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The effect of cultured autologous periodontal ligament cells on the healing of delayed autotransplanted dog's teeth

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Abstract

Introduction: The regeneration of periodontal structure for avulsed teeth extended dry times has been a goal of dentists. The aim of this study was to investigate a new strategy of delayed replantation for avulsed teeth that were not suitable for immediate replantation. **Methods**: Extracted dog's premolar teeth were maintained in a dry environment for a month after isolation and proliferation of the PDL cells. Then tooth roots coated with 1×10^6 cultured autologous PDL cells were autotransplanted in artificial sockets created in the mandible. The dogs were sacrificed 60 days post-transplantation. Histological analyses showed that a root-PDL-bone complex was found in all cases of the PDL cell-loaded samples. **Results**: The new PDL-like connective tissue was located between the alveolar bone and the transplanted roots, with fibers inserting into newborn cementum on one end and alveolar bone on the other. For the control samples, no PDL-like tissue was found and ankylosis was commonly observed. **Conclusions**: The results indicated that cultured autologous PDL cells assist the reestablishment of periodontal architecture of autotransplanted teeth that is devoid of viable periodontal cells.

Key Words: Periodontal ligament; Replantation; Periodontal regeneration; Delayed autotransplantation

Introduction

Dentoalveolar traumas are unexpected events and may occur at any time of life, although these seem to be more prevalent in children and adolescents. Avulsion is probably the most severe form of dentoalveolar injuries; the tooth is completely displaced out of its socket affecting not only the pulp but also the periodontal apparatus including periodontal ligament (PDL), alveolar bone and gingival tissues. PDL is the layer of connective tissues that connect the cementum (of tooth root) and

the alveolar bone. It “fixes” the tooth in the alveolar socket attenuates any occlusal loads acting on the tooth (1). All fibers of the PDL are severed in avulsion injury; the survival of the PDL cells on the root surface following injury plays a critical role in the healing of the replanted tooth. Successful replantation is dependent upon the reestablishment of the PDL, thus preventing ankylosis or any replacement root resorption (2). Great efforts in the shortening the extra-alveolar time and selecting the appropriate storage media have been made to maintain the viability of the remaining PDL cells (3-9). A review of published clinical trials (4, 8, 10-14) indicated variable rates of survival for replanted teeth. A success rate of 4 to 50% has been reported (15). Ankylosis seems to be the most frequent complication associated with the avulsed but replanted teeth, with the ultimate result of replacement resorption and failure of the replanted tooth (16).

The development of tissue engineering and periodontal regeneration sheds some light on the management of avulsed teeth. Several experimental studies have demonstrated that the PDL has a high natural ability of regeneration, contributing positively to the healing process after replantation (17-20). Herr *et al.* reported that fibroblasts originating from both the remaining PDL and alveolar bone compartments are responsible for the repair of the periodontium (21). Nowadays, it has been confirmed that PDL cells have the characteristics of stem cells that are able to differentiate and form all the components of periodontium (22-24). The purpose of this study was to examine the effect of delayed autotransplantation in combination with the periodontal tissue engineering using autologous PDL cells on periodontal healing.

Materials and Methods

Reagents

The following reagents were obtained for use in this study: ketamine hydrochloride (Gutian Pharmaceutical, Fujian, China); Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA); various antibiotics agents (Sigma-Aldrich, Colorado, USA).

Isolation and Proliferation of Dog's PDLCs

This study was approved by the Institutional Animal Care and Use Committee of the Peking Unversity School of Stomatology. 1-year-old mongrel dogs (weight 8~12kg) were used for this study. The dogs were anaesthetized using intramusclar injection of ketamine hydrochloride (Gutian Pharmaceutical, Fujian, China) at 2mg/kg body weight before operation. Under a sterile condition, both the left and right third and fourth mandibular premolars were extracted by using an elevator and dental forceps, and immediately immersed into ice-cold phosphate buffered saline (PBS) solution containing 100 U/mL penicillin and 100μg/mL streptomycin. The extracted teeth were transferred to laboratory for PDL cell culture. The wounds were sutured and the dogs were fed on a soft diet. Before isolation of PDL cells, the dental pulp and any gingival tissues near the cervical region of the tooth were completely removed in order to avoid contamination by other tissue types. The PDL cells were harvested by six sequential digestions of PBS containing collagenase (1 mg/mL) and dispase (2 mg/mL). Cells isolated in runs 2-6 were pooled and cultured until confluence in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (DMEM complete medium) at 37 ºC in 5% CO_2 atmosphere. The sixth to eighth passage of PDL cells was used for the periodontal reconstruction. After

obtaining the PDL cells, the teeth were kept in a dry environment for 30 days, until autotransplantation.

