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Pulp Regenerative Cell Therapy for Mature Molars: A Report of 2 Cases

SIGNIFICANCE

The pulp regenerative therapy harnessing autologous DPSCs with G-CSF after complete disinfection may be a favorable treatment option also in mature multirooted molars for preservation.

ABSTRACT

Regenerative cell therapy using autologous dental pulp stem cells (DPSCs) in mature single-rooted teeth is a potential alternative to traditional endodontic treatment. However, there is no evidence supporting the use of DPSCs in multirooted teeth. This case report aimed to demonstrate the feasibility and outcomes of pulp regenerative cell therapy in mature multirooted molars, which typically have a higher prevalence of apical deltas. A 26-year-old male and a 29-year-old male were referred for the pulp regeneration of their maxillary molars. After access preparation and establishing apical patency, root canal preparation and disinfection were performed. Autologous DPSCs were isolated from extracted third molars, cultured according to the guidelines of good manufacturing practice, and transplanted into the prepared root canals with granulocyte colony-stimulating factor in atelocollagen. The access cavity was sealed with Biodentine and composite resin. Clinical evaluation during the follow-up period of 48 weeks and laboratory evaluation after 4 weeks revealed no adverse events or evidence of systemic toxicity. After 48 weeks, radiographs and cone-beam computed tomography showed no periapical radiolucency. The teeth showed a positive response to electric pulp testing in 4 weeks in both cases. The signal intensities on magnetic resonance imaging of the regenerated pulp tissue in the affected teeth were comparable to those of the normal pulp in adjacent teeth after 24 weeks. This report of 2 cases demonstrates the utility of DPSCs and the feasibility of pulp regenerative cell therapy in multirooted molars. *(J Endod 2022;48:1334–1340.)*

KEY WORDS

Case report; cell therapy; dental pulp stem cells; electric pulp test; multirooted teeth; pulp regeneration

Several procedures widely used for the revitalization of immature teeth are now being used for mature teeth[1]. However, histological studies indicate that these procedures are reparative, not regenerative processes, and that ectopic tissues, such as the periodontal ligament, cementum, and bone, formed in the canal space cannot function as pulp tissue[2]. In a preclinical study, we demonstrated complete pulp regeneration with adequate vascularization and reinnervation using pulp regenerative therapy with dental pulp stem cells (DPSCs) in mature teeth after pulp extirpation[3]. Natural dental pulp has defense mechanisms against bacterial invasion into the dentinal tubules and pulp inflammation, including the outward flow of dentinal fluid, reactionary/reparative dentin formation, immune defense reactions, pulp microcirculation, sensory and autonomic nerves, and neuropeptide release from sensory nerves[4]. Therefore, restoration of the pulp vitality in mature teeth through pulp regenerative cell therapy may prevent progression of caries and periapical inflammation and further prevent tooth fracture. Thus, pulp regenerative cell therapy has the potential to prolong the life of treated teeth. Our previous clinical study demonstrated the safety and efficacy of pulp regenerative therapy using DPSCs in treating irreversible pulpitis in mature single-rooted teeth[5]. However, pulp regenerative therapy in multirooted molars remains a challenge. A 5-log reduction in bacterial number (99.999%) is necessary for the root canal microenvironment to be conducive to pulp regeneration[6]. Apical deltas, which are an intricate system of root canal spaces divided into several ramifications near the apex, are more commonly present in molars than in anterior teeth and premolars[7,8]. Therefore, the outcomes of pulp regeneration in mature molars might differ from those in anterior teeth and premolars, as complex root canal systems, including the presence of apical deltas, might harbor residual infection. To prevent this, nanobubbles (100–200 nm in diameter) loaded with antibiotics were used for irrigation and as intracanal medicaments. In a canine

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0099-2399

https://doi.org/10.1016/j.joen.2022.07.010

model of intractable apical periodontitis, nanobubbles with triple antibiotic paste provided complete disinfection of the complex canal system and removed the biofilm and smear layer effectively[9].

Therefore, in this case report, we aimed to demonstrate the potential clinical use and outcome of pulp regenerative cell therapy in mature multirooted molars.

CASE REPORTS

Our provisional plan of using pulp regenerative cell therapy as a clinical treatment was approved by the Category II Certified Committee for Regenerative Medicine and further officially accepted by the Japanese Ministry of Health, Labour and Welfare (MHLW) (#PB5200005) based on the Act on Securement of Safety of Regenerative Medicine.

