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Bioactivity of Photoactivated Functionalized Nanoparticles Assessed in Lipopolysaccharide-contaminated Root Canals *In Vivo*

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Abstract

Introduction: The persistence of dentin-bound lipopolysaccharides (LPS) in disinfected root canals impedes treatment outcomes of endodontic procedures. This study assessed the effects of photoactivated rose bengal–functionalized chitosan nanoparticles (CSRBnps) on LPS-contaminated root dentin *in vivo* using an intraosseous implantation model and neotissue formation as a marker. **Methods:** Fifty human, 3-mm-long root segments with a 1.2-mm canal lumen were divided into 5 groups ($n = 10$): group 1, canals not contaminated; group 2, canals contaminated with *Pseudomonas aeruginosa* LPS; group 3, canals contaminated and disinfected with sodium hypochlorite (NaOCl); group 4, canals contaminated and disinfected with NaOCl and calcium hydroxide; and group 5, canals contaminated and disinfected with NaOCl and CSRBnps (300 μg/mL) with photoactivation ($\lambda = 540$ nm, 40 J/cm^2). Specimens were implanted into mandibles of guinea pigs, block dissected after 4 weeks, and the canal content evaluated histologically and immunohistochemically. The ingrown neotissue interface (50 μm) with dentin was characterized for fibroblasts, osteoclasts, inflammatory markers, dentin resorption, mineralization, and angiogenesis and dichotomized as type 1 (no inflammation and resorption, indicative of LPS inactivation) or type 2 (inflammation and resorption). The frequency of the observed parameters was analyzed using the Fisher exact test. **Results:** The outcome was categorized as type 1 in groups 1 and 5, type 2 in group 2, and mixed type 1 and 2 in groups 3 and 4. The outcomes in groups 1 and 5 ($P > .05$) differed significantly ($P < .05$) from those in groups 2, 3, and 4. **Conclusions:** Disinfection of LPS-contaminated root canals with photoactivated CSRBnps *in vivo* supported ingrowth of neotissue without signs of inflammation or resorption, suggestive of effective inactivation of dentin-bound LPS. *(J Endod 2017;■:1–7)*

Key Words

Chitosan, functionalized, guinea pig, intraosseous, lipopolysaccharide, nanoparticles

Significance

Persistence of bacterial endotoxins could impede the treatment outcomes of conventional and regenerative endodontic procedures. Root canal dentin treated with photoactivated functionalized nanoparticles supported healthy neotissue formation *in vivo*, suggestive of effective inactivation of dentin-bound LPS.

Disinfection of an infected root canal is directed at eliminating/inactivating bacteria and their by-products to provide a favorable environment for healing (1). Although conventional root canal disinfection protocols significantly lower bacterial loads (2), they are less effective in inactivating bacterial modulins such as lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria (3). The lipid moiety in LPS renders a strong negative charge that can be neutralized with positively charged molecules (4). The positively charged calcium ions released by calcium hydroxide ($Ca[OH]_2$) can inactivate LPS *in vitro* (5); however, an *in vivo* study (6) reported residual LPS in root dentin 1 week after medication with $Ca(OH)_2$.

A positive correlation has been reported among LPS concentration, clinical signs/symptoms of root canal infection (3, 7), and persistence of inflammation after root canal treatment (6). Also, in regenerative endodontic procedures, residual bacteria and LPS may impair healthy neotissue formation in the root canal (8–10). When stem cells are exposed to LPS *in vitro*, there is a marked expression of inflammatory mediators, and cell apoptosis occurs proportional to LPS concentration/duration of exposure (11, 12). Topical antimicrobials commonly used in endodontics can cause cell injury, alter the physical properties of dentin, and compromise neotissue integration with dentin (13, 14). The adverse impacts of residual LPS in both conventional and regenerative endodontic procedures warrant the pursuit of therapeutic modalities that would effectively inactivate bacterial LPS in infected root canals.

Chitosan is a natural biopolymer that can be synthesized into nanoparticles (15). The free hydroxyl and amino groups within the chitosan structure can be functionalized with the carboxyl group of proteins and photosensitizers, such as rose bengal (15, 16). Previous *in vitro* studies (16–18) have shown that rose bengal–functionalized chitosan nanoparticles (CSRBnps) possess potent antibiofilm efficacy, enhance the mechanical properties and chemical stability of dentin collagen, and are nontoxic to eukaryotic cells. Importantly, CSRBnps also inactivate highly concentrated *Pseudomonas aeruginosa* LPS and significantly reduce the expression of inflammatory markers

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from macrophage cells (18). The cumulative effects shown *in vitro* strongly suggest that dentin conditioning with CSRBnps may be effective in the inactivation of LPS-contaminated dentin *in vivo*.

Different clinical and animal models have been used to assess the effects of LPS and its inactivation *in vivo* (6, 19). LPS activity can be indirectly assessed using the expression of inflammatory markers by immune cells (18–20). The expression of inflammatory markers in neotissue formed within LPS-contaminated and subsequently disinfected root canal lumens, as well as dentin resorption, could be measures of LPS activity in an *in vivo* implantation model. Implantation of cups into the jaws of guinea pigs was previously reported for testing the cytotoxicity of endodontic materials (21, 22).

The aim of this study was to examine the effects of photoactivated CSRBnps on LPS-contaminated root dentin *in vivo* using neotissue formation as a marker in an intraosseous implantation model. We hypothesized that root dentin conditioning with CSRBnps would support the formation of healthy neotissue, which would indicate effective inactivation of dentin-bound LPS.

Materials and Methods

The protocols used in this study were approved by the University of Toronto Research Ethics Board (#31330) and the University Animal Research Committee (#20011089).