Preparation of Autograft PDLCs-Alginate Hydrogel Vehicles

Two hours prior to autotransplantation, root canal treatment of the extracted teeth was completed in the laboratory. The canals were obturated with gutta-percha and then their external surfaces disinfected with 70% alcohol, before transferring into a physiological saline solution prior to the autotransplantation procedure. The cultured PDL cells were digested with 0.25% trypsin containing 0.02% ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA), rinsed with PBS once, then re-suspended in 1.2% alginate sodium solution which is a 4:1 mixture of Co^{60}-treated (irradiation at 5 kGy for 24 h) and untreated solution. The cell density was adjusted to 1×10^7 PDL cells/mL. Then, 20μL of 1 mol/L $CaCl_2$ solution was added to 100μl of PDL cell suspension to form an alginate hydrogel with 1×10^6 PDL cells.

Autotransplantation of Autologous Tooth Roots

A mucoperiosteal flap was raised from the first to the fourth premolar on each side of the mandibule under general anaesthesia. Artificial sockets matching the shape of the extracted autologous tooth roots were formed by low-speed sterile round bur under 0.9% cold physiological saline irrigation. Two artificial sockets were created in each side. The roots were divided into two groups: the experimental group - autotransplanted roots with alginate hydrogel containing about 1×10^6 PDL cells. For the control group - roots with alginate hydrogel only. Samples in the former group were transplanted into the artificial sockets at the right side of mandible, then covered

with a restorable membrane (Puros Pericardium Membrane, Zimmer Dental, Inc, CA) and the wound closed with 4-0 silk suture. The same procedures were carried out for the control group in the contralateral side.

Histological Analysis

Two months post-transplantation, the dogs were sacrificed with an overdose of ketamine hydrochloride. Mandibular block segments containing the autotransplanted roots were resected from the jaw and immersed into 10% neutral buffered formalin for three days. The specimens were decalcified in 14% EDTA solution (pH 7.0), and embedded in paraffin after dehydration in a graded alcohol series. Histological sections were cut perpendicular to the long axis of the roots at a thickness of 5 μm at 30μm intervals and stained with hematoxylin and eosin (HE), and were examined under an optical microscope (Olympus DP, Tokyo, Japan) for the formation of periodontium-like architecture. Based on a modified version described by Kirakozova (25), two blinded examiners assessed the type of periodontal healing in each section. Sample with the presence of PDL-like structure was scored as favorable healing, whereas sample without PDL like structure but with root resorption or ankylosis in the artificial periodontal space was scored as unfavorable healing.

Statistical Analysis

The measurements for favorable and unfavorable healing of roots were collected as percentages of total number of roots in each group. Chi-square test was used to determine whether the periodontal healing pattern was different in the two groups. MedCalc statistical software (MedCalc 9.2.1.0, Frank Schoonjans, Mariakerke, Belgium) was used to analyze all data at $P < 0.05$.

Results

The PDL cells derived from the extracted teeth were successfully isolated and expanded using the method described above. After 1 month, the extraction wounds healed and covered with healthy mucosa. Favorable healing after auto-transplantation in the newly created socket was observed in the experimental group. No periradicular infection was found clinically and the dogs recuperated very well for the two months post-operatively before they were sacrificed for histological examination.

In the experimental group, there were clear formation of root-PDL-bone complex in all histological sections; the "newborn" PDL-like structure was located between alveolar bone and the transplanted root (Fig. 1A). The root surface was lined with a thin layer of "new" cementum, into which Sharpey fibers were attached (Fig. 1B). These PDL-like fibers were found extending into the alveolar bone (Fig. 1C). The still-intact scaffold (alginate hydrogel) was also found in the PDL-like connective tissue (Fig. 1A) and in the restored alveolar bone (Fig. 1C). There were some patchy areas of ankylosis in this group of samples (Fig. 1D).

The control group showed an absence of PDL-like connective tissue. Ankylosis (Fig. 2A) and root resorption (Fig. 2B) were virtually noticed in all specimens. Table 1 showed the distribution of the positive roots per groups for the statistical analysis. Experimental group had significantly more favorable healing and less unfavorable healing than control group ($P < 0.05$).

