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Dental Pulp Stem Cells in Pulp Regeneration

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Abstract

Background: Dental loss and deformities caused by diseases and trauma are a major worry that have a negative impact on one's health and quality of life. Treatments for oral disorders now available can enhance clinical diagnostic criteria but do not restore damaged tissue. The pulp of the teeth is a unique source of stem cells. Human Dental Pulp Stem Cells (hDPSCs)-based regeneration techniques have showed considerable promise for treating dental abnormalities during the last few decades. The goal of this initiative is to use hDPSCs as a scaffold-base method for regenerative endodontics (hydroxyapatite: HA).

Methods: In this project, 8 incisor teeth of 4 rabbits were used that divided into 2 groups of case and control. In 2 cases hDPSCs injected (group A) and in 2 cases DPSC + hydroxyapatite (HA) injected (group B). All cases were checked in morphology of regenerated tissue (vascularization, innervation, intact odontoblastic layer, regenerative dentine) and side effects (degree of inflammation, necrosis) after 3 and 10 days.

Result: In 2 case groups(A, B) comparing with control group, the rate of inflammation was less and the morphology of regenerated pulp was improved, but no statistically significant difference between group A and control at the 3th and 10th day. Thus, in group B showed significant difference compare with control group in morphology and inflammation at 3 and 10 days (p=0.0132).

Conclusion: Our study suggests that the DPSCs+ HA transplanted to root canal decrease inflammation and promote cell proliferation and pulp regeneration. So, application of HA as a scaffold can significantly improve the findings rather than using DPSC alone.

Keywords: Dental Pulp; Stem Cells; Regeneration; Transplantation

Introduction

Diseases and injuries cause bone and dental loss and deformities, which is a major problem with a high occurrence [1,2]. Autogenous bone transplantation is currently the gold standard treatment for bone abnormalities, however it is severely limited due to a lack of sources, difficulty harvesting grafts, and donor site morbidity. Other oral disease treatments currently available can only improve clinical diagnostic markers but not repair destroyed tissue [3,4]. In the nineteenth century, researchers focusing on embryonic development suggested the concept of stem cells. Following that, stem cells of various sorts were discovered in many tissues, including dental pulps. Dr. Irina Kerkis discovered dental pulp stem cells (DPSCs) as adult stem cells in 2005 [5]. Because of their differentiation capacity and angiogenic capabilities, DPSCs have the potential to regenerate dentin and dental pulp tissue [6]. Based on their characterization, such as expression of specific markers and multi-differentiation capacity, the separated cells may be called stem cells, which have shown to be promising for use in tissue regeneration [7]. In vivo and in vitro models, DPSCs-HS (Human Serum) produced angiogenic growth factor concentrations comparable to DPSCs-FBS (Fetal Bovine Serum). DPSCs and stem cells from the apical papilla (SCAPs) were injected subcutaneously into immunocompromised mice in an in vivo model. All stem cell constructions showed osteogenic/odontogenic differentiation after

8 weeks, and regeneration of vascularized pulp-like tissue and mineralized tissue development after 12 weeks [8]. For 4, 6, and 8 weeks, cultured DPSCs on human treated dentin (hTD), implanted in a mouse model. As a result, DPSCs combined with hTD in vivo, trigger the regeneration of dentin-like tissues such as dentin sialophosphoprotein and dentin matrix protein-1 [9]. To use of dental pulp stem cells for pulp regeneration, in our experimental interventional study, 8 incisor teeth from 4 mature rabbits were used. Human DPSCs were cultured in proper culture medium and injected into the cleaned and washed canal area. In 2 case groups DPSCs injected (group A) and in 2 case groups DPSCs + Hydroxyapatite injected (group B). After 3 and 10 days, in 2 steps, each time, H&E staining was done to evaluate vital pulp tissue, inflammation, necrosis, vascularization, innervation, dentine quality (Osteodentine or Tubular dentine formation) and intact odontoblast layer.

Materials and Methods

In this interventional experimental study, 8 incisors teeth of upper jaw as clinical cases, from 4 male mature albino rabbits were used. The rabbits were 1-2 years old with 2.5 kg average weight. All the procedures and materials were performed at Pasargad Tissue and Gene Knowledge Company (Histogenotech, Tehran, Iran).

1-Culture of DPSCs

DPSCs line was obtained from the National Center for Genetic Resources of Iran. Cells were cultured in DMEM medium containing penicillin / streptomycin antibiotics and Fetal Bovine Serum (FBS) and under a luminar hood. The cell culture medium was changed every two days. Once a week, by briefly trypsinizing for 5 minutes with trypsin 0.25%, the cells were separated and cultured at a density of 1× 105 cell per square centimeter in a single flask. The new flasks were stored in 37 °C and 5% CO2 (Figure 1).

