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ORIGINAL ARTICLE

Clinical and microbiological performance of resin-modified glass-ionomer liners after incomplete dentine caries removal

Cristiane Duque · Thais de Cássia Negrini · Nancy Tomoko Sacono · Denise Madalena Palomari Spolidorio · Carlos Alberto de Souza Costa · Josimeri Hebling

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Abstract The aims of this study were to evaluate clinically and microbiologically the effects of two resin-modified glass-ionomer cements (RMGICs) used as liners after incomplete dentine caries removal and to identify *Streptococcus mutans* and *Streptococcus sobrinus* strains isolated from dentine samples, before and after indirect pulp treatment. Twenty-seven primary molars with deep carious lesions, but without signs and symptoms of irreversible pulpitis, were submitted to indirect pulp treatment. Treatment consisted of incomplete excavation of the carious dentine, application of one of the RMGICs (Vitrebond or Fuji Lining LC) or calcium hydroxide cement (Dycal), and sealing for 3 months. Clinical evaluation (consistency, color, and wetness of dentine) and carious dentine collects were performed before temporary sealing and after the experimental period. Microbiological samples were cultivated in specific media for subsequent counting of mutans streptococci (MS) and lactobacilli (LB). MS colonies were selected for identification of *S. mutans* and *S. sobrinus* by polymerase chain reaction. After 3 months, the remaining dentine was hard and dry, and there was a significant decrease in the number of MS and LB, in all groups, although complete elimination was not achieved in 33% and 26% of the teeth for MS and LB, respectively. From 243 MS colonies selected, 216 (88.9%) were identified as *S. mutans* and only 2 (0.8%) as *S. sobrinus*. The use of resin-modified glass-ionomer liners after incomplete caries removal, as well as a calcium hydroxide cement, promoted significant reduction of the viable residual cariogenic bacteria in addition to favorable clinical changes in the remaining carious dentine.

Keywords Dental caries · Indirect pulp treatment · Glass-ionomer cements · Liners · *Streptococcus mutans* · Polymerase chain reaction

C. Duque · N. T. Sacono · J. Hebling
Department of Orthodontics and Pediatric Dentistry,
Araraquara School of Dentistry, São Paulo State University (UNESP),
Rua Humaitá, 1680,
Araraquara, São Paulo, Brazil

T. C. Negrini · D. M. P. Spolidorio · C. A. de Souza Costa
Department of Physiology and Pathology,
Araraquara School of Dentistry,
São Paulo State University (UNESP),
Rua Humaitá, 1680,
Araraquara, São Paulo, Brazil

J. Hebling (✉)
São Paulo State University/UNESP,
Rua Humaitá, 1680,
14.801-903 Araraquara, São Paulo, Brazil
e-mail: jhebling@foar.unesp.br

Introduction

In the contemporary dental practice, selective removal of dentine tissue has been recommended for teeth with deep carious lesions and absence of irreversible pulpal or periapical diseases, in order to preserve a maximum of dental structure and to reduce the possibility of pulpal exposure. Although encouraging clinical success rates have been reported in a number of studies [13, 14, 16, 24, 25, 27, 29, 31, 35], there is still some resistance whether intentionally leaving carious dentine behind is safe.

Clinical procedures involving incomplete caries removal such as indirect pulp treatment have been advocated based on the concept that deep carious dentine lesions are comprised of two distinct layers [17]. The outer layer, regarded to as infected dentine, is highly contaminated with

cariogenic bacteria, structurally disarranged, and presents poor mechanical properties [37]. Since these characteristics render this layer irreversibly damaged, it has been recommended its complete removal during dentine lesion excavation. The inner layer or affected dentine is only partly demineralized and prone to undergone remineralization [27] since apatite crystals are still bound to collagen fibers having crossbands similar to those in normal dentine [17]. This layer, therefore, should be maintained on the cavity floor to avoid the exposure of the pulpal tissue.

However, even after removal of the infected layer, viable bacteria have been consistently found in the remaining affected dentine [4, 12, 24, 34]. Because of that, it would be beneficial that liners provided some antibacterial activity in order to promote a more immediate inactivation of the remaining viable microorganisms. The in vitro inhibitory effects of glass-ionomer cements on the growth of cariogenic bacteria have been proven effective [10, 23]. Moreover, these materials have more suitable mechanical and physical properties [28] when compared to those of calcium hydroxide cements, associated with acceptable biocompatibility when applied on deep cavities [7, 11].

