be useful in dental tissue engineering.[14]

New approaches of dental pulp formation rely on materials such as collagen, polyester, chitosan, and hydroxyapatite. The aspects to be considered in engineering the dentin-pulp complex are angiogenicity, cell-matrix interactions, combining growth factors, matrix analysis, mineralization, and infection control.[13] The current study focused on identifying the performance of a combination of specific schemas of collagenous and bone scaffolds, as well as an environment of main growth factors in a population of stem cells extracted from an animal sample. It was done to evaluate the possibility of inducing differentiation of these cells into dentin or pulpal tissue after transplantation of each complex in the oral cavity of the same receiver. The aim behind these struggles was to offer stimulatory, biochemical, and mechanical environments with appropriate scaffold structures for stem cells. It is hoped that employing this schema in tissue engineering helps to develop the possibility of implanting odontogenic structures in future.

MATERIALS AND METHODS

In this experimental animal study, a dog was enrolled considering all the ethical principles related to animals. All procedures performed in the current study were approved by Shiraz University of Medical Sciences research and Animal Ethics Committee (#8794127) in accordance with Animal Research Reporting of *in vivo* Experiment guideline for reporting animal researches.[15]

Based on the radiology image, the dental pulp was decided to be extracted from the maxillary left canine. By using a disc, the tooth was cut slightly above the gingiva. Pulp pieces were extracted from the pulp chamber by using broach file. They were collected in test tube containing Phosphate-buffered saline - ethylenediaminetetraacetic acid (PBS-EDTA), 1% penicillin or streptomycin, and 1% fungizone. The pulp tissue was sectioned into smaller pieces under sterile conditions and was subjected to enzymatic digestion and periodic shakes. The obtained single-cell suspension was passed through a cell strainer, centrifuged to eliminate the enzymes, and the cells were resuspended in the medium.[5]

Isolated cell culture

The obtained single-cell suspension was plated in culture flasks which were enriched with mM4 Glutamax, 100 V/mL penicillin, 100 μg/mL streptomycin, and 20% fetal bovine serum (all by Gibco Invitrogen; USA) through α-minimum essential medium. The flasks were then incubated in 5% carbon dioxide and 90% moisture for 48 h. After that, the unadhered cells and debris were removed, and a new medium was added to the attached cells. The culture medium was replaced twice weekly until the accumulation of cells reached 80% in the flask. The adherent cells were released with trypsin-EDTA and were passaged again. To cultivate cells in designed scaffolds, a suspension of 200,000 cells in 200 μm medium was prepared and centrifuged in microtube. The cells were then transferred into the scaffold.[7]

Preparing the platelet-rich plasma

Blood sample was taken from the cephalic vein of the dog's right hand and was poured into 10 test tubes containing sodium citrate. The tubes were centrifuged and the transparent plasma concentrated at the top of the tube was isolated by using pipette and placed in four other tubes. They were centrifuged once more; then, the top half of the tube was removed, and the remaining half was collected in pipette. The obtained PRP was stored in the freezer until the media were prepared.[16]

Preparing three-dimensional scaffolds

In this study, 10 types of scaffolds were designed as follows;

1. Cancellous bone cube (5 mm × 5 mm × 5 mm cube-mineralized bone, Tissue Regeneration Corporation, Tehran, Iran), with no PRP injection, no stem cell or membrane, soaked in the culture medium
2. Cancellous bone cube soaked in PRP with no surrounding membrane
3. Collagenous cube (5 mm × 5 mm × 5 mm collagen foam, Tissue Regeneration Corporation, Tehran, Iran) with no PRP injection, no stem cell or membrane, soaked in the culture medium
4. Collagenous cube combined with PRP injection, with no surrounding membrane
5. Cancellous bone soaked in culture medium and surrounded with 1 mm thickness 2 cm × 2 cm membrane (Ceno Membrane, Tissue Regeneration Corporation, Tehran, Iran)
6. Collagenous cube soaked in culture medium and surrounded by the membrane
7. Collagenous cube combined with stem cells injection
8. Cancellous bone cube combined with stem cells injection
9. Sandwich 1: Stem cells injected into the central cancellous bone cube and surrounding it with rectangular pieces of cancellous bone cube soaked in PRP, and covering it with membrane
10. Sandwich 2: Soaking the cancellous bone cube in the center in PRP, surrounding it with cancellous bone rectangular cubes which were injected with stem cells, and finally covering it with membrane.