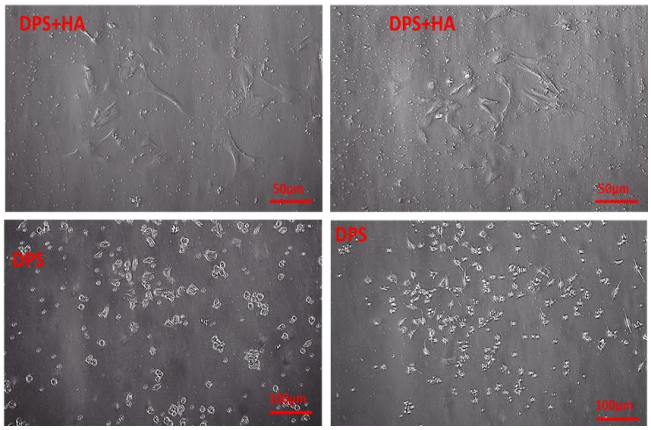

Figure 1: *Cells cultured in two different groups with or without hydroxyapatite.*

Determining the percentage of living cells

Trypan blue dye was used to determine the percentage of living cells. Trypan blue is a vital dye, so living cells do not allow it to pass through and repel it if it enters. But the membranes of dead cells are not able to remove vital dye from the cytoplasm. Therefore, dead cells turn blue and are easily recognizable from living cells that do not stain. For this purpose, first, 10 µl of trypan blue solution of 0.4 wt% by volume was added to 90 µl of cell suspension (trypsinized cells suspended in 1 ml of culture medium) and pipetted several times. Then, 10 microliters of the above solution was poured under a neobar slide and the percentage of stained (blue) cells was determined as the percentage of dead cells by light microscopy.

Cellular step

To perform most of the daily cell culture processes such as freezing, passage, etc., it is necessary to know the number of cells. Using the right number of cells and constant improves cell growth and helps to standardize and repeat the tests in the cell culture process. One of the methods of cell count in a cell solution (suspension) is the use of a neobar slide (homocytometer). After trispinizing and separation of both cell lines from the bottom of the culture dishes, the cell suspension was centrifuged at 1000 rpm at 4 ° C for five minutes. The supernatant was then discarded and the cells were homogenized in one milliliter of culture medium. For cell count, 10 µl of the cell suspension was mixed with 90 µl of methyl green solution and counted under a neobar slide (homocytometer) under a microscope. Before pouring the cells onto the slide, the cell suspension was homogenized several times with a sampler to prevent the formation of cell clamps and cell deposition and to increase the accuracy of the work.

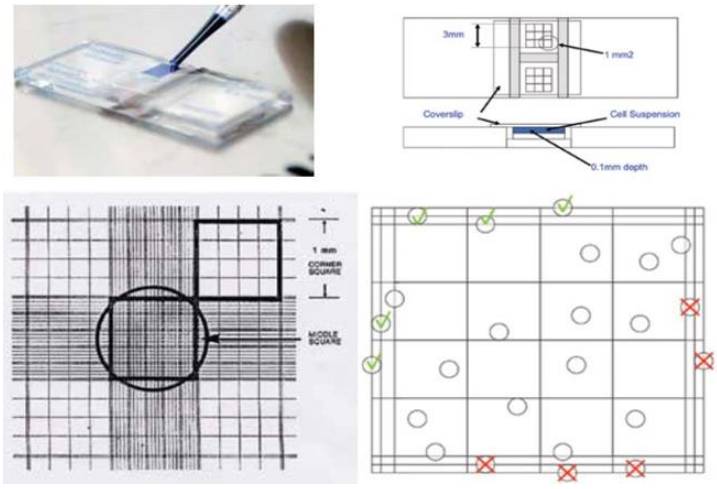

Figure 2: *Cell count using neobar slide.*

When filling the counting chamber, the cells were randomly distributed in the counting chamber, and to determine the total number of cells, the cells in the 4 squared corners of the scaled slide of the homocytometer were counted and the number of living cells was calculated according to the following equation:

Number of viable cells per milliliter of cell solution = Mean of cells counted in 4 neobar slide areas × Percentage of viable cells ×Slice dilution coefficient (10) × Slide number (104) (Figure 2).