Case 1

A 26-year-old male was referred to our clinic for pulp regenerative cell therapy in a maxillary right second molar. Initially, the tooth was diagnosed with asymptomatic irreversible pulpitis with no signs of apical periodontitis and had been devitalized using formocresol one week prior by an outside dentist. Clinical examination revealed caries on the distal and buccal surfaces (Fig. 1*Aa*), with no clinical symptoms. The tooth showed a negative response to electric pulp testing (EPT) (Vitality Scanner/Pulp Tester, Yoshida, Tokyo, Japan). Periapical radiographic examination and cone-beam computed tomography (CBCT) revealed no periapical radiolucency (Fig. 1*Ba-c*) and the presence of 2 mesiobuccal canals that were curved and fused (Fig. 1*Bb-d*). After obtaining informed consent from the patient, the medical history was reviewed and the presence of any serious systemic diseases and infectious diseases was assessed by performing blood and urine tests and serological tests for common infectious agents.

The patient's maxillary right third molar (Fig. 1*Bb, d*) was extracted since it was deemed to be nonfunctional and was sent to the cell processing center facility (Aeras Bio Inc, Kobe, Japan). Enzymatically isolated DPSCs were cultured under stable hypoxic conditions, termed hpDPSCs, as previously reported[10], and bacterial colonies were formed after 7 days (Fig. 2*Aa*). The hpDPSCs (Fig. 2*Ab*) were cryopreserved at the fourth passage of culture, and the results of their quality and safety examinations were matched to our standard of quality control (Table 1).

The K-file #10 (MANI) was used to determine the canal length using an electronic apex locator (Root ZX, Morita, Tokyo, Japan) and establish patency. Canal preparations were performed using WaveOne Gold glider (#15/02 Dentsply Sirona, Tulsa, Oklahoma, United States) followed by ProTaper (Dentsply Sirona) S1, S2, F1, and F2 up to the apex. In this report, "apex" is defined as Root ZX digital display 0.5 on digital scale. This reference point (0.5 on the digital scale) indicates canal length that is 0.2–0.3 mm beyond physiologic apical foramen. Furthermore, the 2 mesiobuccal canals, which joined in the middle and apical thirds (Fig. 1*Ab, B*), were prepared manually with K-files from #30 to #45, while the distobuccal and palatal canals (Fig. 1*Ab, B*) were prepared also manually with K-files from #30 to #55 up to 0.5 mm from the apex. The canals were irrigated first with 6% sodium hypochlorite and then with nanobubbles (Aeras Bio Inc) containing triple antibiotic paste, which was used as an intracanal medicament (Fig. 2*B*).

At the next appointment a week later, the patient was asymptomatic. Samples obtained from the root canals were cultured in PLADIA liquid anaerobic culture media (Showa Yakuhin Kako Co, Ltd, Tokyo, Japan) for 5 days under anaerobic conditions to ascertain the presence of aseptic conditions in the root canals (Fig. 2*B*). The bacterial culture was positive for the palatal root canal and negative for the mesiobuccal and distobuccal root canals (Fig. 2*Ca*). The root canals were further irrigated and sealed using the same procedure as in the previous appointment (Fig. 2*B*). One week later, additional samples were obtained and cultured per the same protocols in the previous appointment; these samples were negative for all canals (Fig. 2*Cb*).

One week later, 3×10^6 cryopreserved autologous hpDPSCs were transported from the cell processing center facility to the clinic and transplanted as previously described[5]. In brief, after irrigation with 3% ethylenediaminetetraacetic acid (for 2 min) and saline, cell suspensions with granulocyte colony-stimulating factor (G-CSF) in atelocollagen were transplanted into the well-dried root canals using a 27G syringe tip (Fig. 2*Da, b*). After cell transplantation, a gelatin sponge (Spongel, LTL Pharma Inc, Tokyo, Japan) was placed over the root canal orifices and the floor of the pulp chamber. In addition, the cavity was sealed with Biodentine (Septodont, Lancaster, USA) and a composite resin (Fig. 2*Dc*).

