Pulp Regeneration: Current Approaches, Challenges, and Novel Rejuvenating Strategies for an Aging Population

ABSTRACT
We showed the safety and efficacy of pulp regenerative therapy by the autologous transplantation of mobilized dental pulp stem cells with granulocyte colony-stimulating factor in a pilot clinical study of young and middle-aged pulpectomized teeth. An experimental study in dogs further demonstrated an age-dependent decline in the amount of regenerated pulp tissue. In our society, in which people will soon live beyond 100 years, this therapy should be efficacious for contributing to the functional survival and endurance of the tooth not only for pulpectomized young teeth but also for aged teeth with periapical disease. However, there are challenges: 1 is enhancing pulp regeneration in aged teeth, and another is complete disinfection before cell transplantation. Thus, this review presents trypsin pretreatment for the former and a novel irrigant, nanobubbles with antibacterial nanopolymers, for the latter, thus demonstrating potential utility for pulp regenerative therapy in aged teeth with periapical disease. (J Endod 2020;46:S135–S142.)

KEY WORDS
Aged teeth; dental pulp stem cells; nanobubbles; pulpectomy; pulp regeneration; trypsin

LIMITATIONS OF PULP REGENERATION THERAPY IN AGED TEETH
There are several pathologic changes that develop with age and serve as the main characteristics of aged tissue. Consequently, there are age-related changes in the composition and function of the dental pulp tissue. In our society, in which people will soon live beyond 100 years, pulp regenerative therapy should be efficacious not only for noninfected pulpectomized cases but also for infected root canals in order to provide the functional survival and longevity of the tooth. However, complete disinfection before cell transplantation by irrigants and intracanal medication is a challenge. We recently developed negatively charged nanobubbles containing pressured air, which have the ability to remove the smear layer and enhance the delivery of medications to dentinal tubules, thus demonstrating the potential utility of nanobubbles with antibacterial nanopolymers for the successful treatment of infected root canal and pulp regeneration. Therefore, enhanced pulp regeneration by trypsin pretreatment and complete disinfection by nanobubbles with antibacterial nanopolymers are discussed in this article.

SIGNIFICANCE
Pulp regenerative therapy is a promising approach to preserve the function and endurance of teeth. However, age-dependent decline in pulp regeneration and complete disinfection before cell transplantation are challenges. We demonstrated enhanced pulp regeneration by treatment of aged teeth with trypsin before MDPSC transplantation and complete disinfection by antibacterial nanopolymers with nanobubbles in a periapical disease model in dogs. These results suggest their potential utilities to ensure pulp regenerative therapy.

From the Departments of Stem Cell Biology and Regenerative Medicine and Oral Disease Research, National Center for Geriatrics and Gerontology, Research Institute, Oobu, Aichi, Japan; Air Water Group, Aeras Bio Inc, Kobe, Hyogo, Japan; and Department of Animal Surgery, School of Veterinary Medicine, South Valley University, Qena, Egypt

Address requests for reprints to Dr Misako Nakashima, Department of Stem Cell Biology and Regenerative Medicine, National Center for Geriatrics and Gerontology, 7-430 Morioka, Oobu, Aichi 474-8511, Japan. E-mail address: misako@ncgg.go.jp 0099-2399

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and tooth structure. With aging, the pulp tissue changes into dense fibrous tissue from a loose connective tissue by increasing collagenous fibers and decreasing cell density and volume. Secondary dentin and cementum deposition are increased in the apical portion of the tooth, leading to constriction of the apical foramen, hypercementosis, and stenosis and fibrosis of the periodontal ligament. Therefore, these changes can prevent the migration of resident stem cells from the surrounding tissue into the root canal. With apical constriction, the blood vessels, lymphatics, and nerve supply, which normally enter the root canal from the apical foramen, are reduced. Moreover, the number of blood vessels and nerves are decreased in the coronal pulp because of mineralization. Odontoblasts and fibroblasts are also reduced in number in aged pulp, resulting in a decline of their ability to respond to injury.

On the other hand, lipofuscin clustered deposits, a general phenomenon with aging, increase in aged odontoblasts, which affects the function of odontoblasts through the inhibition of lysosomal degradation capacity. Additionally, odontogenic markers; dentin sialophosphoprotein, dentin matrix protein 1, and osteogenic markers; bone morphogenetic protein 2 and 7; and osteopontin are and osteogenic markers; bone morphogenetic protein 2 and 7; and osteopontin are increased in the apical portion of the tooth, leading to constriction of the apical foramen, hypercementosis, and stenosis and fibrosis of the periodontal ligament. Therefore, these changes can prevent the migration of resident stem cells from the surrounding tissue into the root canal. With apical constriction, the blood vessels, lymphatics, and nerve supply, which normally enter the root canal from the apical foramen, are reduced. Moreover, the number of blood vessels and nerves are decreased in the coronal pulp because of mineralization. Odontoblasts and fibroblasts are also reduced in number in aged pulp, resulting in a decline of their ability to respond to injury.

