Original Article

Comparative evaluation of remineralization potential of novel bioactive agents on eroded enamel lesions: A single-blinded *in vitro* study

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Abstract

Objective: The escalating prevalence of noncarious tooth wear stands as a critical concern in the backdrop of evolving lifestyles and dietary patterns. Dental erosion, a progressive condition induced by both endogenous and exogenous acidic influences, directly impacts enamel integrity, resulting in surface loss. The contemporary surge in carbonated beverage consumption further exacerbates this erosive milieu, underscoring the urgency for dental practitioners to adopt meticulous treatment strategies. Existing literature underscores a noteworthy 94% reduction in tooth erosion risk for individuals abstaining from sweetened soft beverages, emphasizing the imperative for a well-devised remineralization protocol to counter demineralized surfaces.

Methodology: Seventy-three enamel specimens were taken. Forty samples were subjected to pre-operative hardness testing, and five samples were subjected to baseline EDX evaluation followed by grouping of samples (Group 1 = control Group; Group 2 = casein phosphopeptide–amorphous calcium phosphate fluoride [CPP-ACPF] Group; Group 3 = Biomin F Group; and Group 4 = self-assembling peptide [SAP] P-114 Group). A demineralization–remineralization cycle was carried out for 5 days followed by testing through Vickers Microhardness Tester, EDX Evaluation, and Scanning Electron Microscopy (SEM) Imaging. Statistical analysis was performed using one-way analysis of variance followed by intergroup analysis using Tukey's *post hoc* test with SPSS software 25.0 version.

Results: The mean percentage change in microhardness values was 30.05% in Group 1, 24.21% in Group 2, 18.85% in Group 3, and 12.08% in Group 4. The mean Ca/P ratio of samples tested through EDAX was 2.20 at baseline, 1.40 in Group 1 (Control Group), 1.62 in Group 2 (CPP-ACPF), 1.82 in Group 3 (Biomin F), and 2.01 in Group 4 (SAP-P114). Postintervention values were statistically significant from baseline values in both parameters.

Conclusion: Curodont Protect exhibits superior efficacy, offering valuable insights for future *in vivo* studies and clinical applications. The multifaceted evaluation, encompassing microhardness testing, SEM analysis, and EDXS assessment, contributes to a nuanced interpretation of the agents' impact, paving the way for informed decisions in clinical practice and future research endeavors.

Keywords: Biomin F; casein phosphopeptide–amorphous calcium phosphate fluoride; dental erosion; remineralization; self-assembling peptides

INTRODUCTION

Noncarious tooth wear has increasingly become a major concern due to changing lifestyle and dietary patterns. Dental erosion is a progressive condition, in which acids present inside the body and the one's introduced from outside through the consumption of food and beverages

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directly affect enamel, causing tooth surface loss.[1] The Center for Science in the Public Interest has reported that the total consumption of carbonated beverages in 2004 for every human was approximately 68 gallons per annum.[2] This erosive oral milieu should be of concern to the dental practitioner. Literature reports a 94% lower chance of tooth erosion for individuals who had never used sweetened soft beverages compared to daily consumers.[3] This calls for a meticulously planned treatment protocol for remineralization of demineralized surfaces.

In accordance with the principles of modern dentistry, optimal conservative strategies must be favored for the treatment of early erosive lesions such as the use of remineralizaing agents namely fluroride-containing toothpastes (NaF), nanohydroxyapatite dentifrice and saliva.[4-6] Hence, treatment entails a detailed study of remineralizing agents.

Casein phosphopeptide (CPP) is a milk-derived bioactive agent which stabilizes clusters of amorphous calcium fluoride phosphate on the tooth surface.[7,8] This protein has been evaluated in several *in vitro* and clinical trials for its excellent remineralizing properties and hence may be considered the gold standard for comparison with novel agents. Biomin F is another bioactive agent that has been earlier used to remineralize incipient caries where fluoride has been integrated into the originally used bioglass component rather than being used as an additional component.[9] Hence, it may prove to be beneficial in remineralization of eroded surfaces. In the pursuit of new treatment strategies, biomimetic self-assembling peptides (SAPs) stand out as a promising option due to their high affinity to calcium and diffusion capability into the subsurface micropores of enamel.[10] However, literature-based evidence that point toward its properties in the prevention of demineralization, in addition to repair of demineralized subsurface lesions is a lacunae yet to be filled.