Sample Size

Assuming a 20% difference in mean outcomes between groups, a power analysis suggested that a minimum sample of 8 specimens per group would support analysis with 90% power and 5% significance. Two specimens were added to each group to account for possible loss to processing.

Nanoparticles and Specimen Preparation

CSRBnps were synthesized as previously described (16). Nanoparticles were 60 ± 20 nm in size with a positive charge of 29.9 ± 0.2 mV. All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

Extracted single-rooted anterior teeth ($N = 50$) without patient identifiers were stored in 0.5% sodium azide solution at 4°C immediately after extraction and used within 1 month. With tooth crowns and apical thirds sectioned off, root cylinders were created using a water-cooled diamond bur at low speed to measure 3-mm long and 3 mm in diameter (13). Root canals were enlarged with ISO size 20 K-type files (Kerr Corporation, Orange, CA) followed by a #3 Peeso reamer (Edenta Dental, Southfield, MI) at low speed to a standardized lumen diameter of 1.2 mm. Canals were irrigated with 2.5 mL 2.5% sodium hypochlorite (NaOCl), 5 mL 17% EDTA, 2.5 mL 2.5% NaOCl, and 2 mL sterile saline as the final rinse. Cylinder specimens were autoclaved and stored at 4°C before LPS inoculation.

LPS Contamination

Canals of specimens were sealed at one end with sticky wax. Forty specimens allocated to inoculated groups (see later) had canals inoculated with 20 μL *P. aeruginosa* LPS at a concentration of 50 EU/mL (23). Care was taken to ensure that LPS did not contact the outer surface of any specimen. Inoculated specimens were incubated for 24 hours in 100% humidity at 37°C.

Groups

The 50 specimens were divided into 5 treatment groups ($n = 10$): group 1, no inoculation and no disinfection (negative control); group 2, inoculation and no disinfection (positive control); group 3, inoculation and disinfection with 10 mL each of 1.5% NaOCl, 17% EDTA, and 0.9% sodium chloride delivered over 1 minute; group 4, inoculation and disinfection as per group 3 followed by the placement of $Ca(OH)_2$ paste into the canal with a 29-G NaviTip needle (Ultradent, South Jordan, UT), 7-day incubation in 100% humidity at 37°C, and removal of $Ca(OH)_2$ using 10 mL 17% EDTA and 0.9% NaCl; and group 5, inoculation and disinfection as per group 3 followed by the placement of CSRBnps into the canal with a 10-μL micropipette tip and photoactivation with a noncoherent light source (Lumacare-LC-122M; LumaCare, Newport Beach, CA) with an interchangeable fiber bundle and 30-nm bandpass filters (540 ± 15 nm) for 1 minute for a total dose of 40 J/cm^2. All specimens had canals sealed at the coronal end with fast-setting mineral trioxide aggregate (MTA) (MTA Angelus, Londrina, PR, Brazil) before implantation.

Implantation of Root Specimens

Twenty-five Hartley guinea pigs (male, white acromelanic albino, 601–650 g) were anesthetized with 5% isoflurane and 2% isoflurane via a nose cone supplemented by a submandibular infiltration of bupivacaine. Two specimens were implanted into either side of the mandibular symphysis (21) (Fig. 1). Animals were placed on a warm mat (37°C) in the supine position during the surgical procedure. External surfaces were shaved and decontaminated with 10% iodine solution and 70% ethanol. A 1.5-cm-long incision was made through the skin in the midline along the lower border of the mandible and the symphysis. The superficial tissues were incised, the muscles dissected down to the bone, and the periosteum reflected. Under copious saline irrigation, bilateral bone cavities (3-mm diameter and 2-mm depth) were prepared with twist drills (Nobel Biocare, Richmond Hill, ON, Canada) at 2000 rpm. Blood was collected from the prepared bone cavity using a syringe fitted with a 27-G needle and injected into the specimen canal before implantation. The specimens were inserted into the bone cavities with the open canal end facing the marrow space, leaving the sealed end extending 1 mm above the cortical bone as a guide for subsequent histologic sectioning. Specimens from 2 different groups were implanted in each mandible. After placement, the muscle and skin were repositioned and sutured using absorbable chromic gut sutures (Ethicon, Somerville, NJ). Animals were given postoperative analgesics (0.05 mg/kg buprenorphine) for 2 days.

Histology and Immunohistochemistry

Four weeks after implantation, the guinea pigs were reanesthetized with isoflurane and euthanized by an intracardiac injection of T-61 (Merck Animal Health, QC, Canada). Mandibles were surgically removed, dissected free of soft tissue, implants and surrounding bone blocks dissected, prefixed in 10% buffered formalin, demineralized with 17% Tris EDTA (pH = 7) for 8 to 10 weeks, and embedded in paraffin. Serial thin sections (5 μm) were obtained and stained with hematoxylin-eosin, Masson trichrome (connective tissue/bone) (Masson Kit, Sigma-Aldrich), Giemsa stain (inflammatory cells), and standard tartrate-resistant acid phosphatase (TRAP) stain (osteoclasts/odontoclasts).

Immunohistochemical stains were used to assess the migrant cells present at the interface of dentin and neotissue as per manufacturer's instructions (Abcam, ON, Canada). Primary antibodies were rat monoclonal antibodies for CD68 cells and mouse monoclonal antibodies for CD14 cells. Goat polyclonal antibody to mouse immunoglobulin G H&L (Santa Cruz Biotech, Heidelberg, Germany) was used as the secondary antibody. In brief, sections with primary antibodies were incubated overnight in a humidity chamber at 37°C,

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