The reduction of immune cells in the aged pulp tissue, the defense reaction to irritants decreases and necrosis increases. Increased inflammation in the aged tissues and surrounding niche may result in inhibition of the regenerative process as well as reduction of stem cell function. Consequently, the progenitor cells, especially primary mesenchymal stem cells, may alter their proliferation, differentiation, and therapeutic potential because of the effect of immunosenescence. Furthermore, it has been shown that impaired intrinsic beneficial functions and misbalanced regulatory events alter the immunoregulatory potential of mesenchymal stem cells. As a consequence, all of the previously mentioned changes make pulp regeneration in aged teeth challenging.

We previously related high expression levels of trophic factors with high migration, proliferation, and antiapoptotic activities in aged MDPSCs, and these activities were similar to those observed in young MDPSCs. However, there was little difference in the regenerative potential between aged MDPSCs and young MDPSCs in an ischemic hind limb model and an ectopic tooth root model. As an alternative, in vitro comparison between aged and young MDPSCs in dogs did not show differences in their stem cell properties or trophic effects. The well-vascularized, innervated pulp regenerated after autologous transplantation of MDPSCs with G-CSF was similar to normal pulp in aged dogs. However, the volume of the regenerated tissue was smaller in aged dogs compared with young dogs. This difference was possibly caused by apical constriction and a decreased number of resident stem/progenitor cells with decreased migration abilities migrating from the surrounding tissues, such as the periodontal ligament, into the root canal. Associated fibrous tissue and chronic inflammation in the apical region as well as apoptosis and impaired function of resident stem cells might also be strong causative possibilities. These hypotheses suggest that specific targeting of the microenvironment/niche and resident stem/progenitor cells, with effects on cell migration, anti-inflammation, cytokine/growth factor expression, and cell survival activity, should be considered to enhance pulp regeneration in aged teeth.

**POTENTIAL STRATEGY TO ENHANCE PULP REGENERATION BY TREATMENT OF PULPECTOMIZED TEETH WITH TRYPsin BEFORE CELL TRANSPLANTATION IN AGED DOGS**

The application of stem cells together with some factors, such as cytokines or biomatrices, could establish a healthy paracrine environment and promote the generative ability of the transplanted cells. Enzymes can play an important role in the enhancement of cell function through changes in histone and/or DNA methylation. Enzymes can also prevent integrin damage and improve endothelial cell adhesion. Trypsin is a proteolytic enzyme that has been used clinically to facilitate tissue repair in different tissues. Trypsin shows anti-inflammatory, antiedematous, anti-infective, and fibroinotic properties. The activation of protease-activated receptors by trypsin is the main process involved in tissue regeneration in which the inhibition of inflammation, vascular changes, and release of cytokines occur. Trypsin-activated receptor 2 (PAR2) is a G protein–coupled receptor activated mainly by trypsin that cleaves its N-terminus. Our canine preliminary study showed that trypsin pretreatment of the root canal before MDPSC transplantation could enhance pulp regeneration in aged teeth (5–6 years old). In the preclinical efficacy test, the amount of regenerated tissue was significantly increased by 0.05% trypsin pretreatment for 10 minutes in nanobubbles compared with control nonpretreatment in aged dogs at 14 days (Fig. 2A and B). Angiogenesis was also increased in regenerated pulp tissue and peripulpal tissue. However, no significant effect of trypsin was demonstrated in the young dogs (8–10 months old). The root canal was filled with well-vascularized, innervated pulp tissue and was considerably enclosed by newly formed dentinlike mineralized tissue at 36 weeks. There were no abnormalities, no infiltration of inflammatory cells, and no internal or external resorption of the teeth at 36 weeks, indicating the safety of trypsin pretreatment in pulp regeneration. Serum and urine chemistry tests showed that values were within normal ranges 12 weeks after trypsin pretreatment.

