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Novel antioxidative nanotherapeutics in a rat periodontitis model: Reactive oxygen species scavenging by redox injectable gel suppresses alveolar bone resorption

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ABSTRACT

The excessive production of reactive oxygen species (ROS) has been implicated in a variety of disorders, but to date, ROS scavengers have not been widely used for local treatment of inflammation, because they are rapidly eliminated from the inflamed site. We have designed a novel redox injectable gel (RIG) that is formed at 37 °C after disintegration of nano-assembled flower micelles allowing nitroxide radicals to act locally as specific ROS scavengers for the treatment of periodontitis. In the present study, we have confirmed retention of the RIG in the periodontal region, along with its antioxidant-related anti-inflammatory effects, and we have subsequently evaluated the inhibitory effect of the RIG against *Porphyromonas gingivalis* (*P. gingivalis*)-induced alveolar bone loss attributed to ROS. Alveolar bone loss was estimated by morphometry, gingival blood flow was measured using laser Doppler flowmetry, and osteoclast differentiation was evaluated by tartrate-resistant acid phosphatase staining. The results show that the RIG can inhibit *P. gingivalis*-induced bone loss by antioxidant-related anti-inflammatory actions, and this suggests that the RIG is a promising novel therapeutic agent for the treatment of *P. gingivalis*-induced periodontitis.

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1. Introduction

Periodontitis is a chronic inflammatory disease characterized by gingival bleeding, formation of periodontal pockets, destruction of connective tissue, and alveolar bone resorption leading to early tooth loss [1,2]. The primary etiological agent in periodontitis has been identified as oral bacteria in dental plaque [3], and *Porphyromonas gingivalis* (*P. gingivalis*) lipopolysaccharide is considered to be a major influence [4]. An imbalance between the amount of

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bacterial pathogen and the host immune response to infection has been shown to contribute to the initiation and progression of periodontitis [5,6]. The treatments currently available for periodontitis include mechanical debridement, antibiotics and anti-inflammatory medications, and regenerative surgery for severe cases. However, because of the potential for adverse effects associated with the use of anti-inflammatory drugs, safer anti-inflammatory agents and osteoclastic inhibitors are desired.

The production of reactive oxygen species (ROS) is an essential part of normal cellular metabolism as a host defense mechanism against bacterial pathogens [7,8]. Oxidative stress occurs when the production of ROS exceeds the cell's capacity for detoxification of these potentially injurious species by endogenous antioxidant defense mechanisms. Increased oxidative stress generated by polymorphonuclear leukocytes because of interactions with chemoattractants, endotoxins, cytokines, and adhesion molecules has been shown to cause cell injury in periodontal tissue [9,10]. Although recent studies have shown an association between oxidative stress and periodontal inflammation leading to alveolar bone loss [1,11–15], ROS scavengers have not yet been widely used in the treatment of local inflammatory reactions such as periodontitis because they are rapidly eliminated from the inflamed site, which results in a low therapeutic effect. Therefore, to enhance the local retention time of ROS scavengers, we have developed a redox injectable gel (RIG) system for treatment of periodontitis. We have already demonstrated that redox nanoparticles can effectively eliminate excess ROS at local inflammatory reaction sites in a colitis mouse model [16]. Additionally, it has been demonstrated that redox nanoparticles tend to scavenge ROS outside of cells and do not interfere with normal redox reactions inside of the normal cells, which should avoid possible adverse effects relating to interference with normal intracellular redox reactions [17,18]. Here, we have produced a novel RIG that is formed after partial disintegration of nano-assembled flower micelles at 37 °C and crosslinked between them, allowing nitroxide radicals to act locally as specific ROS scavengers in order to serve as a redox therapy for periodontitis. This RIG was developed by using poly[4-(2,2,6,6-tetramethylpieridine-*N*-oxyl)aminomethylsyrene]-*b*-poly(-ethylene glycol)-*b*-poly(4-(2,2,6,6-tetramethylpiperidine-*N*-oxyl) aminomethylstyrene) (PMNT-PEG-PMNT) triblock copolymer, which possesses ROS scavenging nitroxide radical as side chains at the PMNT segment. It has been shown that the cationic PMNT segment in PMNT-PEG-PMNT forms polyion complexes with anionic poly(acrylic acid) (PAAc) to produce a flower-like micelle (ca. 79 nm) that exhibits in situ thermo-irreversible gelation under physiological conditions [19,20]. In the present study, the prolonged retention of the RIG at periodontitis sites was confirmed, and the ROS-scavenging antioxidant-related anti-inflammatory and anti-*P. gingivalis*-induced bone loss effects of the RIG were subsequently evaluated in a rat periodontitis model. The *in vitro* inhibition of osteoclast differentiation by the RIG was also investigated.