The patient was followed up at 1, 4, 12, 24, and 48 weeks after cell transplantation. Blood and urine tests performed after 4 weeks and clinical examination at all follow-up appointments were within normal limits. The patient remained asymptomatic. The tooth initially did not exhibit any response to hot or cold stimuli. However, it exhibited a positive response to EPT after one week, indicating the initiation of functional reinnervation. After 48 weeks, periapical radiographs and CBCT showed no significant changes in the periapical region (Fig. 1*C, Da-d*), and radiopaque mineralized tissue deposition was apparent in the apical part of the root canals (Fig. 1*Dd*). CBCT images analyzed using OsiriX medical software (Pixmeo SARL, Geneva, Switzerland) showed a reduced volume of the regenerated pulp in the affected tooth, while no changes were observed in the normal pulp of the control (Fig. 1*E*, Table 2), indicating lateral dentin formation in the affected tooth. Furthermore, magnetic resonance imaging (MRI) was performed using a 1.5 Tesla (T) scanner under the conditions and parameters shown in Supplemental Table S1. According to the results of our previous MRI study, the high signal intensity (SI) of the regenerated pulp is comparable to that of the normal pulp within the root canal, while the SI of necrotic tissue after collagen transplantation alone is very low[11]. In this case too, the high SI of the regenerated pulp tissue in the affected tooth was comparable to that of the normal pulp in the adjacent teeth after 24 weeks (Fig. 1*F*), indicating complete pulp regeneration, as previously demonstrated[5].

Case 2

A 29-year-old male was referred to our clinic for pulp regenerative cell therapy in the maxillary right first molar. The patient exhibited pain in response to cold and hot stimuli and discomfort after undergoing direct pulp capping 4 months prior. Clinical examination revealed deep caries from the mesial to distal surfaces (Fig. 3*Aa*) under an existing onlay (Fig. 3*Ba*). The tooth showed a response to cold and hot sensitivity tests, no tenderness on percussion, and a positive response to EPT. Radiographs and CBCT revealed no periapical radiolucency (Fig. 3*Ba-c*) and the presence of 2 mesiobuccal root canals (Fig. 3*Bd*). The tooth was diagnosed with symptomatic irreversible pulpitis, with no signs of apical periodontitis.

Autologous hpDPSCs were prepared (Table 1) after obtaining informed consent from the patient. Pulpectomy and canal instrumentation and irrigation were performed similar to case 1. The apical patency of all canals was established by the manual use of K-files from #10 to #25 up to the apex; as shown in the electronic apex locator, the mesiobuccal and distobuccal root canals (Fig. 3*Ab, B*) were prepared with WaveOne Gold #35 (Dentsply Sirona), and the palatal