Inserting the media into the animal's oral soft tissue: The designated media were stored in PBC-EDTA solution with 1% penicillin, streptomycin, and 1% fungizone. On the operation day, the dog was prepared for the surgery, the media were inserted into different parts of the animal's oral soft tissue, including the buccal vestibules of both upper and lower jaws and lip commissures with at least 3 cm far from each other.

The dog was taken care of for 4 months to evaluate the results based on the Animals (Scientific Procedures) Act 1986. Dog was housed in 10 m^3 cage with shelter and natural light of day and night; given 3 meals of soft diet for the first 2 weeks and natural diet for the rest of this period. With an emphasis on the animal survival after 4 months, the soft tissue was re-operated and the media were removed. They were fixed in formaldehyde and sent to the oral and maxillofacial pathology laboratory of Shiraz Dental School for preparing tissue sections and microscopic assessment.

RESULTS

Microscopic evaluation of the dog's oral tissue slides revealed no sign of cellular differentiation and regeneration in models which used collagenous scaffolds. There were only foci of inflammatory cells, granulomatous reactions, including accumulations of lymphocytes, histiocytes, and multinucleated giant cells. On the contrary, in all models in which a combination of bone scaffold and PRP or stem cells was used, definite structures composed of hard tissue in the center and surrounding layers of mesenchymal spindle cells were observable [Figure 1a and 1b].

These structures were numerous and elliptical and huger in sandwich designs [Figure 2]. Mesenchymal spindle cells were aligned around the hard tissues in

the form of 4–6 layers; the cells in the innermost layer were long with the cytoplasmic processes at the apical side and the nucleus at the base [Figure 3a and b]. The central hard tissue had the tubular pattern with round-to-oval-shaped openings in regular parallel arrangement in some areas and completely irregular in some others [Figure 4a and b]. The hard tissue was less mineralized at periphery and generally indicated tubular dentin, peripheral predentin, and cells of odontoblastic layer [Figure 5]. The surrounding mesenchymal cells had loose myxomatous arrangement indicating that pulp tissue was developing below the odontoblastic layer [Figure 6a and b]. No response was observed to the mere bone scaffold. In sandwich models, odontoblastic differentiation and broad dentin regeneration were observed in addition to granulomatous reaction to the external membrane.

DISCUSSION

Regenerative dentistry is currently considered as an effective treatment to restore the tooth function. When the tooth is damaged, dental pulp starts dentin regenerating during which new cells, micro-environments, and matrices are created to restore the damaged area.[1] This is possible due to the presence of precursor stem cells in the dental pulp that can produce odontoblasts under appropriate conditions. Routine endodontic surgeries depend considerably on the elimination of damaged tissue and subsequently restoration with inert filling materials. However, the statement of the presence of precursor stem cells in adults' dental pulp and their successful cultivation allowed regenerating dentin-pulp complex based on stem cells.[7]

The current study employed collagen pad with cotton consistency as scaffold in half of the models, which was never investigated before. They did not show favorable structural strength in regenerating dentin-pulp complex *in vivo* after 4 months. In a similar *in vitro* study, Donzelli *et al*. used the culture of bone marrow mesenchymal stem cells of adult mice in a sort of collagenous scaffold made of gingistat (a type of hemostatic surgical dressing). They used osteogenic supplements to induce *in vitro* bone differentiation.[17] It was found that although the medium was disintegrated after 4 weeks, bone differentiation was observed and

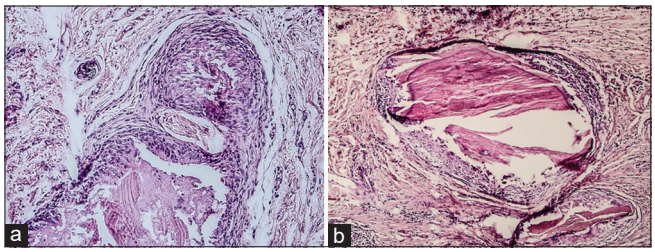

Figure 1: In models with combination of bone scaffold and platelet-rich plasma (a) or stem cells (b), definite structures composed of hard tissue in the center and surrounding layers of mesenchymal spindle cells were observable (H and E staining, ×100).

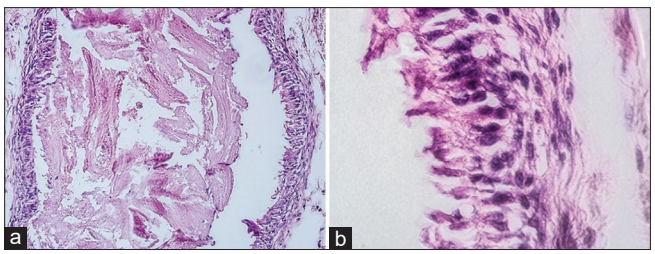

Figure 3: Mesenchymal spindle cells were aligned around hard tissue in 4–6 layers (a) (H and E staining, ×200), innermost cells were similar to odontoblasts (b) (H and E staining, ×400).