Discussion

The "reconstruction" of the periodontium after delayed replantation mediated by cultured PDL cells is based on the idea of the periodontal tissue engineering. As the

success rate of traditional replantation procedure (for teeth with various extra-alveolar time after avulsion) is rather unpredictable, varying from 4 to 50% (15), it is necessarily to develop a new strategy for the treatment. Delayed autotransplantation combined with cultured autologous PDL cells, as was demonstrated above, is just a novel regime to tackle this clinical problem. PDL cells, typically, will remain viable for a finite period of time on the root surface after the tooth is avulsed; these cells play an important role in the periodontal healing of the replanted transplanted teeth (26). Regeneration of the PDL architecture is effective in preventing ankylosis and replacement resorption of the transplanted tooth roots (26).

In this present study, to avoid any influence on the outcome due to any remaining PDL cells surviving on extracted teeth roots were deliberately stored in a dry environment for one month. An artificial bony socket was also created to house the tooth. It is well known that the extraoral time and storage medium are critical for periodontal healing of replanted teeth – storage of less than 1 hour in an appropriate medium is recommended. In the present study, a PDL-like tissue was successfully regenerated by using this new strategy of replantation together with cultured autogenous PDL cells, despite the dry storage of the avulsed tooth for an extended period.

Alginate hydrogel was used as the scaffold here, because the hydrogel allows even distribution of the PDL cells within the material to fill the space in the artificial sockets. Alginate hydrogel has been used as a scaffold in hard (bone) tissue engineering (27, 28). To control the degradation rate of this scaffold, an irradiated (by Co^{60}) sodium alginate solution was mixed with an untreated solution at the ratio of 4:1. Gradual degradation of the alginate hydrogel left spaces for the newly formed periodontal tissue (although we could not match the rates of hydrogel degradation and

periodontal regeneration rate perfectly). In some specimens of the experimental group, there was only a small amount of un-degraded hydrogel sporadically found in the PDL-like connective tissues or the reconstructed alveolar bones, suggesting that the degradation rate of the alginate hydrogel could be controlled further.

Our results showed that the PDL-like structure was found in samples of autotransplantation with PDL cells, but not in the control group. Specimens showing a normal appearance of the architecture were found in some areas of the autotransplanted teeth of the experimental group. In contrary, ankylosis and resorption were commonplace for the control group. Since the newly formed PDL-like connective tissues were embedded into cementum on one end and alveolar bone on the other, it is clearly demonstrated that the cultured PDL cells have the potential to regenerate the periodontal tissues.

While a regenerated PDL-like structure had been found in this study, there were no newly formed fiber bundles with functional orientation of a healthy periodontium. This may be due to the followings: (1) that the autotransplanted roots were embedded and covered by the mucoperiosteum, and thus the root did not receive any mechanical stimuli from occlusion; (2) the mismatch in the rates of alginate hydrogel degradation and periodontal regeneration that might affect the organization of PDL fibers in that space.

The new strategy shed some lights on the treatment of avulsed teeth. But more studies need to be done before patients with avulsed teeth might benefit from this approach. One viable source of PDL cells can be from the stem cell bank derived from extracted the third molar or the first premolars owing to the need for orthodontic treatment.

In conclusion, this preliminary study has demonstrated the beneficial effects of cultured autologous PDL cells on periodontal healing of avulsed teeth following a delayed autotransplantation in dogs. The results indicated that periodontal ligament cells have the potential to regenerate periodontal tissues in artificial alveolar socket. Delayed autotransplantation combined with cultured autologous PDL cells might be a viable alternative management for the avulsed teeth that have left extraorally for an extended period of time. Further studies with a larger sample are necessary.