Furthermore, the underlying mechanism of enhanced pulp regeneration by trypsin pretreatment in aged teeth was elucidated by molecular biological and protein chemical analyses. Our first hypothesis is that trypsin has a direct effect on rejuvenation via PAR2 signaling, which enhances the antiapoptosis, anti-inflammatory, and migration potential of the resident stem cells in the surrounding tissue, especially the periodontal ligament. The second hypothesis is that the indirect effects of trypsin-treated cementum and dentin modify the microenvironment and stimulate migration of resident stem cells into the root canal. The cementum or dentin extract may also enhance vascularization and reinnervation and induce pulp tissue phenotype and odontoblast differentiation. For the first hypothesis, we showed that periodontal ligament cells (PDLCs) isolated from aged dog teeth exhibited a higher expression of PAR2 using Western blot analysis compared with PDLCs from young dog teeth. The genes with higher expression levels were identified in the aged PDLCs after direct trypsin treatment in vitro upon comparison with the nontreated aged PDLCs by microarray analysis, thus revealing the genes related to senescence, antiapoptosis, anti-inflammatory, and the extracellular matrix. Reverse transcription polymerase chain reaction further confirmed that after trypsin treatment, the aged PDLCs significantly increased the gene expression of tissue repair–associated factors, anti-inflammatory factors, and the pulp tissue marker thyrotropin releasing hormone degrading enzyme. Moreover, the cell survival protein Bcl-2 was increased in the aged
PDLcs receiving trypsin treatment, thus indicating its cell survival effect. For the second hypothesis (the indirect effect of trypsin), more proteins were found using proteomic analysis to be extracted from the cementum of aged dogs that received trypsin treatment than from dogs receiving the phosphate-buffered saline control, demonstrating the potential role of many cytoskeleton and extracellular matrix proteins, including fibronectin, in the regeneration process. It has been shown that fibronectin is important in tissue regeneration, and its supplementation could support the regenerative capacity of old muscles. By treating cementum extract with 3% EDTA for 2 minutes followed by 0.05% trypsin for 10 minutes, our study found that aged teeth contained more fibronectin than young teeth. Similar to cementum, fibronectin was also released from dentin by trypsin, and more was released by treatment with 0.05% trypsin for 30 minutes compared with 10% EDTA for 30 minutes from both aged and young dentin after treatment with 3% EDTA for 2 minutes.

Dentin extract by trypsin treatment from aged teeth significantly increased the expression of anti-inflammatory markers, such as indoleamine 2,3-dioxygenase, transforming growth factor beta, and prostaglandin E synthase, as well as the pulp tissue marker thymotropin releasing hormone degrading enzyme compared with EDTA or the phosphate-buffered saline control in aged DPSCs. The cells attached to the dentinal wall significantly increased in cell number and more differentiated into odontoblasts by treatment with 0.05% trypsin for 2 minutes compared with 10% EDTA for 2 minutes. Moreover, both dentin and cementum extract significantly increased angiogenesis, neurite extension, and migration ability, similar to fibronectin. Thus, these results suggested the role of trypsin in the stimulation of pulp and dentin regeneration (Fig. 3); trypsin has a direct effect on the aged resident stem cells that enhances cell survival activity and increases the expression of tissue repair–associated factors via PAR2. Regarding the indirect effect, both cementum and dentin extracts, including fibronectin, stimulate the migration of resident cells into the root canal and enhance angiogenesis and neurite extension. The dentin extract has effects on cell attachment, anti-inflammation, pulp induction, and odontoblastic cell differentiation. EDTA treatment has previously been shown to release growth factors, especially transforming growth factor beta 1, from dentin and to promote the adhesion, migration, and differentiation of DPSCs into odontoblastlike cells on dentin. EDTA treatment before tooth root transplantation with fibrin induced chemotaxis and pulplike tissue formation in a cell homing model in mice. However, the present results showed a higher cell attachment and odontoblastic differentiation along the trypsin-treated dentinal wall compared with the EDTA-treated dentinal wall, a higher expression of anti-inflammatory factors in aged DPSCs supplemented with dentin extract by trypsin treatment, and more release of fibronectin from aged dentin after trypsin treatment compared with EDTA treatment. Therefore, our results show that trypsin pretreatment might be superior to EDTA pretreatment and is a promising candidate to stimulate pulp regeneration in aged teeth.