A number of studies have investigated the effects of conventional glass-ionomer cements on carious dentine after incomplete caries removal procedures [12, 27]. However, there is still a lack of information regarding the clinical and microbiological performance of resin-modified glass-ionomer cements (RMGICs) used in the same circumstances. Therefore, the aims of this randomized clinical trial were (1) to evaluate the effectiveness of two RMGICs used as liners in deep carious dentine after incomplete caries removal and (2) to identify *Streptococcus mutans* and *Streptococcus sobrinus* strains isolated from dentine samples, before and after indirect pulp treatment.

Materials and methods

After approval by the Ethics Committee of the Araraquara School of Dentistry, UNESP (protocol no. 05/04), 30 deciduous molars were selected from 20 children, both genders, aged 4 to 8 years. A signed informed consent was obtained from the legal guardians. The inclusion criteria for the selection were children presenting no systemic problems and that were not currently taking any medication, absence of spontaneous toothache (irreversible pulpits), swelling or fistula, and tooth mobility. Radiographic criteria were caries lesions at the internal half of the dentine thickness, absence of pulp contact with the lesion, absence of internal or external root resorption, and other alterations suggestive of degenerative pulp conditions such as radiolucencies at the furcation or periapical regions or enlargement of the periodontal spaces.

Clinical procedures and dentine sampling Indirect pulp treatment was performed in two sessions by the same investigator to standardize the clinical procedures and the dentine collects. In the first session, after taking a bitewing radiography using a standardized positioner, anesthesia was delivered and a rubber dam applied to isolate the tooth. Pumice-slurry dental prophylaxis and anti-sepsis of operative area using 0.2% chlorhexidine digluconate was performed. Access to infected dentine was gained using a high-speed sterile carbide bur (#245, KG Sorensen, Barueri, São Paulo, Brazil) to remove the undermined enamel when necessary. After removal of superficial necrotic dentine with a spoon excavator, a sterile round steel bur compatible to the cavity size, at low speed, was used to clean all carious tissue from enamel-dentine junction and laterals walls, leaving a layer of soft dentine on the cavity floor to avoid pulp exposure. After washing and air-drying the cavities to remove debris, an initial collect (baseline: collect 1, C1) of carious dentine was sampled from the mesial portion of the cavity floor and inserted in 5 ml of brain heart infusion medium (BHI; Difco Laboratories, MI, USA). In order to obtain similar amounts of carious tissue in different collects, a standardized cavity was prepared in the active extremity of an amalgam plugger using a spherical bur at high speed. This cavity was completely filled with the dentine samples (ca. 0.6 mg) removed from each tooth with a sterile spoon excavator. Subsequently, the pulpal wall was entirely covered with one of the tested liner materials, randomly selected, Vitrebond (VB; 3 M ESPE, St. Paul, MN, USA), Fuji Lining LC (FL; GC, Tokyo, Japan), or the hard-setting calcium hydroxide cement Dycal (DY, Dentsply, Milford, DE, USA) as a control group. The materials were handled according to the manufactures' instructions. The RMGICs were light-activated for 30 s using a HQT unit (Optilux 500, Demetron Research Co., Danbury, CT, USA; 450 mW/cm^2), and the cavities were temporarily restored with a zinc-oxide eugenol cement (IRM; Dentsply, Milford, DE, USA). After 3 months, the second session of the indirect pulp treatment was carried out after a new bitewing radiograph had been taken. Under the same initial conditions of anesthesia and rubber dam placement, the teeth were reopened, and the liner materials were carefully and completely removed. At this moment, a second collect (reentry: collect 2, C2) of carious dentine was sampled from the distal portion of the cavity floor, as previously described. Finally, when necessary, softened remaining carious dentine was removed, and the teeth were permanently restored with silver amalgam after a new placement of the initial liner material.