General anesthesia was done with 100mg/kg ketamine and 5mg/kg Xylazine IP (Intra Pritoneal) injection. Then the access hole was made on palatal surface of each tooth with round carbide bur #2 and high speed hand piece, to appear the pulp. For initial access to dental apex, K-file#6 was used. After complete cleansing of the pulp tissue from the roots and crown (pulpectomy) with barbed broach and K-file, number 6-30, and washing with normal saline and sodium hypochlorite, we passed the K-file#30 through the canal apex to induce bleeding. Then the canal cleaning would continue with K-file#30 to dilate the apical foramen to 0.5 mm diameter. During preparation steps, the tooth canal was washed with sodium hypochlorite 5.25% and was dried with sterile paper points. 0.06 gr hydroxyapatite as a scaffold was dissolved in 200 landa PBS, and finally 10 landa in 105 cells is obtained. In each rabbit, one upper incisor tooth was in an interventional (case) group. In 2 case groups DPSCs injected (group A) and in 2 case groups DPSCs + final obtained hydroxyapatite injected (group B). Another upper incisor tooth was in control group that after canal cleaning, we didn't put anything inside it. Then all these incisors were sealed with Self-cured Glass Ionomer (GC Company) (Figure 3).

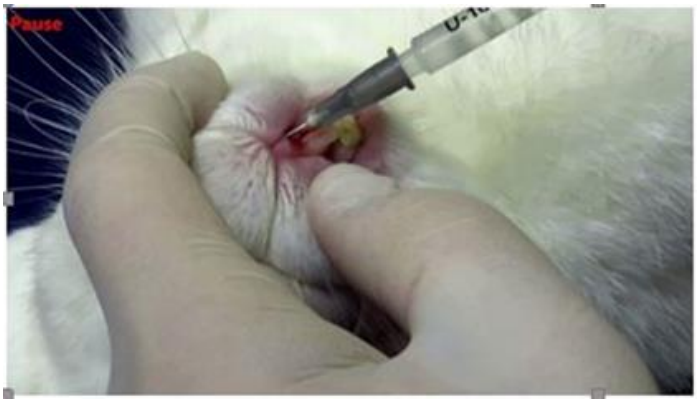

Figure 3: *Cell transplant into the opened dental canal.*

So, 4 teeth were in case group and 4 teeth in control group. After passing 3 and 10 days, in two steps, each time 2 rabbits were taken from the samples. By observing ethical standards, general anesthesia was done with 100mg/kg ketamine and 5mg/kg Xylazine IP (Intra Pritoneal) injection. Then the infiltrated injection was done in the depth of incisor tooth vestibule. The tooth with elevator and dicidious teeth forceps was extracted. The extracted teeth were put in formalin solution 10% immediately for 24-72 hours for fixing the tissue. Then dehydration with ethyl alcohol was done. This helps paraffin to penetrate into the tissue for cutting easily. Then put in xylol solution for removing extra alcohol and paraffin. Then samples were colored with Hematoxylin and Eosin (H&E) staining. The samples were observed with light microscope to compare and check in inflammation, necrosis, vascularization, innervation, dentine quality (osteodentine or tubular dentine formation) and intact odontoblast layer (Figure 4).

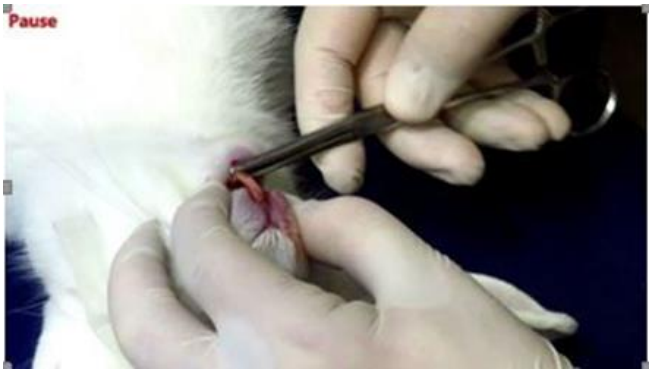

Figure 4: *Tooth extraction.*

In H&E staining, the degree of inflammation was classified according to the following score:

- There are one or more scattered inflammatory cells in the pulp below the injection site.
- Polymorphonuclear leukocytes (acute) or mononuclear lymphocytes (chronic) in an inflammatory lesion
- Severe inflammatory lesion that appears as an abscess or dense infiltration in one-third or more of the crown pulp
- The pulp is completely necrotic

The pulp soft tissue morphology was classified according to the following score:

- Morphology of normal or relatively natural tissue below the injection site and throughout the pulp, with vascularization, innervation, tubular dentine formation and intact odontoblast layer
- Lack of morphology of normal pulp tissue below the injection site, with a relatively normal appearance in deeper areas
- Loss of general pulp morphology and cellular organization below the injection site
- Without vascularization, innervation and intact odontoblast layer, with osteodentine dentine formation

Results

Comparison of the group A and the control group according to the inflammation degree after 3 days

Based on the inflammation scoring that mentioned in method, in both groups, the rate of inflammation (as an abscess in one third of the cronal pulp area) was almost high (Mean degree in the group A was 3.000±0.5774 and mean degree in the control group was 3.667±0.3333). So there was no statistically significant differences between control group and the group A with DPSCs transplantation at 3 day (P value = 0.3739) (Figure 5).