References

1. Ho SP, Marshall SJ, Ryder MI, Marshall GW. The tooth attachment mechanism defined by structure, chemical composition and mechanical properties of collagen fibers in the periodontium. Biomaterials 2007; 28: 5238-45.
2. Van Hassel HJ, Oswald RJ, Harrington GW. Replantation 2. The role of the periodontal ligament. J Endod 1980; 6: 506–8.
3. Schwartz O, Andreasen FM, Andreasen JO. Effects of temperature, storage time and media on periodontal and pulpal healing after replantation of incisors in monkeys. Dent Traumatol 2002; 18: 190-5.
4. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 4. Factors related to periodontal ligament healing. Endod Dent Traumatol 1995; 11: 76-89.
5. Krasner P, Person P. Preserving avulsed teeth for replantation. J Am Dent Assoc 1992; 123: 80-8.
6. Oswald RJ, Harrington GW, Van Hassel HJ. A postreplantation evaluation of air-dried and saliva-stored avulsed teeth. J Endod 1980; 6: 546–51.
7. Blomlof L, Lindskog S, Andersson L, Hedstrom KG, Hammarstrom L. Storage of experimentally avulsed teeth in milk prior to replantation. J Dent Res 1983; 62: 912–6
8. Pohl Y, Filippi A, Kirschner H. Results after replantation of avulsed permanent teeth. I. Endodontic considerations. Dent Traumatol 2005; 21: 80-92.

9. Andreasen JO. Effect of extra-alveolar period and storage media upon periodontal and pulpal healing after replantation of mature permanent incisors in monkeys. Int J Oral Surg 1981; 10: 43–53.
10. Andreasen JO, Andreasen FM, Mejare I, Cvek M. Healing of 400 intra-alveolar root fractures. 2. Effect of treatment factors such as treatment delay, repositioning, splinting type and period and antibiotics. Dent Traumatol 2004; 20: 203-11.
11. Andreasen JO, Paulsen HU, Yu Z, Ahlquist R, Bayer T, Schwartz O. A long-term study of 370 autotransplanted premolars. Part I. Surgical procedures and standardized techniques for monitoring healing. Eur J Orthod 1990; 12: 3-13.
12. Andreasen JO, Paulsen HU, Yu Z, Bayer T. A long-term study of 370 autotransplanted premolars. Part IV. Root development subsequent to transplantation. Eur J Orthod 1990; 12: 38-50.
13. Andreasen JO, Paulsen HU, Yu Z, Bayer T, Schwartz O. A long-term study of 370 autotransplanted premolars. Part II. Tooth survival and pulp healing subsequent to transplantation. Eur J Orthod 1990; 12: 14-24.
14. Andreasen JO, Paulsen HU, Yu Z, Schwartz O. A long-term study of 370 autotransplanted premolars. Part III. Periodontal healing subsequent to transplantation. Eur J Orthod 1990; 12: 25-37.
15. Krasner P, Rankow HJ. New philosophy for the treatment of avulsed teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995; 79: 616-23.
16. Caliskan MK, Turkun M, Gokay N. Delayed replantation of avulsed mature teeth with calcium hydroxide treatment. J Endod 2000; 26: 472-6.
17. Gebbardt M, Murray PE, Namerow KN, Kuttler S, Garcia-Godoy F. Cell survival within pulp and periodontal constructs. J Endod 2009; 35: 63-6.
18. Andreasen JO. Interrelation between alveolar bone and periodontal ligament repair after replantation of mature permanent incisors in monkeys. J Periodont Res 1981; 16: 228-35.
19. Lekic PC, Pender N, McCulloch CA. Is fibroblast heterogeneity relevant to the health, diseases, and treatments of periodontal tissues? Crit Rev Oral Biol Med 1997; 8: 253-68.
20. Lang H, Schuler N, Arnhold S, Nolden R, Mertens T. Formation of differentiated tissues in vivo by periodontal cell populations cultured in vitro. J Dent Res 1995; 74: 1219-25.

21. Herr Y, Matsuura M, Lin WL, Genco RJ, Cho MI. The origin of fibroblasts and their role in the early stages of horizontal furcation defect healing in the beagle dog. J Periodontol 1995; 66: 716-30.

22. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004; 364: 149-55.

23. Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. J Periodont Res 2001; 36: 71-9.

24. Yang ZH, Zhang XJ, Dang NN, Ma ZF, Xu L, Wu JJ, Sun YJ, Duan YZ, Lin Z, Jin Y. Apical tooth germ cell-conditioned medium enhances the differentiation of periodontal ligament stem cells into cementum/periodontal ligament-like tissues. J Periodont Res 2009; 44: 199-210.

25. Kirakozova A, Teixeira FB, Curran AE, Gu F, Tawil PZ, Trope M. Effect of intracanal corticosteroids on healing of replanted dog teeth after extended dry times. J Endod 2009; 35: 663-7.