Clinical evaluation criteria Before all dentine collects, dental cavities were copiously washed and carefully air dried, and the color, consistency, and humidity of the

carious dentine were blindly evaluated by a second investigator based on the following criteria [4]: dentine consistency: 0=hard (similar to normal dentine), 1=leathery (dentine spoon removes carious tissue when forced), and 2=soft (tissue easily removed by dentine spoon); dentine color: 0=dark brown, 1=light brown, and 2=yellow; and dentine humidity: 0=dry and 1=humid.

Microbiological procedures Dentine samples immersed in 5 ml of BHI were homogenized in a tube agitator (Vortex, Phoenix AT 56, Munising, MI, USA) for 1 min and six decimal dilutions (10^{-1} to 10^{-6}) in 0.9% saline solution were carried out. Subsequently, aliquots of 25 μl obtained from the dilutions were cultivated in duplicate on the surface of two selective media: trypticase, yeast, cystine agar supplemented with 20% (*w*/*v*) sucrose and bacitracin (TYCSB; 0.1 U/ml) for isolation of mutans streptococci (MS) and Rogosa agar (Oxoid, Basingstoke, Hampshire, England) for lactobacilli (LB). All plates were incubated in microaerophilic environment at 37°C for 48 h. After incubation, the total number of colony-forming units per milliliter (CFU/ml) was counted from a representative area of each agar plate yielding 50–300 colonies using a stereoscopic microscope. Additionally, six to eight representative colonies of streptococci, recognized on the basis of the colonial morphology in TYCSB medium, were collected and inoculated individually in 5 ml of BHI medium for 24 h. The purity of the cultures was confirmed using Gram technique, and aliquots of the subculture were frozen at −20°C in 10% glycerol BHI for further molecular analysis of bacterial isolates.

Polymerase chain reaction (PCR) In order to prepare DNA samples, overnight cultures in BHI medium were centrifuged, followed by washing twice with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and boiled for 10 min. After centrifugation, 60 μl of supernatant were collected and used as templates for the PCR reactions. The DNA samples from MS isolates were identified by PCR using specific primers for portions of the glucosyltransferase (GTF) gene. In order to confirm *S. mutans* identity, the bases sequences of primer pair were (GTFB-F): 5′-ACT ACA CTT TCG GGT GGC TTG G-3′ and (GTFB-R): 5′-CAG TAT AAG CGC CAG TTT CAT C-3′, to amplify a 517-bp DNA fragment. For *S. sobrinus*, base sequences were (GTFI-F): 5′-GAT AAC TAC CTG ACA GCT GAC T-3′ and (GTFI-R): 5′-AAG CTG CCT TAA GGT AAT CAC T-3′, to amplify a 712-bp DNA fragment. Each PCR mixture contained 5 μl of the DNA template, 5 μl of X 10 PCR amplification buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 0.2 mM of dNTPs (DNA polymerization mix), 3.0 mM $MgCl_2$, 1 μM of each primer and 2.5 U de Taq DNA polymerase, and sterile distilled water to make a final volume of 25 μl. All PCR reagents were obtained from Invitrogen, Life Technologies, São Paulo, Brazil. Positive and negative controls of PCR reactions were purified genomic DNA of *S. mutans* (ATCC 25175) or *S. sobrinus* (ATCC 27607) and sterile water, respectively. The amplification of DNA was performed in a thermocycler (GeneAmp PCR System 2400, Perkin Elmer, Applied Biosystems, Foster City, CA, USA) with a initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min, besides the final extension at 72°C for 7 min. The PCR amplification products were separated by electrophoresis in 1% agarose gels in Tris-borato-EDTA running buffer (pH 8.0) at 75 V for 2 h. Gels were stained with 0.5 μg of ethidium bromide/milliliter and visualized under ultraviolet light illumination (UltraLum, Labtrade do Brazil). The photographed images (Camera Kodak Digital Science) were analyzed in specific software (Kodak Digital Science 1D). A 100-bp DNA ladder was included as a molecular-size marker in each gel.

Statistical analysis Wilcoxon's non-parametric test was used to compare the differences in consistency, color, and humidity of dentine, before and after indirect pulp treatment. The baseline (C1) and reentry (C2) counts of MS and LB were compared within each material group using the same test. Medians and ranges of bacterial counts were expressed as (log (CFU+1)). The constant 1 was added to CFU since many samples showed zero counts, after the experimental period. Complementary Mann–Whitney tests were applied to identify differences among the materials. All statistical tests were considered at 5% level of significance.