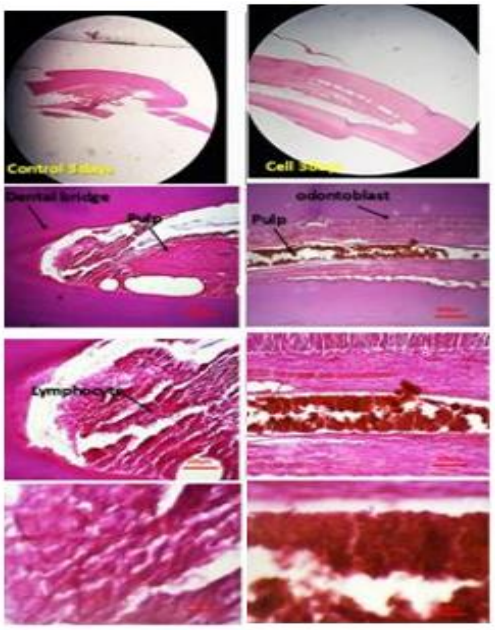

Figure 5: *Histological view of the inflammation degree of the group A and the control group after 3 days.*

Comparison of the group A and the control group according to the inflammation degree after 10 days

Histological examination showed that the inflammation degree was higher in the control group (Mean = 3.333±0.3333) than the group A (Mean = 2.333±0.3333). In the control group, severe inflammatory lesion was observed as an abscess in one third of the coronary pulp area, while in the 10-day cell group, polymorphonuclear leukocytes (acute) or mononuclear lymphocytes (chronic) were observed at the transplant site. So, there was no significant difference between two groups in terms of inflammation (P value = 0. 1012). According to comparison result in each group (Table 1), with time passing, there was no significant difference in inflammation rate in the control group (P value = 0.5185). In the group A, the inflammation rate decreased with time passing but it wasn't statistically significant differences (P value=0.3739). In the 3rd day, both groups had no significant difference in inflammation (P value = 0.3739). In the 10th day the inflammation rate in group A was less than control group but there is no statistically significant differences (P value=0.1012).

Comparison of the group B and the control group according to the inflammation degree after 3 days

Based on the inflammation scoring, the rate of inflammation in the case group B with DPSCs+HA transplantation at 3 days (Mean = 1.667±0.3333) was less than the control group (Mean = 3.667±0.3333) and in the control group the pulp was completely necrosed. So the difference was statistically significant (P value = 0.0132) (Table 1). In the group B, it was observed some polymorphonuclear leukocytes (acute) or mononuclear lymphocytes (chronic) at the surgical sites (Figure 6).

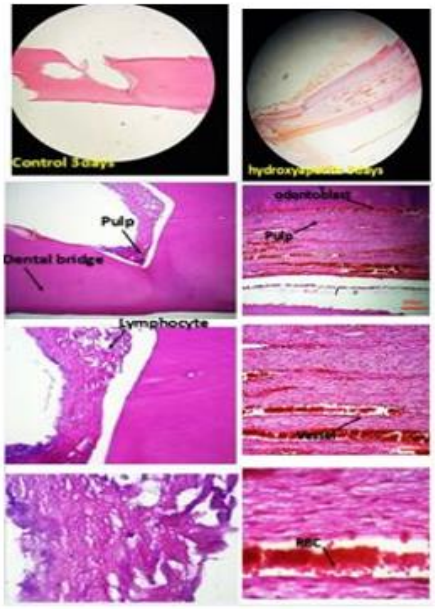

Figure 6: *Histological view of the inflammation degree of the group B and the control group after 3 days.*

Comparison of the group B and the control group according to the inflammation degree after 10 days

Histological examination in the control group and the group B showed that the inflammation degree (as an abscess lesions in one third of the coronary pulp area) was higher in the control group (Mean = 3.333±0.3333) than the group B (Mean = 1.333±0.3333), with statistically significant difference between these two groups (P value = 0. 0132). According to comparison result in each group (Table 1), in the both groups with time passing, the inflammation rate decreased but it wasn't statistically significant differences (P value = 0.5185). In the 3rd day and 10th day the inflammation rate in the group B was less than control group with statistically significant difference (P value = 0.0132).