The effect of the bioactive agents on the physical properties of demineralized tooth may be assessed using surface microhardness testing through Vickers hardness tester, surface ultrastructure analysis through scanning electron microscopy (SEM) and assessing change in calcium/phosphorous ratio through Energy Dispersive X-ray Analysis (EDXS).[11]

In line with the best of our knowledge, there are no previous studies that have compared these bioactive materials for the remineralisation of erosive lesions. Hence, the present study aimed to comparatively evaluate the remineralizing effects of certain novel agents on eroded enamel lesions.

Sample size calculation

The following formula was used to compute the sample size.[12]

$$n = \frac{2\sigma^2\left(Z_{1-\alpha/2\tau} + Z_{1-\beta}\right)^2}{\left(\mu A - \mu B\right)^2}$$

where

μA is the mean of group $A = 85.2$

μB is the mean of group $B = 106$

σ is pooled standard deviation $= 12.49$

τ is the number of pairwise comparisons to be made $= 10$

α is Type I error $= 0.05$

β is Type II error, meaning $1-\beta$ is power (99% power) $= 0.01$

Substituting these values from the previous study, the sample size determined was $n = 18$. Hence, there were $n/2 = 9$ samples in each group.

Ten samples were assigned for the hardness of every group. In addition, 5 samples were added for baseline EDX evaluation and 5 samples per group for postintervention EDX evaluation. Further, two samples per group were added for SEM evaluation.

Therefore, the total sample size was 73.

METHODOLOGY

After obtaining ethical approval from the institutional ethical clearance committee under protocol number IIEC/2020-23/CONS/04, 73 human mandibular premolars that were extracted for orthodontic or periodontal reasons were chosen for this study. Inclusion criteria of teeth samples included noncarious, intact crowns, and teeth free from restorations. After confirming the inclusion criteria by Radio Visio Graphy (Acteon, France), samples were inspected under an operating microscope (10×) to exclude the possibility of cracks, fractures, and anatomical developmental anomalies. Root canal-treated teeth were also excluded. Teeth were disinfected by autoclaving at 121°C at 15 lbs psi, thereby storing in freshly prepared artificial saliva (pH 6.8: 0.0625 g KCl, 0.059 g $MgCl_2.6H_20$, 0.166 g $CaCl_2.2H_2O$, 0.804 g K_2HPO_4, 0.326 g KH_2PO_4 in 2 g/l methyl-hydroxybenzoate, and 10 g/l of sodium carboxymethylcellulose) until further use.

Specimen preparation

The crowns were separated from the roots using a diamond disc in a slow speed handpiece under copious water irrigation, and each crown was sectioned in two halves in the mesiodistal direction to obtain lingual surfaces of premolars. Tooth samples were embedded in acrylic

resin with the lingual surfaces facing up, and enamel was abraded with silicon carbide abrasive papers of grit size 1000 followed by 600 and then 320 to obtain flat surfaces. Further, a 3 × 3 wax sheet was placed on the labial or lingual surfaces to prepare a window, and acid-resistant nail varnish was painted on the rest of the enamel. Samples were randomly divided into four groups of 15 samples each depending on the remineralizing agent used after every demineralizing cycle as follows:-

- Group 1 (n = 17): No remineralization agent was applied (control group)
- Group 2 (n = 17): Sample was smeared with CPP-amorphous calcium phosphate fluoride (ACPF) paste (GC Tooth Mousse Plus, Japan) for 3 min
- Group 3 (n = 17): Biomin F paste (BioMin Technologies, UK) was applied and left for 3 min
- Group 4 (n = 17): SAP P11-4 (Curodont Protect remineralizing tooth gel, Switzerland) was applied for 2 min, and excess material was removed without washing.

Ten samples from each group, single blinded by numbering were assigned for surface microhardness measurement, two from each group for SEM analysis and three samples from each group for EDXS analysis.