**COMPLETE DISINFECTION BY NANOBUBBLES WITH ANTIBACTERIAL NANOPOLYMER**

Nanobubbles are produced by a nanoscale porous polymer film (Monotrape film; Nac Corp, Siki, Japan) with pressurized gas from a nanobubble generator (FOAMEST B; Nac Corp). Nanobubbles contain pressurized air; these bubbles are approximately 100–200 nm in diameter and were found to have a concentration of $0.7–4 \times 10^8$ particles/mL, as determined via the nanotracking method using the Nanosight instrument (NS500; Quantum Design, San Diego, CA). Their surface is negatively charged, whose zeta potential is $–18$ to $–22$ mV by Zetasizer Nano ZS (Malvern Panalytical Ltd, Worcestershire, UK), and their pH is 6.6–7.0, as determined by a pH meter with an electrode that can measure ultrapure water (Orion Star A210 Series; Thermo Fisher Scientific KK, Tokyo, Japan). The enhanced delivery of medication into dentinal tubes and complex root canals and removal of the smear layer were demonstrated by an experimental model using porcine tooth roots in vitro (Fig. 4A). To examine the effect of nanobubbles in infected root canal treatment, an intractable periapical disease model was constructed in dogs after opening the root canal for 1 month followed by closing the access without any medication for more than 2 months. This root canal could not be completely disinfected, even after irrigation and administration of intracanal medication with a final concentration of $10^7$ particles/mL nanobubbles and $35 \mu g/mL$ doxycycline hydrochloride hydrate (Vibramycin; Pfizer, New York, NY), which was repeated more than 5 times. Thus, further irrigation and administration of intracanal medication were performed with $10^7$ particles/mL nanobubbles and antibacterial cyanoacrylate nanopolymers (kindly provided from Nano CAME Co, Ltd, Yokohama, Japan). The efficacy of disinfection was examined using the bacterial anaerobic culture method. These antibacterial nanopolymers have high affinities for the sugar chain peptide surface layer of the bacterial cell wall. The synthesis of the cell wall is inhibited in the local areas to which the nanopolymer has adsorbed, which makes it impossible to maintain the internal pressure and leads to bacterial cell autolysis. The antibacterial nanopolymers repeatedly cause bacteria to burst, showing substantive antimicrobial activity. The antibacterial nanopolymers...
applied with nanobubbles significantly reduced the number of bacterial colonies under the limit of detection at the third application (Fig. 4B)35. In the tooth refractory to root canal treatment, biofilm was demonstrated by Gram staining of the outer surface of the root and lacunae in the cementum and periapical tissue when treated with antibacterial nanopolymers only (Fig. 4Cb–d). When treated with antibacterial nanopolymers and nanobubbles, little biofilm was demonstrated in dentin, cementum, and periapical tissue (Fig. 4Cf–h). Cone-beam computed tomographic (Veraview; Morita, Osaka, Japan) examination demonstrated that the periapical lesion size was significantly reduced by treatment with the antibacterial nanopolymers with nanobubbles within 2 months (Fig. 4Da and b). On the other hand, treatment with antibacterial nanopolymers alone resulted in little change (Fig. 4Db). The complete disinfection of teeth before cell transplantation could induce regeneration of the pulp and periapical tissue. Cone-beam computed tomographic examination demonstrated that the periapical lesion was further reduced after cell transplantation (Fig. 4Db). These results showed that antibacterial nanopolymers with nanobubbles have a potential application for complete disinfection.

**FIGURE 2** – Enhanced pulp regeneration by trypsin pretreatment in the root canal before transplantation of MDPSCs with G-CSF in atelocollagen in aged pulpectomized teeth in dogs on day 14. (A) Pretreatment with trypsin for 10 minutes. (B) Pretreatment with saline for 10 minutes as a control. Hematoxylin-eosin staining.