Comparison of the group A and the control group according to the morphology of regenerated pulp after 3 days

Based on the morphology of regenerated pulp scoring that mentioned in method, it was found that in the control group of 3 days without cell injection, in the third part of the pulp crown,

necrosis was observed (Mean=3.667±0.3333). In the 3-day cell injection group, normal pulp morphology was lost and cell disruption was observed in the injection site (Mean=3.333±0.3333). In general, no significant difference was observed between the two groups according to the morphology of regenerated pulp (P value=0.5185).

Comparison of the group A and the control group according to the morphology of regenerated pulp after 10 days

Histological examination showed that in the 10-day control group, normal pulp morphology and cell cohesion in the surgical site were lost (Mean=3.333±0.3333). In the 10-day cell injected group, the normal morphology of the pulp was lost, but in some deeper areas, it was observed normally (Mean=2.667±0.3333). So no significant difference was observed between the two groups (P value=0.2302).

According to comparison result in each group Table 2, with time passing, there was no significant difference in the morphology of regenerated pulp in the control group (P value = 0.5185). In the group A, the morphology of regenerated pulp was improved but it wasn't statistically significant differences (P value=0.2302). In the 3rd day, both groups had no significant difference in the morphology of regenerated pulp (P value = 0.5185). In the 10th day the morphology of regenerated pulp in group A was more improved than control group but there is no statistically significant differences (P value=0.2302).

Comparison of the group B and the control group according to the morphology of regenerated pulp after 3 days

Based on the morphology of regenerated pulp scoring, in the control group of 3 days without injection, at the third part of the pulp crown, necrosis was observed (Mean=3.667±0.3333). In the 3-day DPSCs+HA injection group, normal pulp morphology was lost but it was normal in the deeper areas (Mean= 1.667±0.3333). Significant difference was observed between two groups according to the morphology of regenerated pulp (P value= 0.0132).

Comparison of the group B and the control group according to the morphology of regenerated pulp after 10 days

In the 10-day control group without hydroxyapatite transplantation, the general morphology of the pulp and cell cohesion at the transplant site were lost (Mean=3.333±0.3333). In the hydroxyapatite-transplanted group, normal or relatively normal pulp morphology was observed below the injected site and throughout the pulp (Mean=1.333±0.3333). There was also a significant difference between the two groups in morphology of regenerated pulp (P value = 0.0132). According to comparison result in each group Table 2, with time passing, there was no significant difference in the morphology of regenerated pulp in the control group (P value = 0.5185). In the group B, the morphology of regenerated pulp was improved by time passing but it wasn't statistically significant differences (P value = 0.5185). In the 3rd and 10th day, significant difference in the morphology of regenerated pulp between control and case group (P value = 0.0132) showed the significant role of DPSCs with hydroxyapatite helping in pulp regeneration. The group B had more normal and natural tissue compare with control group. (Figure 5-12) (Table 1,2).

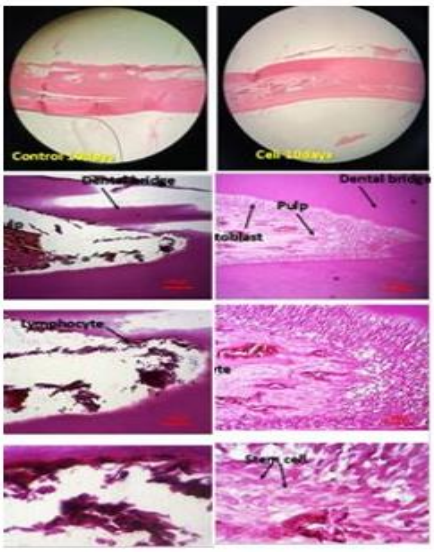

Figure 7: *Histological view of the morphology of regenerated pulp of the group A and the control group after 3 days.*