Evaluation of baseline surface microhardness and EDX values

Ten samples from each group assigned for enamel microhardness were measured with a Vickers hardness tester (Banbros, India) equipped with a diamond indenter. A standardized load of 200 g was applied to the surface for a dwell time of 15 s. In addition, five samples were subjected to EDX analysis to evaluate the Ca/P ratio of sound enamel and tabulated as baseline values.

Demineralization–remineralization cycle

This cycle comprised demineralization by immersion of each of the 68 samples in 30 mL of unstirred cola drink (pH 2.74), for 5 min followed by storage in artificial saliva (pH 7.0) for 6 h.

After 6 h of storage in artificial saliva, pea-sized remineralizing agents of Groups 2, 3, and 4 were applied on enamel samples as per the manufacturer's instructions, again followed by storage in artificial saliva for 6 h. This cycle was repeated twice a day. Hence, two demineralizations and two remineralizations were performed per day with storage in artificial saliva for the intermediate time.

For the control group, after each demineralization, samples were immersed in artificial saliva until the next demineralization.

This process was repeated for 5 days.

Post intervention evaluation

After 5 days, 40 samples (10 samples from each remineralizing group) that were previously evaluated for baseline surface microhardness were tested with a Vickers hardness tester (Banbros, India) equipped with a diamond indenter in a similar way, in which preintervention microhardness was tested. Changes in the hardness of enamel were recorded. Eight samples (two from each remineralizing group) were sonicated for 10 min, rinsed with deionized water, air dried, sputtered with gold, and evaluated under SEM to examine microstructures of the enamel sample surface. For the remaining twenty samples (five from each remineralizing group), changes in mineral content were evaluated by calculating the calcium and phosphorus weight percentage, and thus the Ca/P ratio with the help of energy energy-dispersive X-ray spectrometer (Bruker, UK). The evaluators were blinded to the grouping of the samples.

Statistical analysis

The data were analyzed using using IBM SPSS (Statistical Package for Social Sciences) Version 25.0 Version (IBM Corp 2013, New York, USA). Paired t-test was conducted for comparison of the baseline hardness with postintervention hardness. The intergroup comparison of percentage change of hardness was conducted using *post hoc* Tukey's evaluation (level of significance <0.05). For comparison of EDX values, one-way analysis of variance test was conducted for intragroup comparisons. Intergroup comparison of Ca/P ratio was done using post hoc Tukey's test (level of significance <0.005).

RESULTS

The mean change percentage in microhardness values was 30.05% in Group 1 (Control Group), 24.21% in Group 2 (CPP-ACPF), 18.85% in Group 3 (Biomin-F), and 12.08% in Group 4 (SAP-P114). Postintervention values were statistically significant from baseline values ($P < 0.05$) [Table 1 and Figure 1a and b].

The mean Ca/P ratio of samples tested through EDX was 2.20 at baseline, 1.40 in Group 1 (Control Group), 1.62 in Group 2 (CPP-ACPF), 1.82 in Group 3 (Biomin F), and 2.01 in Group 4 (SAP-P114) [Table 2 and Figure 1c and d]. Postintervention values were statistically significant from baseline values ($P < 0.05$) [Figure 2].

On examination of the surface morphology under SEM at a magnification of ×2000 [Figure 2], a honeycomb-like structure was seen in samples of Group 1 (control group) due to porosities created in the prismatic structure of enamel. In addition, the appearance of a keyhole type pattern in the interprismatic region suggested demineralization. Samples of Group 2 (Tooth Mousse Plus) represented a

Table 1: Comparison of percentage change in microhardness (using one-way ANOVA followed by *post hoc* Tukey's test)

	Group 1	Group 2	Group 3	Group 4	*P*					
					1 versus 2	1 versus 3	1 versus 4	2 versus 3	2 versus 4	3 versus 4
Baseline hardness	260.34±12.83	261.26±6.61	262.55±8.83	267.6±4.23	0.027*	<0.001*	<0.001*	0.048*	<0.001*	0.008*
Postintervention hardness	182.16±17.57	197.89±9.39	212.87±7.12	235.22±12.04						
Change	78.18	63.36	49.68	32.38						
Percentage change	30.06±5.46	24.21±3.99	18.85±3.41	12.09±4.57						
P	<0.001*	<0.001*	<0.001*	<0.001*						