26. Mine K, Kanno Z, Muramoto T, Soma K. Occlusal forces promote periodontal healing of transplanted teeth and prevent dentoalveolar ankylosis: an experimental study in rats. Angle Orthod 2005; 75: 637-44.

27. Lawson MA, Barralet JE, Wang L, Shelton RM, Triffitt JT. Adhesion and growth of bone marrow stromal cells on modified alginate hydrogels. Tissue Eng 2004; 10: 1480-91.

28. Alsberg E, Anderson KW, Albeiruti A, Franceschi RT, Mooney DJ. Cell-interactive alginate hydrogels for bone tissue engineering. J Dent Res 2001; 80: 2025-29.

Figure legends：

Figure 1. Experimental group (PDL cells-loaded samples) showing fibrous connective tissue in the artificial periodontal space (A), with fibers inserting into newborn cementum on one end (B) and alveolar bone on the other (C). Ankylosis was also found in some area in this group (D). Note: Bone = alveolar bone; S = un-degraded scaffold; CT = fibrous connective tissue; A = ankylotic area; Single arrows: Fibers embedded in the surface of the root surface or the alveolar bone; Double arrow:

Fiber bundle-like structure (HE stain, original magnification: ×200 for Figure. 1A and ×400 for the others, unit of scale bar: μm).

Figure 2. Control group showing areas with ankylosis and the artificial periodontal space filled by newborn bony tissue (A) and the presence of replacement root resorption. Note: B = alveolar bone; A = ankylotic area; (HE stain, original magnification ×400, unit of scale bar: μm).

Figure 1

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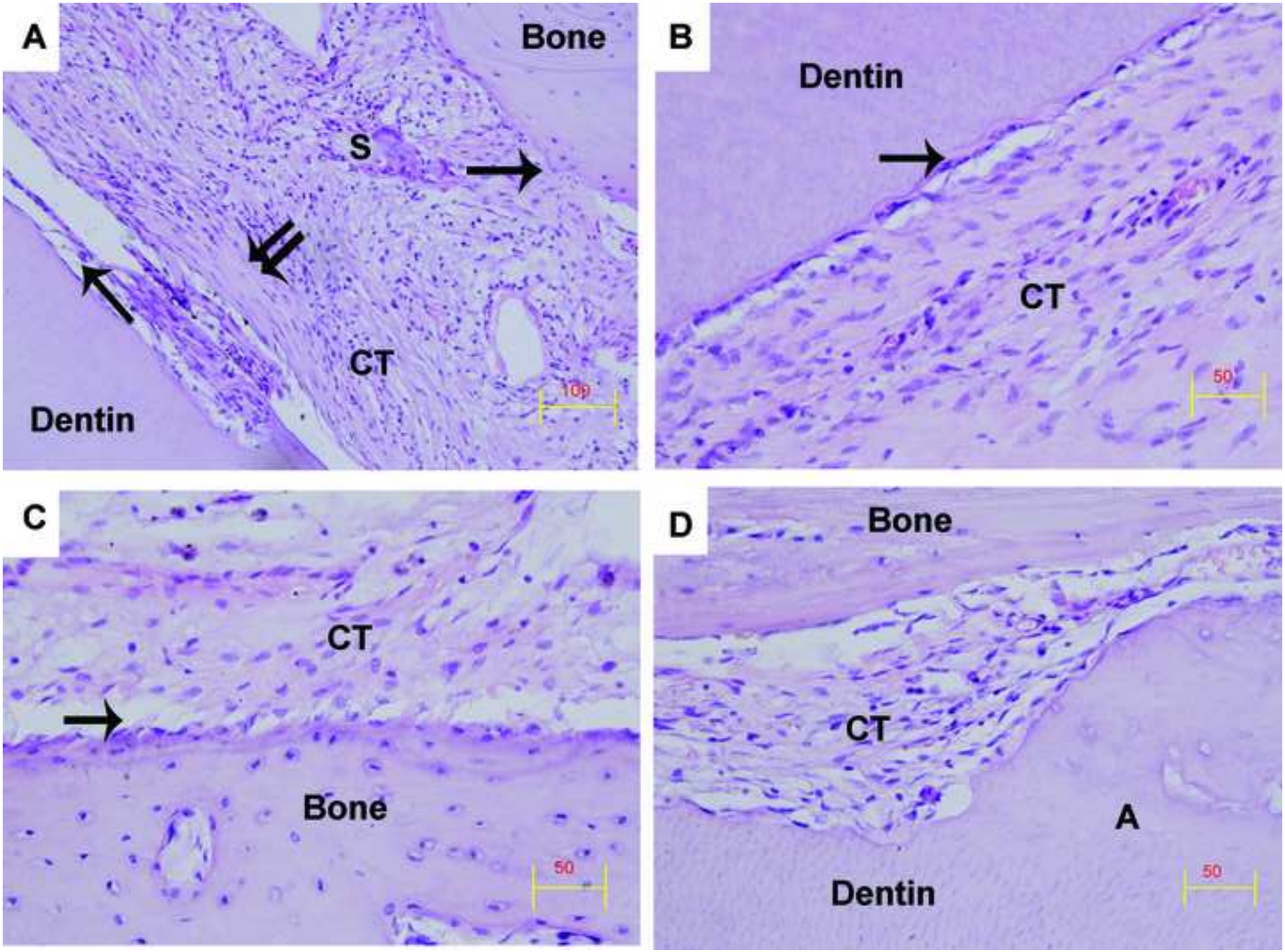