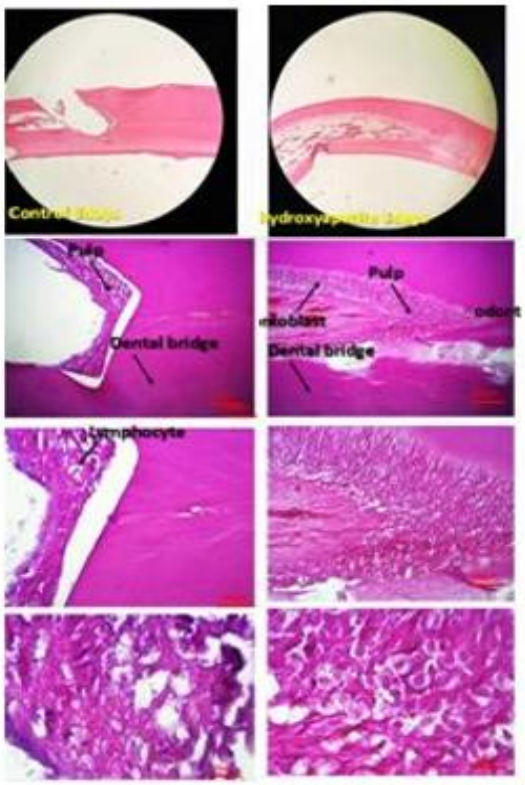

Figure 8: *Histological view of the morphology of regenerated pulp of the group B and the control group after 3 days.*

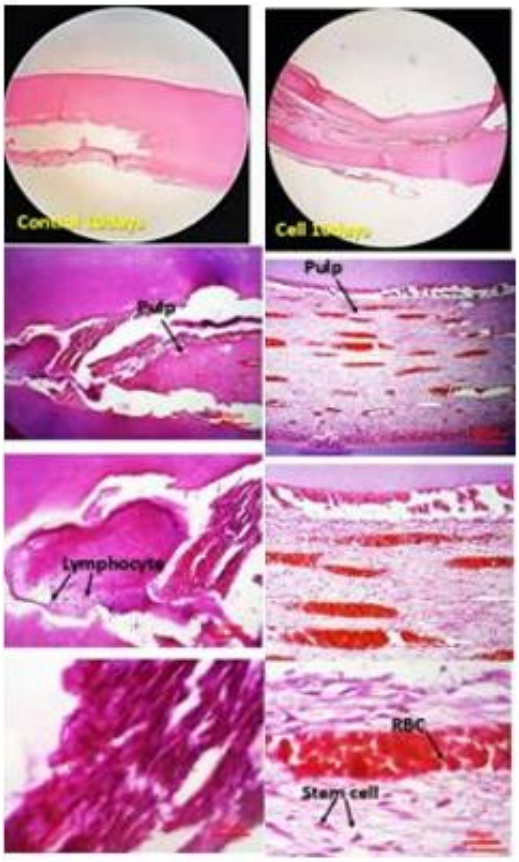

Figure 9: *Histological view of the inflammation degree of the group A and the control group after 10 days.*

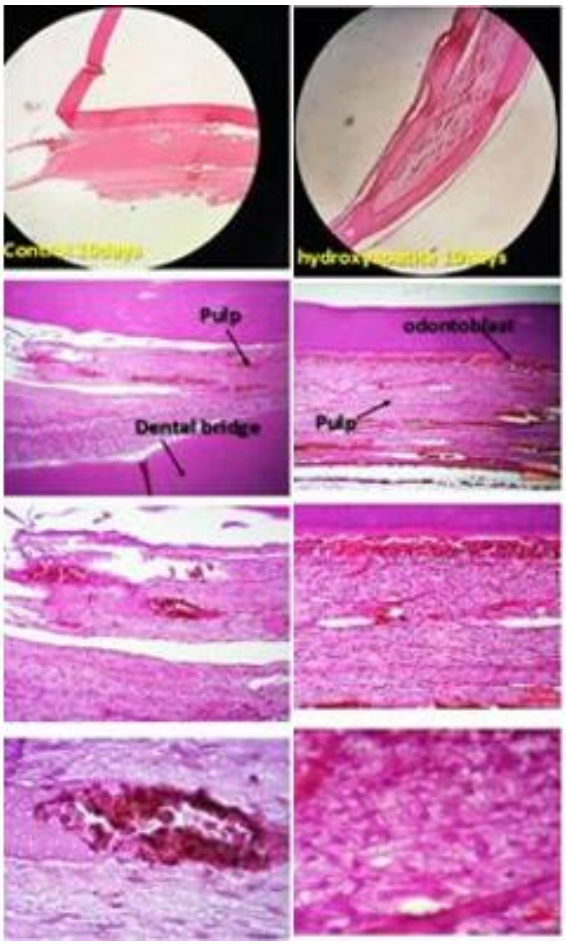

Figure 10: *Histological view of the inflammation degree of the group B and the control group after 10 days*.