Results

Three teeth were lost due to patient failure to show up for the follow-up consultation, one tooth from the VB and two teeth from the DY groups. From the 27 teeth, 14 were primary first molars (three upper and 11 lower) and 13 primary second molars (four upper and nine lower). All teeth had deep dentine carious lesions involving the occlusal surface; however, in eight of them, one approximal surface was also involved. Nineteen teeth had caries-active lesions, and the other eight had chronic lesions, based on clinical characteristics. Nine teeth were capped with VB, ten with FL, and eight with DY.

Clinical observations

None of the teeth presented any clinical symptoms (sensitivity to cold, heat, or sweet or spontaneous pain)

Table 1 Frequency of scores and medians (P25-P75) determined in the clinical evaluation before (baseline) and 3 months after the indirect pulp treatment (reentry)

Criterion	Liners	Baseline (C1) scores			Median (range) baseline (C1)	Reentry (C2) scores			Median (range) reentry (C2)
		0	1	2		0	1	2	
Dentine consistency	VB	2	6	1	1 (1–1) b	8	1	0	0 (0–1) c
	FL	6	4	0	0 (0–1) b	6	4	0	0 (0–1) b
	DY	2	6	0	1 (0–1) b	6	2	0	0 (0–1) c
Dentine color	VB	3	5	1	1 (1–2) a	5	4	0	2 (1–2) a
	FL	6	4	0	2 (1–2) a	7	3	0	2 (1–2) a
	DY	1	6	1	1 (1–1) a	6	2	0	2 (1–2) b
Dentine humidity	VB	3	6	–	1 (0–1) a	9	0	–	0 (0–0) b
	FL	10	0	–	0 (0–0) b	10	0	–	0 (0–0) b
	DY	4	4	–	1 (0–1) a	7	1	–	0 (0–0) b

For the same criterion and liner material (rows), medians followed by the same letters are not statistically different (Wilcoxon test, $p>0.05$)

and radiographic signs of pulpal and periapical pathologies during the study period. The frequency and median/range of the scores for the clinical evaluation, recorded at the baseline and reentry, are shown in Table 1. Comparing the remaining dentine characteristics immediately before (collect 1, C1) the placement of the materials and at the reentry (collect 2, C2), statistical differences were observed in consistency and humidity for DY and VB groups (Table 1). Therefore, 90 days after the treatment, the dentine became harder and dry. No statistical significant difference was evidenced for any clinical criterion evaluated in the FL group, when collects 1 and 2 were compared (Table 1).

Microbiological observations

Medians and ranges for MS and LB counts are shown in Table 2. Statistically significant differences were observed in the CFU counts before and after the indirect pulp treatment for all material groups ($p<0.05$), with an expressive reduction in bacterial counts. After the experimental period, no growth (CFU=0) of MS was exhibited in 18 teeth (eight VB, three FL, and seven DY), while the same was observed for LB in 20 teeth (six VB, eight FL, and six DY). Considering both bacteria, no growth was verified in 14 teeth.

PCR identification

From the 243 MS colonies selected, 216 (88.9%) were identified as *S. mutans* and two (0.8%) as *S. sobrinus*. A total of 25 strains remained unclassified, probably representing other species of the MS group. Table 3 shows the number of MS isolates and *S. mutans* strains, according to the collect period and material group.

Discussion

This study demonstrated the clinical effectiveness of indirect pulp treatments performed in deciduous molars with

Table 2 Mutans streptococci counts (log (UFC+1)) according to the collect period and experimental materials

Microorganism	Material	Median (range)		Difference median (range)
		Collect A (baseline)	Collect B (reentry)	
Mutans streptococci	Vitrebond	7.87 (3.04–8.08) a	0 (0–0) b	6.53 (2.14–8.08) A
	Fuji Lining LC	7.41 (6.73–7.94) a	5.10 (0–6.82) b	1.74 (−0.03–4.07) A
	Dycal	7.29 (5.80–8.08) a	0 (0–0) b	7.29 (4.65–8.08) A
Lactobacilli	Vitrebond	6.08 (2.00–7.72) a	0 (0–0.56) b	2.00 (0–7.19) A
	Fuji Lining LC	6.34 (0–6.91) a	0 (0–1.15) b	1.00 (0–6.67) A
	Dycal	3.54 (0–8.08) a	0 (0–4.43) b	0.54 (0–5.85) A