Figure 2

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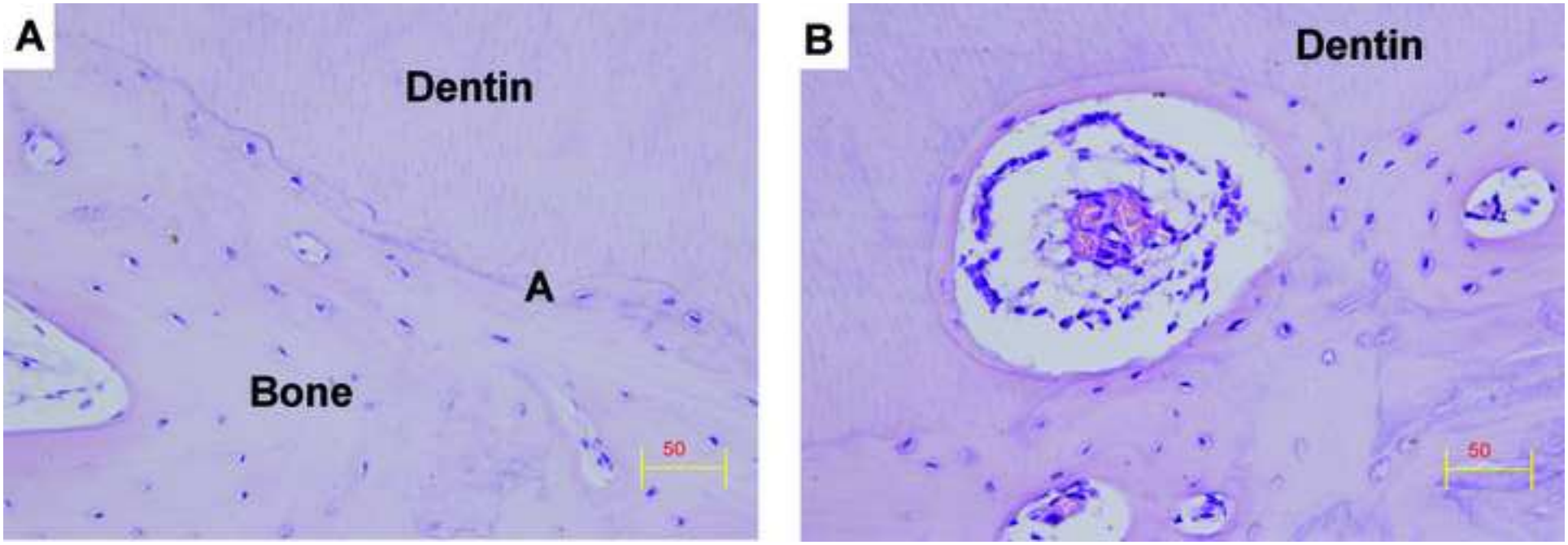

Table 1. Percentage of positive roots per group used for statistical analysis*

	Experimental group Percent (total roots)	Control group Percent (total roots)	Chi-square test *P* value**
Favorable healing	100%(6)	0%(6)	*P* = 0.002
Unfavorable healing: ankylosis	0%(6)	100%(6)	*P* = 0.002
Unfavorable healing: Root resorption	0%(6)	83.3%(6)	*P* = 0.015

* Three sections per root were evaluated.

** P<0.05 was considered statistically significant.