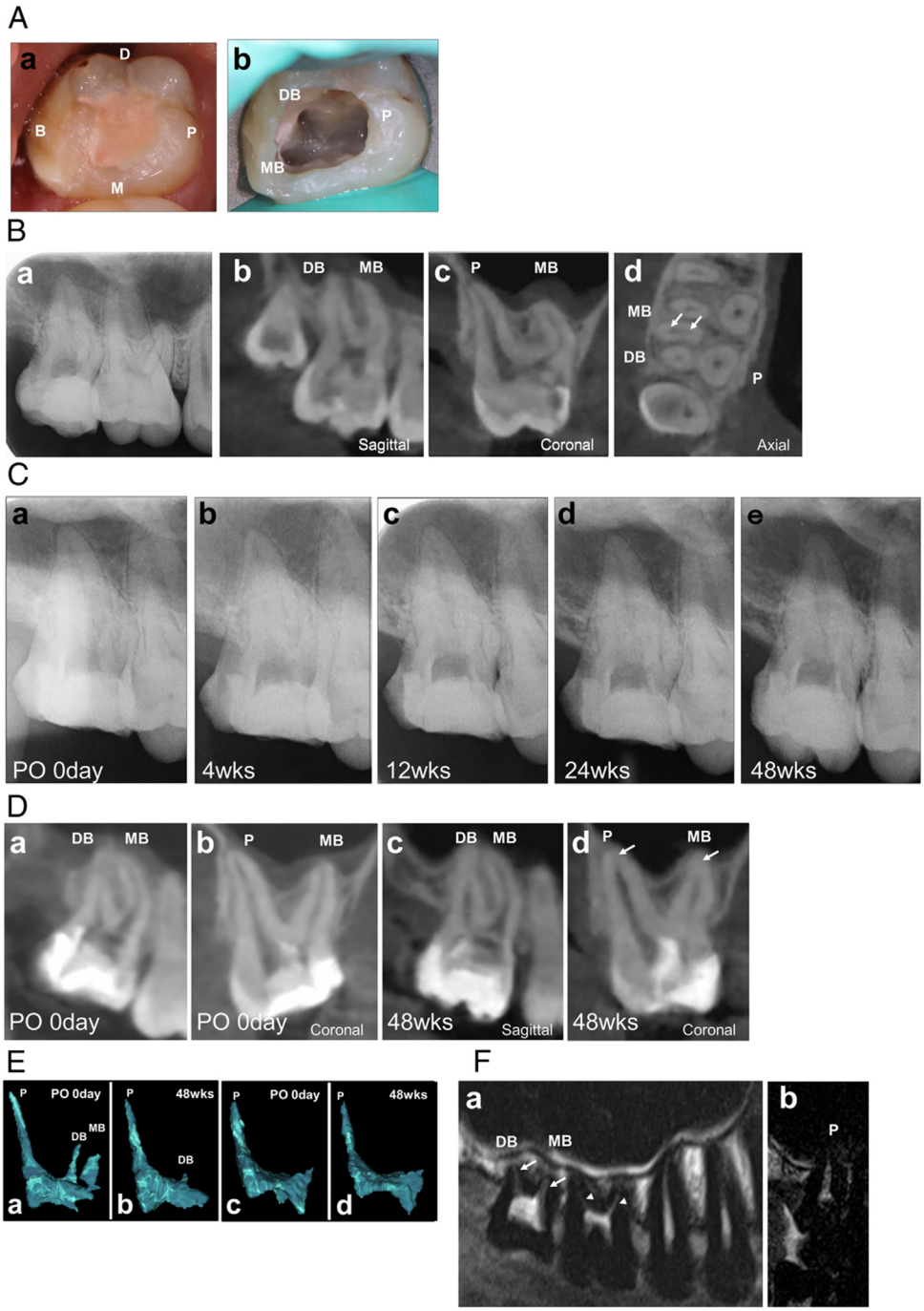

FIGURE 1 – Case 1. Maxillary right second molar. (*A*) Mirror images of the preoperative occlusal view (a) and occlusal view with the access cavity preparation. (*B*) Preoperative radiographs showing caries on the distal surface and a large restoration on the buccal and occlusal surfaces (a) and cone-beam computed tomography (CBCT) showing caries on the distal surface in the sagittal view (b), caries on the buccal surface and curved mesiobuccal root canal in the coronal view (c), and 2 orifices of the mesiobuccal root canals (*arrows*) in the axial view (d). (*C*) Follow-up radiographs after 48 weeks show the absence of periapical radiolucency and no widening of the periodontal ligament space (a–e). (*D*) CBCT immediately after cell transplantation (postoperative [PO] day 0) (a, b) and CBCT after 48 weeks (c, d). Sagittal view (a, c). Coronal view (b, d). Note the absence of periapical radiolucency and lateral dentin-like mineralized tissue formation in the apical part of the root canals (*arrows*) after 48 weeks. (*E*) Analysis of 3D CBCT images using the OsiriX program demonstrates a decrease in low-density areas after 48 weeks (b) compared with immediately after cell transplantation (PO day 0) (a). Cell-transplanted maxillary right second molar (a, b) and the control maxillary right first molar (c, d). (*F*) T2-weighted magnetic resonance images of cell-transplanted root canals after 24 weeks. Sagittal views show high signal intensities (SIs) of the mesiobuccal and distobuccal (a) and palatal (b) root canals. Note the similar SI of the cell-transplanted root canals (*arrows*) with the SI of healthy normal pulp (*arrowheads*).

root canal (Fig. 3*Ab, B*) was prepared with WaveOne Gold #35, #45 and manually with K-file #55 up to 1.0 mm from the apex. Transplantation was performed after confirming the aseptic condition of all 4 root canals (Fig. 2*Cc*).