Difference median (range): difference between collects A and B. For each microorganism, within the material, medians (P25-P75) followed by the same lower letters are not statistically different, according to Wilcoxon test ($p>0.05$). Medians (P25-P75) followed by the same upper case letter are not statistically different, according to Mann–Whitney test ($p>0.05$)

Table 3 Mutans streptococci colonies isolated from SB-20 medium and *Streptococcus mutans* strains identified by polymerase chain reaction

Liner material	MS colonies			*S. mutans* strains		
	Collect A (baseline)	Collect B (reentry)	Total	Collect A (baseline)	Collect B (reentry)	Total
Vitrebond	51	14	65	44 (86.3%)[a]	14 (100%)	58 (89.2%)
Fuji Lining LC	55	47	102	55 (100%)	37 (78.7%)	92 (90.2%)
Dycal	51	25	76	47 (92.2%)	19 (76.0%)	66 (86.8%)
Total	157	86	243	146 (93.0%)	70 (81.4%)	216 (88.9%)

[a] Absolute frequency and percentage of *Streptococcus mutans* strains identified from the total amount of MS colonies for the respective material and collect

deep dentine caries based on the absence of symptoms and radiographic signs of pulpal and periapical pathologies 3 months after incomplete caries removal and temporary cavity sealing. Considering the short period of evaluation, these results are consistent with those reported in other studies, irrespective of the material applied on the residual carious dentine [13, 14, 24, 25, 27, 35].

Clinical changes in color and consistency of the remaining dentine after months of tooth sealing have been associated with the arrest of caries lesion progression [24]. In the present study, dentine became harder and dry after 3 months in contact with VB and DY. For FL LC, no clinical changes in color, hardness, or humidity of dentine were detected which could be explained by the fact that the number of teeth with chronic-like lesions randomly included in this group was higher than in the other two groups. Consequently, dentine lesions were already hard, dark, and dry right after the termination of the initial excavation in contrast to the active-like lesions.

It has been suggested that calcium and fluoride ions could enhance the remineralization process, promoting the hardening of the remaining dentine after partial caries removal [27]. In vivo and in vitro studies have demonstrated an increase on the mineral content in dentine lesions adjacent and/or subjacent to glass-ionomer cement restorations [27, 33], suggesting a significant remineralization potential exerted by these materials. Besides, dental materials such as calcium hydroxide and especially glass-ionomer cements due to their initial low pH setting have superficial solubilizing effects on dentine immediately after their application on the cavity floor [7, 11, 18]. As a consequence, bioactive molecules such as transforming growth factors (TGF-βs) could be released from the dentine matrix and activated to induce odontoblast cells to produce intratubular and reactionary dentine in order to decrease dentine permeability [18] and to pose a barrier against bacterial invasion via dentinal tubules.

Although some clinical aspects of the dentine have differed among the groups, there was a striking reduction in bacterial counts for all the liner materials used. In fact, the relationship between dentine color or consistency and the number of microorganisms detected in caries lesions is still controversial. While some studies have shown a correlation between darkening and hardening of dentine and the reduction of bacterial counts [2, 3, 24], others have not evidenced this association [1, 4]. Therefore, changes in color, consistency, and humidity of dentine should not be considered ultimate parameters to determine the complete decontamination of the dentine tissue. Moreover, the clinical assessment of dentine by visual and tactile methods is subjective and depends on the experience of each examiner.

Expressive reduction in bacterial numbers isolated from the remaining carious dentine was observed for all material groups at the reentry (C2). Despite no detectable growth of both bacteria (CFU=0) had been verified for most of the treated teeth after 3 months (51.8%), positive culture for MS and LB was still observed in 33.3% and 25.9% of the teeth, respectively. Similar results were obtained by other investigators [2, 3, 21, 36]. Only in the study developed by Maltz et al. [24] no streptococci and LB growth was detected from dentine samples collected at the reopening of the dental cavities 6 to 7 months after incomplete caries removal.