*Indicates a significant difference at $P \leq 0.05$. Paired *t*-test, one-way ANOVA test, *post hoc* Tukey's test. Group 1: Control group, Group 2: CPP-ACPF, Group 3: Biomin-F, Group 4: SAP-P114, CPP: Casein phosphopeptide, ACPF: Amorphous calcium phosphate fluoride, SAP: Self-assembling peptide

Table 2: Comparison of energy dispersive X-ray analysis values (one-way ANOVA followed by *post hoc* Tukey's test)

	Group 1	Group 2	Group 3	Group 4	*P*					
					1 versus 2	1 versus 3	1 versus 4	2 versus 3	2 versus 4	3 versus 4
Mean Ca/P (sound enamel)	2.20±0.01	2.20±0.01	2.20±0.01	2.20±0.01	0.017*	<0.001*	<0.001*	0.034*	<0.001*	0.038*
Mean Ca/P postintervention	1.40±0.09	1.62±0.09	1.82±0.07	2.01±0.15						
Change	0.80	0.58	0.38	0.19						
P	<0.001*	<0.001*	<0.001*	0.044*						

*Indicates a significant difference at $P \leq 0.05$. Paired *t*-test, one-way ANOVA test; *post hoc* Tukey's test. Group 1: Control group, Group 2: CPP-ACPF, Group 3: Biomin-F, Group 4: SAP-P114, CPP: Casein phosphopeptide, ACPF: Amorphous calcium phosphate fluoride, SAP: Self-assembling peptide, Ca/P: Calcium/phosphorus

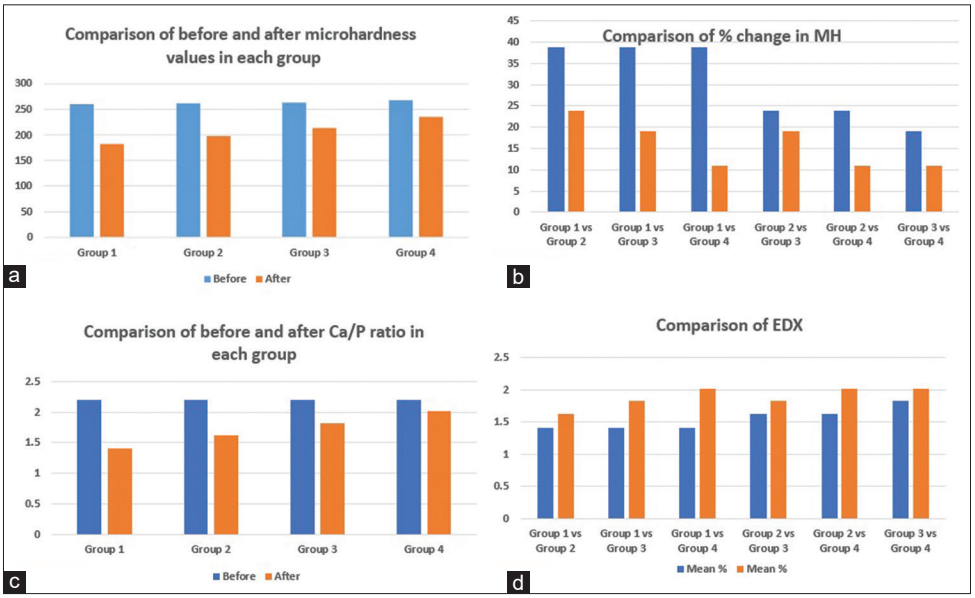

Figure 1: (a) Intragroup comparison of change in microhardness values before and after intervention; (b) Intergroup comparison of percentage change in microhardness (MH); (c) Intragroup comparison of CA/P ratio before and after intervention; (d) Intragroup comparison of CA/P ratio after intervention; Group 1 = Control Group; Group 2 = Casein phosphopeptide–amorphous calcium phosphate fluoride; Group 3 = Biomin-F; Group 4 = Self assembling peptide -P114

thick homogeneous and compact layer, suggestive of remineralization. Samples of Group 3 (Biomin F) revealed a globular structure with calcium particles scattered over the surface. Regarding Group 4 (Curodont Protect), the interprism cavities appeared largely filled. Lines of remineralization were visible along the prismatic borders.