Follow-up examinations performed up to 48 weeks showed that the patient was asymptomatic and exhibited no adverse

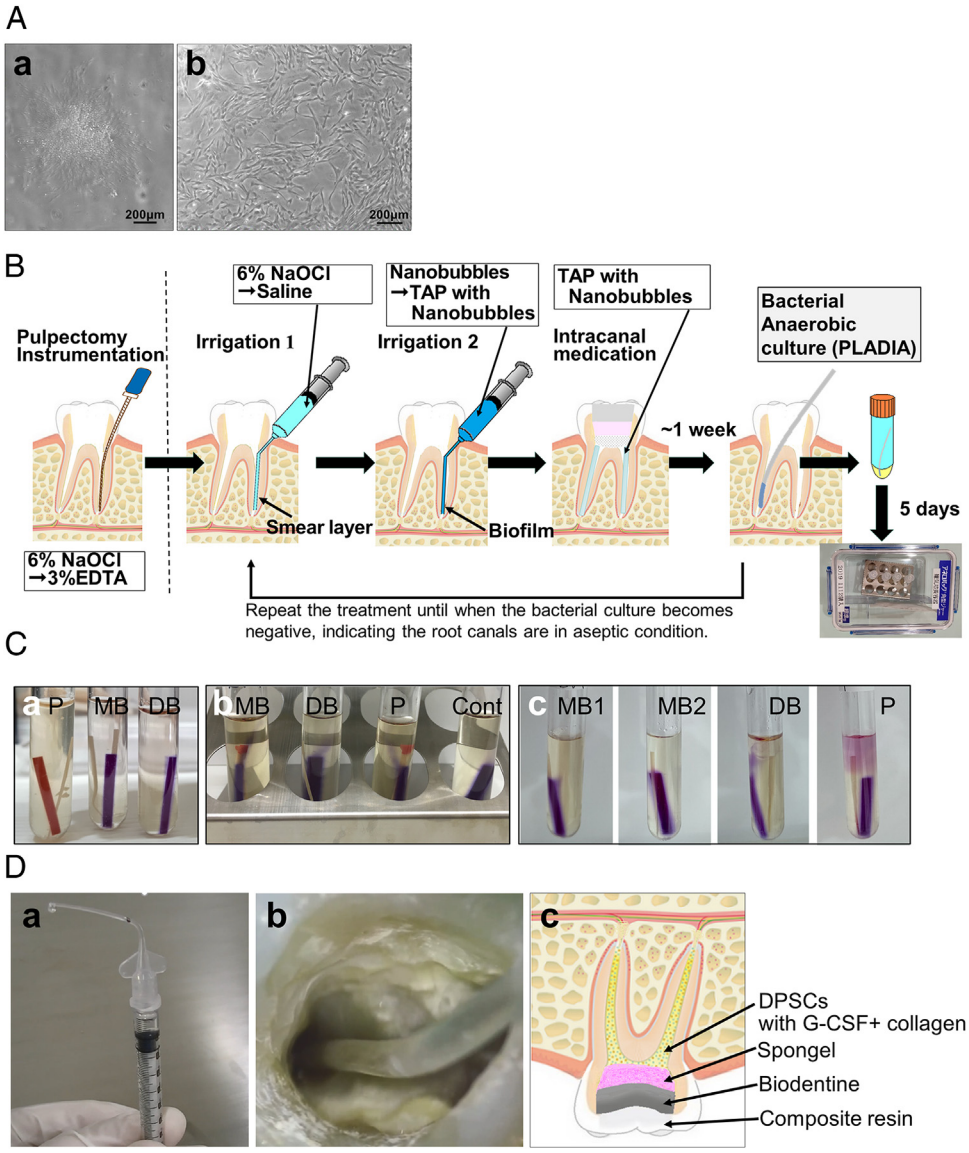

FIGURE 2 – Schematic diagrams and photographs of the endodontic treatment and transplantation procedures and the 48-week follow-up cone-beam computed tomography images. (*A*) Granulocyte colony-stimulating factor with dental pulp stem cells (DPSCs) at primary culture (a) and the DPSCs at the third passage of culture under hypoxic conditions just before cell detachment and cryopreservation for cell transplantation. (*B*) Schematic diagram of the procedures involved in endodontic treatment: irrigation, placement of intracanal medication, and anaerobic bacterial culture. (*C*) Anaerobic bacterial culture showing positive results (red) for samples from the palatal root canal indicated by resazurin paper and negative results (blue) from samples from the mesiobuccal and distobuccal root canals at the third appointment (a). Results of bacterial culture were negative in all root canals at the fourth appointment (b) in case 1. The results of anaerobic bacterial culture were negative for all root canals at the fourth appointment in case 2 (c). (*D*) Cell injection syringe containing the DPSCs and granulocyte colony-stimulating factor in atelocollagen (a), cell transplantation into the root canal using a syringe (b), and diagram showing the method of sealing the transplanted DPSCs (c).