Two important factors could have influenced the reduction of residual bacteria: (1) the adequate cavity sealing [13], which limited the amount and complexity of nutrients available [30], and (2) the antibacterial activity of the liner materials [10]. Several studies have demonstrated the clinical effectiveness of calcium hydroxide cements on residual dentine, after incomplete caries removal [13, 14, 16, 24, 31]. Calcium hydroxide cements have the property to increase medium alkalinity (ca. pH 9.5) at the first 3 h [15], which causes damage to organelles and consequent cellular lyses. However, an in vitro study demonstrated survival of *S. mutans* strains exposed to pH 8.5, suggesting resistance of this species in alkaline environments [5]. Some disadvantageous properties of these cements, such as high solubility, low compression resistance, and no adherence to dental substrates, allied to the recent advances in

adhesive dentistry, have led researchers to investigate the application of glass-ionomer cements on carious dentine, because of their favorable mechanical and biological properties [7, 11] including marked antibacterial activity [10, 23].

RMGICs exhibit effective antimicrobial activity against cariogenic bacteria [10, 20, 23] suggesting that their application on affected dentine could contribute to decrease or to eliminate residual microbiota. This inhibitory activity is attributed to the low initial pH and the release of chemical components such as fluoride and metallic ions [8, 23] VB and FL LC have initial pH about 4.0, which increases to approximately 5.5 in the first 24 h [8, 23]. The entrance of high levels of H^+ protons in the cell cytoplasm result in loss of activity of the relatively acid-sensitive glycolytic enzymes (which severely affects the ability to produce adenosine triphosphate) and structural damage to the cell membrane and macromolecules such as DNA and proteins culminating with cell death [6]. Also, fluoride ions released from the cements, besides playing an important role in the remineralization of the softened residual dentine, contribute to the antimicrobial effect of RMGICs on the remaining bacteria via three principal mechanisms: (1) binding of F^- or HF to specific sites of enzymes or other bacterial proteins, causing their inhibition, (2) formation of fluoride-metal complex with aluminum or beryllium that modulates enzyme activities of bacteria, and finally, (3) the action of HF as a transmembrane proton transporter, interfering on the protons movement out of the cytoplasm and decreasing acid tolerance response [26].

Despite the striking reduction in the number of cariogenic microorganisms achieved by all the liner materials investigated in the present in vivo study, their complete elimination was not warranted. After the experimental period of 90 days, viable bacteria were still found in 48% of the teeth. MS were detected in more teeth (36%) than LB (21%) which is in line with studies that showed the prevalence of MS over LB in deep dentine caries lesions [1, 34]. Among oral streptococci isolated from the lesions, PCR method revealed that *S. mutans* prevailed (88.9% of the analyzed colonies) over other strains such as *S. sobrinus* (0.2%).

The ability of bacteria to survive and persist in a specific environment depends, partially, on their genetic plasticity which determines their response to stress and environment fluctuations [9]. The exposure of oral streptococci and LB strains to pH values between 6.0 and 3.5 may induce an acid tolerance response that enhances the survival of these strains at or below pH 3.5 [32]. A rapid adaptive response is exhibited by *S. mutans* involving synthesis of specific proteins, after 30 min of acid shock [19], which could be induced by acids from chemical reactions of dental materials or from microbial metabolites. The environmental acidification stimulates a variety of self-protectors measures produced by bacteria, including systems which modify cell membrane composition, protons extrusion, and metabolic pathways [6]. The survival of acid-resistant *S. mutans* under restorations could be associated with their ability to liberate carbohydrates from glycoprotein of dentinal fluid, such as sialic acid, galactose, and *N*-acetylglucosamine [22, 30].

In conclusion, considering the short period of evaluation, indirect pulp treatments with RMGICs, as well as with calcium hydroxide cement, were clinically successful and promoted expressive reduction in the counts of the tested cariogenic bacteria; however, their complete elimination was not achieved in 33% and 26% of the teeth for MS and LB, respectively. It is relevant to consider that environmental changes could induce the selection of resistant strains. More studies are necessary to genetically evaluate these resistant isolates and to verify the possibility of generating more virulent strains, after incomplete caries removal procedures.

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Conflicts of interest The authors declare that they have no conflict of interest.

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