Trypsin treated teeth

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<th>Direct effect</th>
<th>Indirect effect</th>
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<td>PDLCs in the surrounding tissues</td>
<td>Microenvironment: Cementum &amp; Dentin</td>
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<tr>
<td>Senescence ↓</td>
<td>Angiogenesis ↑</td>
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<td>Cell survival ↑</td>
<td>Neurite extension ↑</td>
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<td>Anti-inflammation ↑</td>
<td>Odontoblast differentiation ↑</td>
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**FIGURE 3** – A schematic diagram demonstrating direct and indirect effects of trypsin pretreatment before cell transplantation on enhanced pulp regeneration in aged pulpectomized teeth.
FIGURE 4 – Complete disinfection by nanobubbles with antibacterial cyanoacrylate nanopolymers in an intractable periapical disease model in dogs. (A) Removal of the smear layer of the dentinal wall of porcine root canal in vitro by scanning electron microscopic analysis. (Aa) Treatment with nanobubbles for 5 minutes and (Ab) with distilled water for 5 minutes. Note the significant exposure of dentinal tubules in nanobubble treatment compared with distilled water treatment. (B–D) Root canal treatment in an intractable periapical disease model by nanopolymers with nanobubbles compared with nanopolymers only. (B) Statistic analysis of the number of remaining bacteria at the first to the third application. The solid line indicates treatment with nanopolymers with nanobubbles, and the dashed line indicates treatment with nanopolymers only. Data are expressed as the mean ± standard deviation (n = 5) (*P < .05). (C) The removal of biofilm (Ca–Cd) by treatment of nanopolymers only and (e–h) by treatment of nanopolymers with nanobubbles at 4 months after root canal treatment. (Cb and Ch) Dentin, (Cc and Cg) lacunae in the cementum, and (Cd and Ch) periapical tissue. (Ca and Ce) Hematoxylin-eosin staining. (Cb–Cd and Ch–Ch) Gram staining. Note that positively stained bacteria remained in the tooth treated by nanopolymers only (arrows), and no bacteria remained in the tooth treated by nanopolymers with nanobubbles. (D) Cone-beam computed tomographic examination at the time of pretreatment, transplantation, and extraction. (Da) Analysis using the OsiriX program (Pixmeo, Geneva, Switzerland). (Db) The ratio of the volume of periapical lesions at transplantation and extraction compared with pretreatment. The solid line indicates treatment with nanopolymers with nanobubbles, and the dashed line indicates treatment with nanopolymers only. Data are expressed as the mean ± standard deviation (n = 6) (**P < .01). (Reprinted with permission from Ishara K, Nakashima Y. Enhanced delivery of antibacterial nanopolymers with nanobubbles for the complete disinfection of the root canal system in a canine model of intractable periapical disease. Jpn J Conserv Dent 2020;63:73-82.)
of teeth with periapical disease, therefore leading to pulp and periapical tissue regeneration.

**FUTURE PERSPECTIVES**

An aging environment can decline the function of resident stem cells triggered by hormonal disturbances, immunologic disorders, and metabolic alteration. Modulating microenvironments by signaling modulators, anti-inflammatory factors, antioxidants, and metabolic regulators might be significant for the development of strategies other than trypsin pretreatment of the root canal to improve or rejuvenate the endodontic microenvironment in aged teeth. We showed that neutralization antibodies against the C-C motif chemokine ligand 11 (Eotaxin-1) and C-C motif chemokine receptor 3 antagonists as anti-inflammatory factors have the potential to enhance pulp regeneration in an ectopic tooth root model of aged mice and in a pulpectomized tooth model of aged dogs, respectively.

There was little difference in the stem cell properties, including the pulp regenerative potential of MDPSCs between aged teeth and young teeth. However, the autologous MDPSCs derived from aged patients have certain limitations to overcome, such as the limited availability of discarded teeth, the prolonged time to expand the cells in culture because of atrophy or mineralization of aged pulp tissue, and altered intrinsic stem cell properties from patients with certain systemic diseases. We examined the safety and efficacy of allogeneic MDPSCs with mismatched dog leukocyte antigen (DLA) types. There was no evidence of toxicity or adverse events in the allogeneic transplantation of the MDPSCs with mismatched DLA types. Additionally, no adverse events were observed in the dual transplantation of the MDPSCs with mismatched DLA types. Well-vascularized and innervated regenerated pulp tissues were quantitatively and qualitatively similar in the mismatched DLA transplants and the matched DLA transplants, even with dual transplantation. Thus, the allogeneic transplantation of mismatched HLA MDPSCs with G-CSF might be safe and effective for total pulp regeneration.

Another critical challenge is enhanced dentin formation in the crown to fully cover the regenerated pulp tissue. It takes 24–48 weeks or more before full coverage of the regenerated pulp tissue with dentin is achieved. The delay of full coverage might cause microleakage and fracture. We are making 3-dimensional biomimetic scaffolds with dentinal tubule-like pores as a dentin induction device. The pulp cells can differentiate into odontoblast-like cells, extending their process into the dentinal tubule-like pores.

In vivo, a large amount of tubular dentin is possibly regenerated on the regenerated pulp tissue. The tubular structure of dentin is critical for fully covering the regenerated pulp. We are now preparing for clinical-grade biomimetic scaffolds.

In conclusion, it might be possible to regenerate the dentin-pulp complex by autologous or allogeneic transplantation of MDPSCs after complete disinfection and trypsin pretreatment of the root canal, even in aged teeth. It might be useful for patients in a superaged society to maintain and prolong the function and endurance of teeth, which results in improvement in the quality of life.

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The authors deny any conflicts of interests related to this study.

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