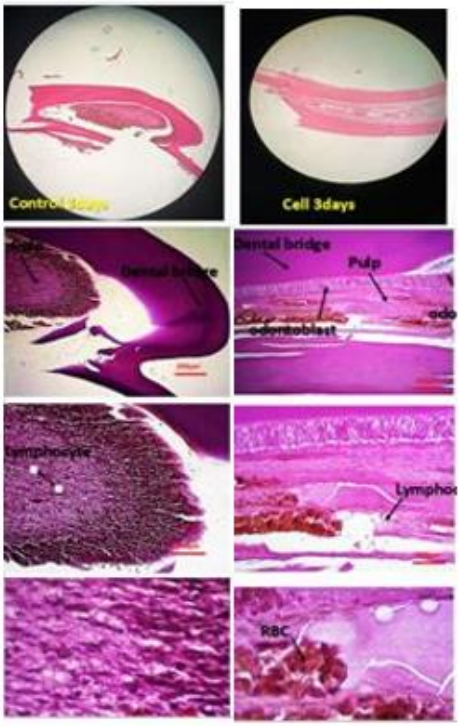

Figure 11: *Histological view of the morphology of regenerated pulp of the group A and the control group after 10 days*.

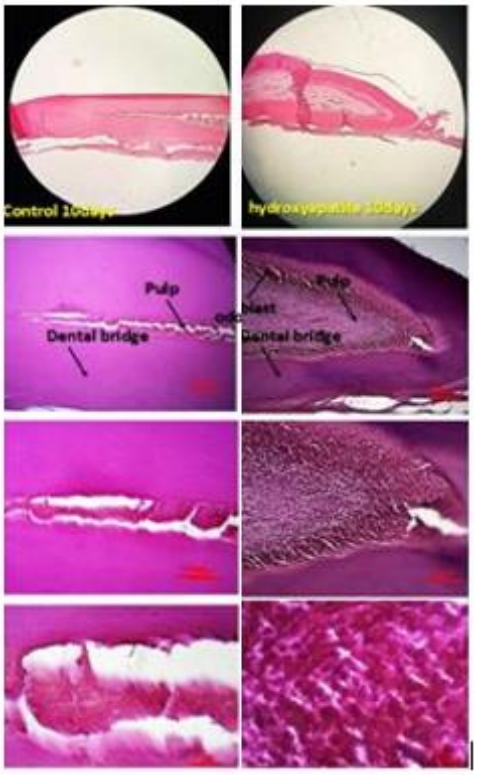

Figure 12: *Histological view of the morphology of regenerated pulp of the group B and the control group after10 days.*

inflammation (Mean degree)	control	Group A	Group B	P value A & control	P value B & control	P value A & B
Day 3	3.667±0.3333	3.000±0.5774	1.667±0.3333	0.3739	0.0132	0.1161
Day10	3.333±0.3333	2.333±0.3333	1.333±0.3333	0.1012	0.0132	0.1012
P value	0.5185	0.3739	0.5185			

Table 1: *Comparison of Mean degree of inflammation between groups A, B and control in 3,10 days.*

Pulp morphology (Mean score)	control	Group A	Group B	P value A & control	P value B & control	P value A & B
Day 3	3.667±0.3333	3.333±0.3333	1.667±0.3333	0.5185	0.0132	0.0241
Day10	3.333±0.3333	2.667±0.3333	1.333±0.3333	0.2302	0.0132	0.0474
P value	0.5185	0.2302	0.5185			

Table 2: *Comparison of Mean degree of the pulp morphology of regenerated pulp between groups A,B and control in 3,10 days.*

Discussion

Synthetic materials traditionally have been used as a choice treatment in prosthetic and restorative dentistry. However, this form of replacement does not regenerate the original biological tissues and other organs, are simply not responsive to mechanical substitution approaches. Thus, tissue engineering (TE) is emerging as a new therapeutic choice for the complete biological regeneration of pulpal and dental tissues. Three critical components are required: (1) stem cells; (2) scaffolds; and (3) stimulating factors or inductive signals [10]. In this study, we explored the potential usage of DPSCs for the regeneration of pulp. We investigated the hDPSC activity and growth behavior on 3 different study groups. Based on data extraction from the different databases, we can conclude that DPSCs may be an ideal choice to be used in experimental models of tissue-engineered reconstruction of organs of the oral cavity. DPSCs are adult stem cells that have several advantages over other types of stem cells, including ease of accessibility and noninvasive collection with low morbidity [11-13]. After 3 days histological comparing based on inflammation degree of group A and control showed that the inflammatory rate had a small difference, but not significant (p=0.3739) and in the both teeth sever inflammatory lesion as an abscess in one third of cronal pulp area , was seen. After 10 days there was no significant difference between two groups in inflammatory rate (p=0.1012), but the amount of inflammatory cells were decreased in group A. Group A showed just some acute and chronic leucocytes, but control tooth had sever inflammatory abscess lesion. This finding also showed in other studies that we can see the proliferation of stem cells in recipient zoon, but it is not significant and helpful enough for regeneration and they need a suitable scaffold to overcoming inflammation [14]. In this study hydroxyapatite was used as a scaffold in group B that was already commercially available for clinical applications. Scaffolds allow recapitulation of the extracellular environment of cells, the extracellular matrix, by providing attachment sites, the ability for cells to grow in 3D shape, and for some of them, rigidity of this environment and associated soluble factors like growth factors and immune system [15].

