

Association between dental pulp stones and calcifying nanoparticles

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Abstract: The etiology of dental pulp stones, one type of extraskeletal calcification disease, remains elusive to date. Calcifying nanoparticles (CNPs), formerly referred to as nanobacteria, were reported to be one etiological factor in a number of extraskeletal calcification diseases. We hypothesized that CNPs are involved in the calcification of the dental pulp tissue, and therefore investigated the link between CNPs and dental pulp stones. Sixty-five freshly collected dental pulp stones, each from a different patient, were analyzed. Thirteen of the pulp stones were examined for the existence of CNPs in situ by immunohistochemical staining (IHS), indirect immunofluorescence staining (IIFS), and transmission electron microscope (TEM). The remaining 52 pulp stones were used for isolation and cultivation of CNPs; the cultured CNPs were identified and confirmed via their shape and growth characteristics. Among the dental pulp stones examined in situ, 84.6% of the tissue samples stained positive for CNPs antigen by IHS; the corresponding rate by IIFS was 92.3 %. In 88.2% of the cultured samples, CNPs were isolated and cultivated successfully. The CNPs were visible under TEM as 200-400 nm diameter spherical particles surrounded by a compact crust. CNPs could be detected and isolated from a high percentage of dental pulp stones, suggesting that CNPs might play an important role in the calcification of dental pulp.

Keywords: calcifying nanoparticles, nanobacteria, pulp stones

Introduction

Frequently found on bitewing and periapical radiographs, pulp stones are discrete calcified bodies that are either located in the pulp tissue or attached to or embedded into dentine. Pulp stones are an oral disease with high incidence. It was reported that in young Australian adults, the prevalence of pulp stones was 46.1% among dental patients who go to clinics and 10.1% of the teeth were affected. Occurrences were rare in premolars (0.4%), but significantly higher in molars (19.7%).¹ The first and second molars were the teeth most commonly affected.²

Pulp stones may occur in one or all of the teeth in one person, even in unerupted or impacted teeth. Located in the pulp cavity or root canal, these stones often narrow or even obstruct the access to the apical point root canal. The calcification of pulp tissue might lead to the failure of root canal therapy and loss of the teeth.

The etiology of pulp stones has been controversial. Etiological factors implicated include aging,³ biological factors,⁴ physical factors,^{5,6} and chemical factors.^{7,8}

The discovery of calcifying nanoparticles (CNPs), also referred to as nanobacteria, led to novel theories for pathological calcification. CNPs first appeared as self-propagating calcifying macromolecular complexes found in bovine and human

blood and blood products. These nanoparticles could produce biogenic carbonate apatite on their cell envelope at all growth phases, which resulted in white biofilm and mineral aggregates closely resembling those found in tissue calcification in the human body.⁹ CNPs are capable of producing nucleate hydroxyapatite; thus, they have been heralded as one potential etiological factor of pathological calcification, such as kidney stones or kidney cyst,¹⁰⁻¹⁸ gall stones,¹⁸⁻²⁰ atherosclerosis,²¹⁻²³ and dental calculus in periodontitis.²⁴ As the lacking of satisfactory 16S rRNA sequence, the controversy exists on the bacteria state on the so-called 'nanobacteria', so CNPs best describes the agent.²⁵

Pulp stones are the characteristic manifestation of physiological or pathological calcification of the human body reflected in the dental pulp tissue. It could be hypothesized that CNPs are involved in the calcification of the dental pulp tissue.

Materials and methods

Collection of pulp stones

Sixty-five randomly collected pulp stones, one from each patient, were scaled out by endodontists during root canal therapy (38 from maxillary molars and 27 from mandibular molars) in the Hospital of Stomatology, Sun Yat-sen University, Guangzhou, China. Ages of the patients ranged from 28 to 60 years. Sample information is listed in Supplementary Table 1. Written informed consent was obtained from each participant prior to enrollment, with approval of the ethical committee of the Hospital of Stomatology, Sun Yat-sen University. The pulp stones were collected over a period of 1 year. All of the dental pulp stones were kept in sterile normal saline before further treatments.