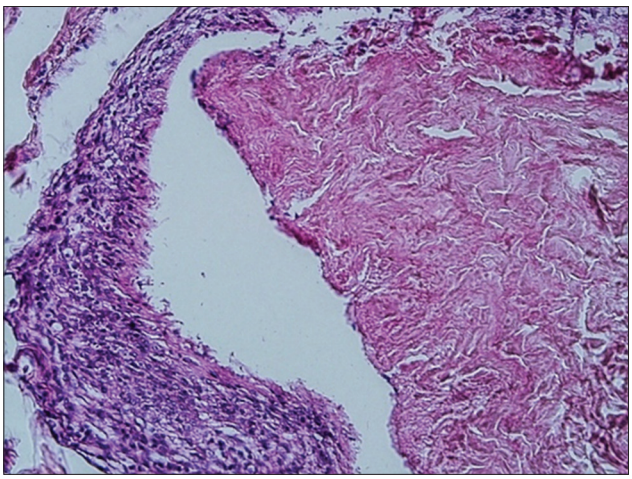

Figure 2: In sandwich designs, huge structures of central hard tissue and peripheral spindle cells were present (H and E staining, ×200).

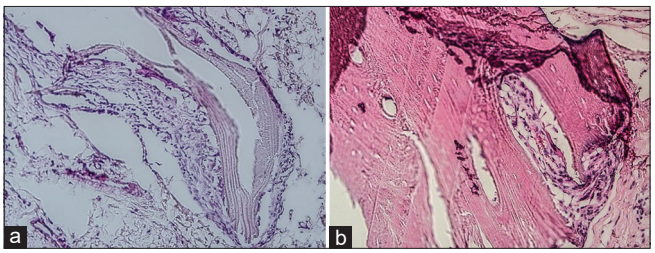

Figure 4: Central hard tissue had tubular pattern (a) with round to oval-shaped openings (b) in regular parallel arrangement in some areas and completely irregular in some others (H and E staining, ×200).

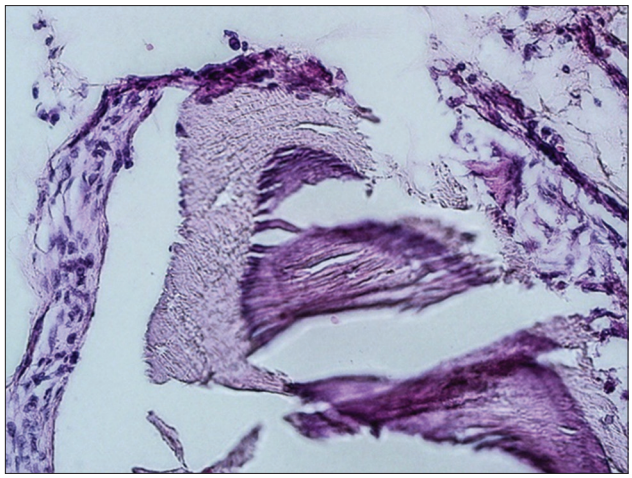

Figure 5: Less mineralized hard tissue at periphery indicated peripheral predentin near odontoblastic layer (H and E staining, ×400).

acted as a suitable source of bone cells for bone regeneration in periodontal lesions. It was concluded that gingistat collagenous scaffold had favorable structural strength to support the distribution and differentiation of mesenchymal stem cells; however, its rapid decomposition restricted its further use in bone regeneration.[17]

Nevertheless, it is far more difficult to chemically and mechanically control the micro-environments around the implanted scaffold *in vivo* (animal samples). Particularly in animals such as dog, there is no control on the animal's behavior and taking care of the surgical site concerning the pressure, mechanical stresses, and infection. Hence, the complete decomposition of collagen scaffolds used in this study was justifiable; although, the precise mechanism of decomposition process requires to be more thoroughly investigated.

Employing the scaffolds of mineralized bone in tissue regeneration was positively effective. Honsawek *et al*. and Shi *et al.* placed the mesenchymal stem cells derived from adipose tissue into the demineralized bone; their scaffold guided the bone differentiation. Similarly, using the mesenchymal stem cells derived from the umbilical cord in this scaffold did show osteogenic differentiation.[18,19] In this regard, a recent *in vitro* research pinpointed that using PRP and demineralized bone scaffold considerably helped to induce the osteogenic differentiation.[20] Nonetheless, none of these studies were conducted on animal models as the present study.