DISCUSSION

A dynamic state of balance is created by a continuous and simultaneous process of demineralization and remineralization to maintain the mineral content of tooth enamel. Any alteration with regard to the duration or intensity of demineralization or remineralization causes a disbalance of the tooth surface's elemental distribution. A true remineralizing approach needs to aim at regenerating hydroxyapatite crystals within the subsurface lesion.[13] Thus, in the current scenario where dietary habits include the intake of carbonated drinks regularly that shifts the dynamics of the tooth more toward the demineralization process, concurrent reminerlization must counteract the acid-driven erosion.

The novelty of this work originated from the testing of novel remineralizing agents such as SAP-Curodont Protect and

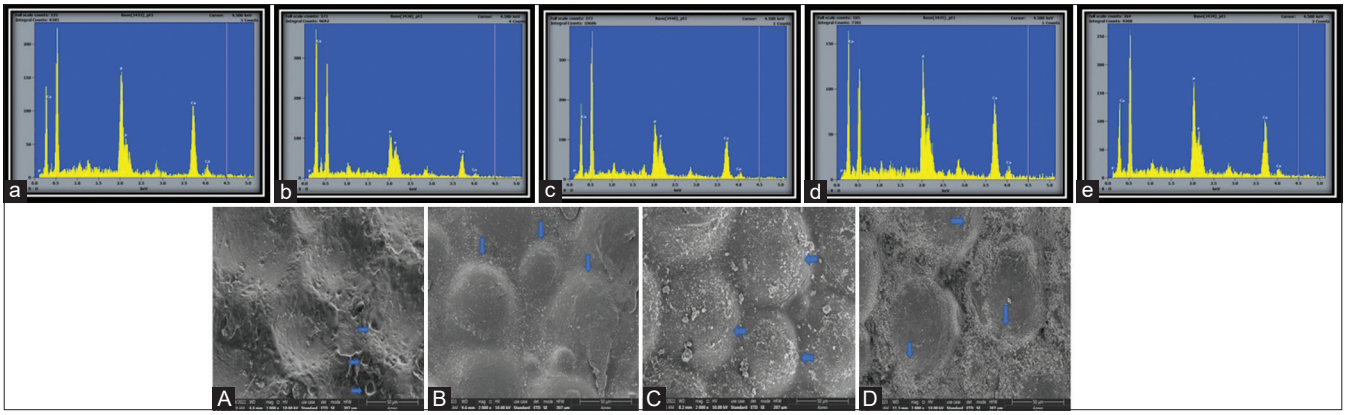

Figure 2: EDX of (a) Sound Enamel, (b) Group 1, (c) Group 2, (d) Group 3, (e) Group 4 scanning electron microscopy images at ×2000; (A) demineralization; (B) Group 2-arrows represent a thick, homogeneous and compact layer suggestive of remineralization; (C) Group 3 – arrows represent globular structure with scattered calcium particles suggestive of remineralization; (D) Group 4 – arrows represent lines of remineralization along prismatic borders

fluoride-incorporated bioglass-BiominF and its comparison with conventionally used CPP-ACPF in a regimen of intermittent erosive demineralization using Coke that simulate a youngsters pattern of beverage consumption. Therefore, the present study aimed to evaluate the remineralization potential of novel bioactive agents on eroded enamel lesions.

On exposure to cola-based beverage for 5 min, the results revealed that the mean microhardness of the enamel surface decreased by 30.05% in comparison to baseline values. This could be attributed to the acidic challenge that creates a surface of low mineral content, thus reducing the surface hardness values.[14] Similar results have been reported by Dionysopoulos *et al*. (2019)[1] and by Hao *et al.* (2018),[15] wherein the microhardness reduction calculated was 43.01% and 38.06%, respectively. In a study by Wongkhantee *et al*.,[16] a decrease of 63% was observed in hardness. The higher loss of hardness on exposure to acidic pH in his study could be attributed to the immersion time which was 8 min in contrast to 5 min in this study.