After 3 days histological comparing of group B and control showed that the control tooth totally necrosed with increasing inflammatory cells (Lymphocyte), but in case tooth the inflammation cells were less, without necrosing. Vessels and RBCs were seen as well (Figure 6). So the difference was significant (P value =0.0132). After 10 days, in the control tooth showed increasing inflammation cells and sever inflammatory lesion as an abscess in one third of coronal pulp area. But in group B, one or more inflammatory cells were seen without necrosis and it was significantly different (P value =0.0132) (Table 1). Our study in this part showed the DPSC proliferation potential with hydroxyapatite helping that stimulate pulp and dentin regeneration. This finding confirmed in another study with hydroxyapatite-based scaffolds on dental pulp stem cell proliferation and differentiation. It showed scaffolds support hDPSC adhesion, proliferation and differentiation [16]. After 3 days histological comparing based on regenerated pulp morphology, the third part of pulp crown, in control tooth necrosis (Lymphocyte) was seen, but in group B, normal pulp tissue existed in the deep area. Also the morphological difference in these two teeth was significant (p=0.132). After 10 days, in the control group general pulp morphology were lost, but in group B the normal pulp morphology was seen in the injected area, even in entire pulp (odontoblast) and the difference was significant between two groups (p=0.132). This finding consists the role of DPSC in regeneration. Comparing our study in this group and cell morphology investigation with previous studies showed the interaction between stem cells and the material applied as scaffold plays a critical role in the generation of a cell-friendly microenvironment, which must be conducive to the regeneration of dental structures that has enough potential [17]. In group A with DPSC without hydroxyapatite After 3 days histological comparing based on regenerated pulp morphology showed that in control group, in the third part of cronal pulp, necrosis was observed. Also in group A, normal pulp morphology was lost and cell disruption was observed in the injection site. In general, no significant difference (p=0.5185) was observed between the two groups according to pulp morphology. Examination of the histology images based on scoring that mentioned in method and material, showed that in the 10-day control group, normal pulp morphology and cell cohesion in the surgical site were lost. Also, in the group A, the normal morphology of the pulp was lost, but in some deeper areas, it was observed normally and no significant difference was observed between the two groups (p=0.2302). In another study demonstrated that to achieve a helpful method to use DPSCs in regeneration, scaffold is important to make a suitable environment. But in this study the result was better than us, maybe because of longer time spending for human in comparison with rabbit (at least 2 weeks for preparing) and 6 months following in human cases [18]. Our study suggested that the DPSCs promote cell proliferation and decrease inflammation. But the role of hydroxyapatite as a scaffold, was significant that without it, we couldn't see the good result of using DPSCs alone. On the other hand, in other research, it was observed that the patients treated with DPSCs had no signs or symptoms of rejection. However, they are ideal cells for tissue engineering and for clinical use. As we saw in comparing in each 3 groups between 3 and 10 days (time dependent), there were no significant difference between these two days according to the inflammation rate and regenerated pulp morphology. So the dental pulp tissue regeneration can performed in rabbits at 3rd day

and showed the acceptable results, because of rapid dental growth and regeneration of dental defects in rodents. There were some restrictions in our study that limited us to get predictable results, such as few numbers of cases. If the number of samples increases, the probability increases significantly. Immoral and non-ethical work on human cases was another limitation. Using growth factor was so costly that restricted us too. As a recommendation, many important factors that can effect on stem cells, like growth factors (Epidermal Growth Factor (EGF), Insulin like Growth Factor (ILGF), and Vascular Endothelial Growth Factor (VEGF)), angiogenic factors, inhibitors and the others, can be examined in further and future studies. The control group could be different. Human cases might be studied, but takes time and legally complicated.

Conclusion

Our study suggests that the DPSCs+ HA transplanted to root canal decrease inflammation and promote cell proliferation and pulp regeneration. But the role of hydroxyapatite as a scaffold, was significant that without it, we cannot see the good result of using DPSC alone but concerning no signs or symptoms of rejection and severe inflammation in tooth treated with DPSCs, they are recommended for dental engineering and for future clinical use.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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