Identification of pulp stones in situ

Thirteen of the dental pulp stones were immersed in 4% paraformaldehyde and nitric acid for demineralization for 24-72 h and neutralized with 5% sodium sulfate. After washing in water for 12 h, each pulp stone, considered as one sample, was paraffin embedded and cut into 5- μ m sections. Three randomly selected tissue sections from each sample were prepared for immunohistochemical staining (IHS) and indirect immunofluorescence staining (IIFS). Each tissue section was deparaffinized, rehydrated, and demineralized and then processed as described earlier.⁹ IgG1 class anti-CNP mAbs, 8D10 (Nanobac Oy, Kuopio, Finland) was used as primary antibody in IHS and IIFS. The samples were examined under a Microphot-FXA microscope (Nikon Corporation, Tokyo, Japan) with fluorescence and

differential interference contrast optics. Negative control sections underwent the same staining process, except that the primary mAb step was replaced with phosphate-buffered saline (PBS) buffer. Those with positive results were further examined with a transmission electron microscope (TEM; Hitachi H-600; Hitachi, Tokyo, Japan) operated at 15 kV, and micrographs were recorded.

Culture of CNPs from pulp stones

The remaining 52 pulp stones were randomly classified into 17 samples, with 3-4 for each sample. Samples were immersed in 0.3 μ g/mL tetracycline and then demineralized in 1 N HCl for 30 min, then powdered and neutralized with 0.5 MTris (pH 10.5; Sigma, St Louis, MO). Suspensions were centrifuged at 14,000 g for 15 min in a Minispin® Centrifuge and sterile filtered through 0.22- μ m millipore filters. The filtrate was cultured in flask containing Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA) with 10% fetal bovine serum (FBS; Sigma) as described in previous studies.²⁶ All FBS was γ -irradiated at a minimum dose of 30 kGy at room temperature for 24 h. DMEM incubated with FBS and Tris but without stone filtrate was used as negative controls. Subcultures were derived with a rubber scraper in serum-contained DMEM after 4 weeks of initial inoculation. All of the cultures were harvested by centrifugation at 20,000 g for 45 min at 4°C, washed with PBS (pH 7.2), and used for identification of CNPs.

Culture examination

The cultures were incubated for 8 weeks and evaluated microscopically for the growth of CNPs during and at the end of the incubation period. After incubation for 2, 4, or 8 weeks, the cultures were washed with PBS and photographed under inverted phase contrast microscope (Axiovert 40; Zeiss, Oberkochen, Germany). The cultures were examined by TEM to verify the detail on the morphology. For negative staining, samples were isolated by centrifugation at 20,000 g for 1 h directly from FBS and diluted 1:5 in PBS. A carbon-coated 400-mesh copper grid was placed on a drop of CNP suspension in PBS for 1 min, washed with water, and stained on a drop of 1% phosphotungstic acid for 90 sec. Photographs were taken using TEM (JEM-100CXII; JEM, Tokyo, Japan) for morphological identification.

Identification of CNPs from cultures

All culture samples were harvested by 20,000 g centrifugation for 30 min at 4°C after 8 weeks of incubation. The pellets were analyzed for IHS and IIFS using CNP