events. In addition, no findings suggestive of systemic toxicity were evident in the laboratory evaluations performed at 4 weeks. Radiographic examinations showed no significant changes in 48 weeks (Fig. 3*C*). The tooth showed a positive response to EPT after 4 weeks. The CBCT showed no radiolucency in the periapical area (Fig. 3*D*), and the formation of mineralized tissue along the dentinal wall in the apical third of the palatal root canal was apparent after 48 weeks (Fig. 3*Dc, d*). The volume of the regenerated pulp had decreased after 48 weeks (Table 2, Fig. 3*E*). Furthermore, the SI on MRI after 24 weeks demonstrated very few differences between the affected and control teeth (Fig. 3*F*).

DISCUSSION

This is the first report to evaluate the feasibility of a protocol for pulp regenerative cell therapy in multirooted molars. All previously reported cell therapies have been performed in single-rooted teeth[5,12–16]. This could be because vitality evaluation with an EPT is easier in single-rooted teeth than in multi-rooted teeth, especially when the large access cavities of multirooted teeth are restored using restorative materials that interfere with the conduction of electricity. Furthermore, multirooted teeth have more anatomic intricacies in the root canal system, including the presence of the apical

TABLE 1 - The Quality Tests Including Doubling Time, Viability, Expression Rate of Stem Cell Markers, and Safety Tests of hpDPSCs at the Fourth Passage of Culture after Thawing

Quality test	Case 1	Case 2
Stem cell markers		
CD29 (%)	96.8	99.3
CD105 (%)	97.6	99.3
CD31 (%)	0.0	0.5
Viability (%)	80.5	98.0
Doubling time (hrs)	11.9	30.1
Bacteria (aerobe, anaerobe, fungus)	(-)	(-)
Endotoxin (pg/ml)	<1.0	<1.0
Mycoplasma	(-)	(-)

delta, leading to unexplainable failure of endodontic treatment[8].

Critical challenges in feasibility of this protocol include government regulatory issues in regenerative medicine, difficulties in stem cell isolation and expansion, limited availability of good manufacturing practice facilities, and poor cost performance[17]. These challenges were partially addressed in the present report. According to the Act on the Safety of Regenerative Medicine, a certified committee for regenerative medicine and the MHLW must approve the treatment protocol before an endodontist begins the treatment, which is not covered by health insurance. The endodontists can contract with good manufacturing practice compliance facilities approved by the MHLW for the cell processing of DPSCs, which is cost-effective and efficient, and thus do not need to isolate and expand DPSCs by themselves in their own facilities. Furthermore, isolation and culture methods for hpDPSCs, which are safer, have higher regeneration potential, and are more cost-effective than the mobilized DPSCs used in our previous clinical study[5], have been successfully developed[10].

In both cases, clinical evaluations performed within 48 weeks did not reveal any adverse events. Laboratory evaluations performed at 4 weeks did not reveal any toxic response, especially with respect to C-reactive protein level, white blood cell count, white blood cell fraction, and total bilirubin level. In addition, CBCT performed after 48 weeks did not show any periapical radiolucency, indicating the safety and efficacy of DPSC transplantation in pulpectomized molars. Positive responses to EPT, starting at 4 weeks, continued during the follow-up duration of 48 weeks. Evaluation of CBCT images using OsiriX showed that the pulp volume had decreased in 48 weeks due to lateral dentin formation, which is a function of the pulp[18]. Previous studies involving MRI and histological analysis of extracted teeth in dogs demonstrated that the high SI of regenerated pulp tissue 24 weeks after cell transplantation in affected teeth was comparable to that of normal pulp in intact teeth. Additionally, the SI was very low both in pulpectomized teeth and in teeth that had undergone collagen transplantation, and there was no evidence of pulp regeneration after 24 weeks[11]. Furthermore, our clinical study on pulp regenerative cell therapy demonstrated that regenerated pulp tissue with a positive response to EPT and lateral dentin-like mineralized tissue formation revealed the high SI similar to that of normal dental pulp in control intact teeth[5], demonstrating the potential usefulness of MRI in evaluating treatment outcomes. In this study, the results of EPT, CBCT, and MRI confirmed the potential efficacy of pulp regenerative cell therapy in multirooted molars, which are similar to the results found in our previous clinical study for single-rooted teeth[5].