By using cancellous bone scaffolds in the dog's oral cavity, dentin-pulp regeneration was observed in all

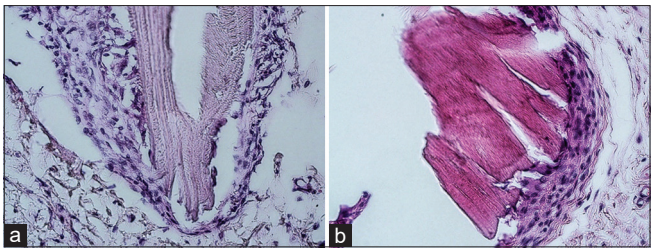

Figure 6: Surrounding mesenchymal cells with loose myxomatous arrangement indicating pulp tissue developing below the odontoblastic layer in bone scaffold with platelet-rich plasma (a) and with stem cells (b) (H and E staining, ×400).

combination designs, including PRP, DPSC cells, and sandwich models. In Yu *et al*. study, using the scaffold *per se* and in association with DPSCs was detected to be effective. In that study, the soluble factors produced by the germ cells of rat incisor induced the differentiation of DPSCs and resulted in regeneration of regularly-shaped dentin-pulp complex. It indicates that DPSCs can also induce differentiation of dentin-pulp complex. Compared with other sources of stem cells such as bone marrow stromal stem cells, the DPSCs functioned more efficiently in bioengineering of dental regeneration.[21]

When the stem cells are placed within a 3D scaffold of the substrate, not only they maintain their physiologic strength but also function better in the regeneration process.[10,11] Moreover, according to the previously performed studies, cultivating mature cells in 3D matrix displayed an altered phenotype, which prevented their inherent proliferation and increased their ability in forming structures of higher level. In addition, growth of mature cells in such a matrix improved the potential of stem cells through providing a dynamic relation that naturally occurs between the stem cells and matrix.[22,23]

Zhang *et al*. used an aqueous silk scaffold and 3D silk scaffold based on hexafluoro-2-propanol (HFIP). Not only did they support the formation of osteodentin but also guided the size and shape of formed osteodentin. Comparing these two scaffolds in terms of interaction with DPSCs revealed that destruction occurred faster in the aqueous scaffold; whereas, the HFIP-based 3D silk scaffold better supported pulp regeneration.[24] Comparing the two-dimensional and 3D scaffolds, the latter considerably altered the behavior of stem cells in terms of primary growth and matrix formation in the target tissue. Furthermore, majority of former studies focused on the growth and differentiation of stem cells *in vitro*; while, our study investigated the

biocompatibility of the scaffolds as well and positive results were achieved.

Regenerative potential of platelets was first introduced in the 1970s, just when they were found to contain growth factors responsible for increasing the collagen production, cell mitosis, blood vessel growth, and induction of cell differentiation. The platelets were increasingly used in tissue regeneration over time.[25] Yamada *et al*. stated that combination use of gelatin, PRP, and mesenchymal stem cells extracted from the pelvic crestal bone marrow in periodontal lesions lead to regeneration of interdental papilla and decreased the depth of dental lesion as observed in radiology images. It indicated the positive effects of PRP on the regeneration of periodontal tissue.[8]

In the present study, regeneration of dentin and pulp tissue occurred in combination models of cancellous bone scaffolds. It indicates the biocompatibility and proper structure strength of these scaffolds, and supports the physiological stability of stem cells and the new cells that enter the environment. Microscopic evaluation of the obtained sections proved the production of spherical or elliptical structures, including odontoblasts, predentin, and tubular dentin in the above-mentioned models. However, despite achieving odontoblastic differentiation, it did not show up in the desired cubic shape. Moreover, no microscopic difference was observed among the models combined with PRP, or DPSCs, and sandwich models. It points out the basic role of transferring a suitable scaffold to the appropriate environment that contains the micro-environment and its target cells; adding PRP or DPSCs made it possible.

Further studies are recommended to assess these models over a longer period of time with more sample size, to design the scaffolds with computer-aided design & computer-aided manufacturing (CAD/CAM), to apply nutritional, behavioral and environmental control with regularly rinse the animal's mouth with disinfectant agents to eliminate the intervening factors, and also to make use of new scaffolds with other compositions.

CONCLUSION

Using all the combination models designed with cancellous bone scaffold (in combination with PRP, DPSCs, and sandwich models) to regenerate dentin-pulp complex in the dog was associated with odontoblastic differentiation and regeneration of pulpal structure and dentin formation.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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