surface-antigen-specific mAb 8D10 to verify the existence of CNPs. In both IHS and IIFS, culture samples were spread on coverslips, fixed with 4% paraformaldehyde for 1 h, incubated at 70°C for 10 min, and blocked in 3% H₂O₂ and 5% bovine serum albumin (BSA)-containing normal serum for 30 min, respectively. In IHS, the particles were incubated with mouse monoclonal antinobacteria antibodies 8D10 (NanoBac Oy) at 4°C overnight; subsequently, the particles were sequentially incubated with biotinylated goat antimouse IgG antibody and streptavidin-biotin-peroxidase complex at 37°C for 30 min and visualized in diaminobenzidine solution for 10 min. In IIFS, the cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antimouse IgG antibody at 37°C for 30 min; slides were then washed, dehydrated, and mounted for observation under a fluorescent microscope. In IHS and IIFS, the primary antibody was omitted in negative controls. Furthermore, von Kossa staining was applied to confirm the presence of mineralization. For von Kossa staining, cultures were harvested and reseeded into wells of a 24-well plate containing medium; after 24-h incubation, von Kossa staining was performed as described previously.²⁷ Energy-dispersive X-ray analysis (EDX) were performed to show the topographic features by a scanning electron microscope (Quanta400; Philip FEI, Amsterdam, The Netherlands) equipped with EDX (INCA, Oxford, UK) as previously described.⁹

Results

Identification of CNPs in pulp stones in situ

Eleven out of the 13 tissue samples (84.6%) stained positive for CNPs antigen for IHS, whereas by 12 positive samples were detected by IIFS (92.3%). Positive staining results for IHS, as a brown-colored precipitate at the antigen site, are shown in Figure 1 A. To negative result is shown in Figure 1a. The red-fluorescence-labeled signals of positive results for IIFS are shown in Figure 1B, and the corresponding negative result is shown in Figure 1b. TEM identified a large number of spherical coccoid particles, from 200 to 400 nm, as shown in Figure 1C, and the negative control is shown in Figure 1c.

Morphology of CNPs in culture

CNPs were successfully cultured from 15 of the 17 samples (see 'Materials and methods') after incubation for 2 weeks, demonstrating a positive rate of 88.2%. The CNPs were first seen as individually separated and shaped typically as coccoid, bacilliform, or dumbbell with a highly refractive crust after 2 weeks, culture (Figure 2A). Those particles were

very tiny with a diameter of 0.125 to 0.05 mm and barely detectable (Figure 2a). In 4 weeks, particles grew bigger and the average diameter was about 0.10 mm (Figure 2B, b). After 6-8 weeks, larger carbonate particles were aggregated in clumps and easily observed under the microscope. They were shown as coccoid particles with a diameter of 0.30-0.40 mm and aggregated in crowds (Figure 3A, B, and C). Some of them nearly fused together to form 'concentric circles' (Figure 3D, F) with a diameter of nearly 1.25 mm. Surrounding the 'concentric circles', smaller size particles could be also seen (Figure 3E) and aggregated around relatively larger particles like 'satellites'.

Culture examination

By IHS, the harvested cultured particles exhibited a clearly visible yellow or brown staining with the anti-CNPs antibody 8D10 (Figure 4A), and no visible positive signal was detected in the negative controls (Figure 4a). Similarly, strong red fluorescent signals were detected by IIFS (Figure 4B). The same view under the light microscope is shown in Figure 4b. In the merged image under fluorescence and light microscope (Figure 4B + b), the thin arrow represents positive signals, and those represented by the thick arrow show the negative control. After von Kossa staining, the particles were colored black or brown, and their surrounding calcifying crust could be distinguished (Figure 4C). TEM image of the CNPs at 20,000x magnification, showing as 200-400 nm spherical particles (Figure 4D), and the topographic features on chemical composition of CNPs, were identified with calcium and phosphate peaks by EDX (Figure 4E).

Discussion

CNPs have spurred a major controversy in modern microbiology. Ambiguity of bacteriologic evidence for CNPs has led to various hypotheses of the nature and origin of CNPs, such as those of primordial life forms²⁸⁻³⁰ and those of inert mineral fetuin complexes.^{16,31,32} However, evidence for a significant role of CNPs in human biology has been accumulating.

CNPs were first identified in human kidney stones.³³ Subsequently, a rat model of CNPs-induced kidney stones was successfully established.¹¹ Since then, the significance of CNPs had extended substantially beyond kidney stones. Now, they are believed to be implicated in a variety of pathological calcifications in the human body.