In the present cases, the closed apices of the mature molars with apical deltas were prepared by canal instrumentation. Thus, in the presence of G-CSF, transplanted DPSCs with a high migratory activity could migrate to the apical part of the root canals, where blood supply was available[3], and survive to release trophic factors. The effects of these trophic factors with G-CSF on vascularization, reinnervation, regulation of inflammation, and enhanced migration of resident stem cells to the root canal are potential mechanisms of success in mature teeth[3].

To protect the regenerated pulp tissues, Biodentine was used as a dentin substitute under the composite resin restoration and over the gelatin sponge[19]. The absence of clinical symptoms of microleakage after 48 weeks in the present cases suggests that Biodentine can be a potential capping agent. However, the formation of only a small amount of dentin bridge-like mineralized tissue indicates that further development of capping agents/materials that induce the formation of a larger amount of dentin, thus providing a physical barrier and protection for the regenerated pulp tissue, is necessary in the future.

In conclusion, this case report demonstrates the regeneration of pulp tissue containing sensory nerves using pulp regenerative therapy with DPSCs in multirooted molars. The technique might be useful in the field of endodontics by expanding the use of DPSCs to include multirooted teeth to maintain the pulpal function, thus preventing further progression of caries and inflammation of affected teeth.

TABLE 2 - Evaluation of CBCT Images of the Pulp Volume by OsiriX Medical Software

	Case 1	Case 2
Affected tooth		
Day 0	0.055 cm^3	0.065 cm^3
48 wk	0.038 cm^3	0.058 cm^3
Reduced volume rate (%)	31.0	10.8
Control tooth		
Day 0	0.041 cm^3	0.038 cm^3
48 wk	0.040 cm^3	0.037 cm^3
Reduced volume rate (%)	2.4	2.6

The low-density areas ranging from 0 to 1,100 and from 0 to 1,000 were considered as the dental pulp at postoperative day 0 and at 48 weeks, respectively, in case 1. The low-density areas ranging from 0 to 1,400 were considered as the dental pulp both at day 0 and at 48 weeks in case 2. The areas with these density ranges were automatically deducted by region growing, and the volumes of the dental pulp were calculated.

ACKNOWLEDGMENTS

The authors thank Prof. Akitoshi Katsumata (Asahi University) for analyses of the CBCT images, Mr. Ryosuke Nasada (Kobe City Medical Center General Hospital) for MRI diagnoses, Dr Iwao Takenono for extraction of the autologous teeth, Drs Hiroyuki Tanaka and Maiko Nakahama for their assistance in prognosis determination, and Dr Nadia Chugal for English editing.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

The authors deny any conflicts of interest related to this case report.