2. Materials and methods

2.1. Materials

Commercial PEG possessing sulfanyl groups at both ends (SH-PEG-SH) (Mn = 10000) (NOF CORPORATION Co., Ltd., Tokyo, Japan), 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (4-amino-TEMPO) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), poly(acrylic acid) (PAAc) (Mn = 5000) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and sulfo-Cy7-NHS (Lumiprobe, Florida, USA) were used without further purification. 2,2′-Azobisisobutyronitrile (AIBN; Kanto Chemical Co., Inc., Tokyo, Japan) was purified by recrystallization from methanol. Chloromethylstyrene (CMS) was kindly provided by Seimi Chemical Co., Ltd. (Kanagawa, Japan) and purified on a silica gel column to remove nitrophenol and the other inhibitors, followed by vacuum distillation under a nitrogen atmosphere.

2.2. Preparation of the redox injectable gel

PMNT-PEG-PMNT triblock copolymer was synthesized by the same method as our previous study [19,20]. PMNT-PEG-PMNT (5.0 mg/mL) and poly (acrylic acid) (PAAc) (5.0 mg/mL) solution were dissolved separately in 100 mM phosphate buffer (PB, pH 6.2). A 10.0 mL PMNT-PEG-PMNT solution was dropped slowly into 1.5 mL PAAc solution while stirring to make nitroxide radicals-containing polyion complex (PIC) flower micelles. The mixing ratio was adjusted to an NH_2^+ (molar unit of cationic secondary amine groups of PMNT-PEG-PMNT): COO^- (molar unit of anionic carboxyl groups) ratio of 1:1. The PIC flower micelles solution was concentrated to prepare an RIG precursor solution (30 mg/mL) to be used *in vivo* experiments. A non-nitroxide radical-containing injectable hydrogel (nRIG) was prepared as the control agent (30 mg/mL). The polycation-b-PEG-b-polycation with similar molecular weights and compositions, similar number of amino groups in polycation segments, and the similar hydrophobicities were prepared without nitroxide radicals as a side chain. Using nRIG we have confirmed the same retention tendency as that of RIG in abdominal cavity in mice [21]. Thus it is expected to show almost the same retention tendency of nRIG as that of RIG even in rat periodontitis model.

2.3. Bacterial strain and growth condition

American Type Culture Collection (ATCC) *P. gingivalis* strain 33277 was used in this study. The *P. gingivalis* was grown in brain heart infusion broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with 5 mg/mL yeast extract, 5 μg/mL hemin, and 0.2 μg/mL vitamin K_1. Bacterial cells were grown at 37 °C for 24 h in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 [22,23].

2.4. Experimental periodontitis

Fifty-six male four-week-old Sprague–Dawley rats (Nihon SLC, Shizuoka, Japan) were obtained and housed in cages throughout the experimental period to facilitate successful isolation. They were given sulfamethoxazole (1 mg/mL) and trimethoprim (200 μg/mL) in their drinking water for 4 days to reduce original oral microorganisms, followed by a 4-day antibiotic-free period before the oral challenge with bacterial infection by *P. gingivalis*. The rats were divided into four groups. The control group (sham group) received only 5% carboxymethylcellulose (CMC). The Pg group was infected orally with *P. gingivalis*. The nRIG@Pg group was infected orally with *P. gingivalis* and given 0.5 mL of nRIG, and the RIG@Pg group was infected orally with *P. gingivalis* and given 0.5 mL of RIG. The *P. gingivalis* (3.0×10^{10} cells/mL, 0.5 mL) was suspended in 5% CMC and administered by oral gavage on days 8, 10, 12, 14, and 16 (Fig. 1). At 1 day and 21 days after final infection, malondialdehyde (MDA) levels were measured as a marker of proinflammatory oxidative stress [24,25]. Rats were euthanized by inhalation anesthesia with diethyl ether (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 21 days after the final infection and alveolar bone loss was measured by morphometry [22,23,26]. The procedures used in this study were in accordance with the guidelines of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and our protocols were approved by the Animal Care Committees of Kanagawa Dental University (Approved #163).

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