Ciftcioglu et al³⁴ suggested observation of mineralized fibers and numerous small lobular on the teeth with pulp stones, and if being cultured with nanobacterias (CNPs),

revealed voluminous mineral formation, resembling dental pulp stones. Based on Ciftcioglu's morphological observation, it could be hypothesized that CNPs might be implicated in the calcification of dental pulp stones. In the present study, in order to test this hypothesis, first, CNPs were identified in the dental pulp stones in situ. Furthermore, CNPs were isolated and characterized from the stone samples derived from cultures. Among the dental pulp stones examined in situ, the CNP-positive rates by IHS and IIFS analysis were 84.6% and 92.3%, respectively. In 88.2% of the cultured samples,

CNPs were isolated and cultivated successfully. Therefore, the results suggested a potential link between the CNPs and human dental pulp stones.

Efforts were made in the present study to rule out of possibility of false positives of CNPs cultured from pulp stones. First, CNPs were reportedly found in 80% of the commercial FBS products.⁹ In this study, high dose of γ -ray (30 kGy) radiation was applied for FBS to destroy the FBS-contained CNPs and thus to avoid false positive results.^{35,36} Second, FBS contained inhibitors on apatite

crystal formation, such as osteopontin, osteocalcin, and fetuin.^{35,37} These inhibitors might interfere with the mineralization and, therefore, the visualization of CNPs. In this study, heat-inactivated FBS was used to remove the inhibitors of apatite crystal formation and avoid false negative results. Third, it was of great importance to ensure the CNPs isolated in this study were from pulp stone samples, not from other contaminating sources. Three anticontamination measures were taken. 1) All dental pulp stones used for the isolation of the CNPs were initially immersed in tetracycline

for 30 min. 2) All reagents and instruments were sterilized, and rubber dam were utilized in clinical treatment. 3) The supernatants of CNPs cultures were filtered through a 0.22- μ m minipore filter, a generally accepted method to remove the common bacteria, fungi, and mycoplasma. Fourth, no other cellular entities were present as proved by microscopy and culture tests, and no cloudiness of color or pH changes were observed in medium. Therefore, it could be concluded that CNPs were, for the first time, isolated and identified from dental pulp stones.

Zhang et al²⁴ described the detection of CNPs in gingival crevicular fluid and dental calculus as refractive aggregates in clumps after 6-week culture and the formation of a white-colored layer visible to the naked eye after 2 months. Our study provided more detailed information on the culture of CNPs in different growing states. CNPs isolated in the present study were coccoid with a highly refractive shell, similar in size and morphology to the CNPs reported

in other pathological calcification tissues in the human body.^{22,38,39} The morphological changes could be observed during the culture period. From the barely detectable tiny coccoid or dumbbell particles with a diameter of 0.025-0.05 mm after 2-week culture, these particles grew gradually to an average diameter of about 0.10 mm after 1 month. Furthermore, clumps aggregated in crowds were generated with average diameter of 0.30-0.40 mm in 6-8 weeks, and particles of all sizes could be observed. These observations indicated that CNPs were in a growing

state and could bud off new particles unceasingly. In addition, two interesting phenomena in the cultures of CNPs from pulp stone were indicated.

First, 'concentric circles' formed after about 6-8 weeks of culture; this morphology has not been reported before for CNPs. It could be speculated that the individually separated CNPs not only aggregated into clusters, but also merged and exhibited this specific appearance. Coincidentally, one of the characteristic features of microcosmic structures observed in the dental pulp stone sections under scanning electron microscopy was also concentric circles.^{40,41} Thus, it is possible that the miniature form of concentric circles could be the very onset of dental pulp stone formation, and the gradual accumulation of calcium and phosphate around CNPs might lead to larger mineralization such as pulp stones.