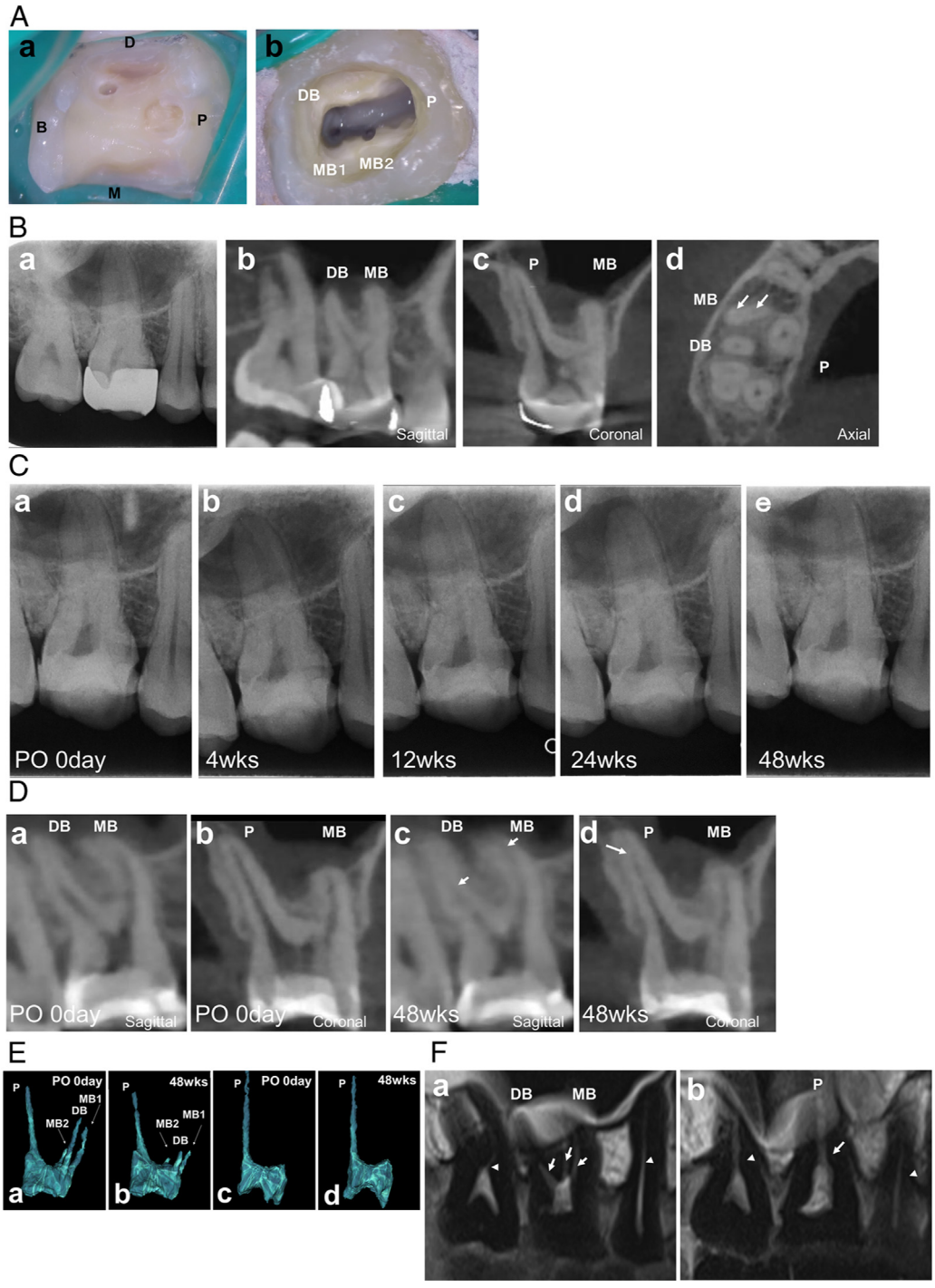

FIGURE 3 – Case 2. Maxillary right first molar. (*A*) Mirror images of occlusal view before (a) and after access cavity preparation (b). (*B*) Preoperative radiographs showing caries under the metal onlay (a) and cone-beam computed tomography (CBCT) showing no abnormal periapical findings in the sagittal (b) and coronal (c) views. Two mesiobuccal root canals (*arrows*) in the axial view (d). (*C*) Follow-up radiographs after 48 weeks showing an absence of periapical radiolucency and no widening of periodontal ligament space (a–e). (*D*) CBCT immediately after cell transplantation (postoperative [PO] day 0) (a, b) and then after 48 weeks (c, d). Sagittal view (a, c). Coronal view (b, d). Note the absence of periapical radiolucency and lateral dentin-like mineralized tissue formation in the apical part of the root canals (*arrows*) after 48 weeks. (*E*) Analysis of 3D CBCT images using the OsiriX program demonstrates a decrease in low-density areas at 48 weeks (b) compared with that immediately after cell transplantation (PO day 0) (a). The cell-transplanted maxillary right first molar (a, b) and the control maxillary right second molar (c, d). (*F*) T2-weighted magnetic resonance image of cell-transplanted root canals after 24 weeks. The sagittal view shows signal intensities (SIs) of mesiobuccal and distobuccal (a) and palatal (b) root canals. Note similar SI of the cell-transplanted root canals (*arrows*) with the SI of healthy normal pulp (*arrowheads*).

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SUPPLEMENTAL TABLE S1 - The Imaging Conditions and Parameters of Magnetic Resonance Imaging (MRI)

MRI	1.5 Tesla (T)
Product name	MAGNETOM Avanto fit, Siemens Healthineers, Erlangen, Germany
Equipped Coil	4-channel special purpose coil
Image	T2-weighted images (T2WI)
Spin echo	Turbo spin echo (TSE)
Repetition time (TR)	4,000 msec
Echo time (TE)	87.0 msec
Slice thickness	4.0 mm
Slice spacing/Slice gap	0.4 mm