Second, minute particles like 'satellites' aggregated around relatively larger particles, which could be observed in the subculture flasks. Interestingly, all these particles always appeared ~24 h later in subcultures than those in primary cultures. It could be hypothesized that the subculturing procedure of filtering through 0.22-µm minipore prevented the passage of larger particles or stripped them off the outer apatite crusts. As a result, CNPs in the subcultures were smaller and needed time to accumulate calcium and phosphate around their outer walls to become visible again.

In conclusion, this study has revealed the prevalence of CNPs in human dental pulp stones. The findings raised the possibility that CNPs might be implicated in the formation or development of dental pulp stones. Further studies are required to test whether CNPs are the causative agents of pulp stones or are simply a marker of the pathologic state. These investigations might lead to novel approaches for preventing or treating pulp stones and consequentially facilitating successful root canal therapy.

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Supplementary material

Table SI Sample information on the selected teeth for pulp stones collection

Sample ID	Age	Gender	Teeth	Teeth state
1	38	Female	16	Chronic pulpitis
2	44	Male	16	Chronic pulpitis
3	45	Male	36	Apical periodontitis
4	28	Male	17	Apical periodontitis
5	29	Female	26	Apical periodontitis
6	60	Female	26	Apical periodontitis
7	60	Female	28	Apical periodontitis
8	45	Female	47	Apical periodontitis
9	39	Male	46	Chronic pulpitis
10	44	Male	17	Necrotic pulp
1 1	44	Male	17	Apical periodontitis
12	42	Female	17	Necrotic pulp
13	37	Male	16	Chronic pulpitis
14	32	Female	47	Chronic pulpitis
15	45	Male	46	Chronic pulpitis
16	56	Female	36	Chronic pulpitis
17	43	Female	18	Chronic pulpitis
18	29	Male	36	Chronic pulpitis
19	28	Male	47	Chronic pulpitis
20	30	Female	47	Cracked tooth & Acute pulpitis
21	40	Female	16	Apical periodontitis
22	37	Female	16	Cracked tooth & Acute pulpitis
23	32	Male	17	Cracked tooth & Acute pulpitis
24	35	Male	17	Apical periodontitis
25	41	Male	27	Cracked tooth & Acute pulpitis
26	50	Male	27	Apical periodontitis
27	54	Female	27	Apical periodontitis
28	52	Female	36	Apical periodontitis
29	49	Male	37	Apical periodontitis
30	51	Female	28	Apical periodontitis
31	46	Male	27	Cracked tooth & Acute pulpitis
32	37	Male	27	Chronic pulpitis
33	39	Male	27	Chronic pulpitis
34	40	Female	38	Chronic pulpitis
35	39	Female	46	Chronic pulpitis
36	60	Female	46	Chronic pulpitis
37	56	Female	27	Necrotic pulp
38	50	Female	47	Apical periodontitis
39	49	Male	47	Apical periodontitis
40	47	Male	16	Apical periodontitis
41	38	Male	27	Chronic pulpitis
42	45	Male	37	Chronic pulpitis
43	37	Male	47	Apical periodontitis
44	38	Male	17	Chronic pulpitis
45	43	Female	27	Acute pulpitis
46	42	Male	47	Apical periodontitis
47	39	Female	47	Acute pulpitis
48	37	Female	46	Acute pulpitis
49	36	Female	16	Acute pulpitis
50	42	Female	17	Necrotic pulp
51	38	Female	26	Apical periodontitis
52	39	Female	26	Apical periodontitis
53	59	Male	27	Apical periodontitis

Table SI *(Continued)*

Sample ID	Age	Gender	Teeth	Teeth state
54	50	Male	37	Apical periodontitis
55	39	Male	36	Apical periodontitis
56	54	Male	36	Acute pulpitis
57	38	Male	27	Acute pulpitis
58	38	Female	37	Apical periodontitis
59	32	Male	28	Acute pulpitis
60	45	Female	16	Necrotic pulp
61	49	Male	37	Apical periodontitis
62	48	Male	36	Necrotic pulp
63	43	Female	18	Necrotic pulp
64	44	Male	17	Apical periodontitis
65	52	Male	17	Apical periodontitis