Application of remineralizing agents in addition to exposure to cola-based beverages resulted in a statistically significant decrease in loss of hardness in all the experimental groups. The highest recovery in microhardness was exhibited by Curodont Protect that might be attributed to its ability to mimic physiological function by the formation of a nucleation site for hydroxyapatite crystals. Small self-assembling molecules diffuse into the subsurface lesion and serve as the ideal building blocks for three-dimensional fibrillar scaffolds guiding the recovery of calcium and phosphate ions from saliva, thus increasing hardness and the Ca/P ratio.[11] Similar remineralizing effects have been witnessed by studies from Jablonski-Momeni[17] and Kind *et al*.[18] for remineralization of artificial caries lesions.

A statistically significant lower recovery percentage has been witnessed by Biomin F when compared to Curodont Protect. This inferior remineralization effect of Biomin F must be predisposed to the low level of soluble fluoride ion release from the fluoro calcium phosphosilicate in presence of artificial saliva as stated by Naumova *et al*. in 2019.[19] However, the remineralization effect of Biomin F was more than that seen from GC Tooth Mousse Plus that contains CPP-ACPF. This can be attributed to the compositional differences between the remineralizing agents. When bioglass is placed in the oral environment, an ionic exchange takes place, and the glass begins to dissolve, leading to the release of calcium ($Ca^{2}+$) and phosphate ($PO4^{3-}$) ions, further leading to the formation of fluorapatite, which is fluoride-rich, carbonate-poor, acid-resistant surface mineral layer. In contrast, fluoride from CPP-ACPF can be washed off quickly by the salivary flow and the amount of fluorapatite thus formed is also questionable.[20] These results are in accordance with the research by Ramadoss *et al*.[21] and Bakry *et al*.[22]

The significantly higher ratio of Ca/P compared to the control group might be due to the sedimentable and amorphous form of calcium and phosphorus ions.[23] On the other hand, saturation with fluoride ions converts the acidic environment of the oral cavity to alkaline making it more favorable to remineralization. Hence, prevention of erosion was achieved using CPP-ACPF, though the level of remineralization was less than the other two bioactive agents. These results are in corroboration with other studies given by Palaniswamy *et al*.[24] and Mehta *et al*.[25]

On examination of the surface morphology under SEM at a magnification of ×2000, a honeycomb-like structure was seen in samples of Group 1 due to porosities created in the prismatic structure of enamel. Samples of Group 2 (Tooth Mousse Plus) represented remineralization similar to the description in the study presented by Poggio *et al*.[26] on remineralization of enamel lesions using CPP. Samples of Group 3 (Biomin F) revealed a globular structure with calcium

particles scattered over the surface similar to the images seen by Aidaros *et al*.[27] These images depict remineralization to an extent that is higher than in Group 2. Regarding Group 4 (Curodont Protect), the interprism cavities appeared largely filled. Lines of remineralization were visible along the prismatic borders. These results are in line with the results given in the study by Ali *et al*.[28] and point toward a higher remineralizing potential of Curodont Protect than Biomin F and CPP-ACPF both. In addition to hardness and EDX results, the higher mineralization along the aprismatic enamel seen in SEM images back up the results directing toward higher remineralization abilities of Curodont Protect followed by Biomin F and least by CPP-ACPF for prevention of erosion.[29]

The major strength of this study is the amalgamation of the different testing parameters as the specificity and accuracy of a single diagnostic method are cited as insufficient. Featherstone *et al*.[30] stated that the quantification of microhardness of the enamel is linearly proportional to the loss of mineral content at a certain depth of decay; hence, Vickers surface microhardness tester was used for assessment of mineral loss which yields a quantitative determination of the mineral content. EDX can precisely analyze the content of various elements, especially calcium and phosphorus.[31] Its absolute sensitivity is high, which helps in quantitative analysis. Furthermore, blinding of the evaluators regarding the group to which the samples belonged to minimized the chances of bias.

Inevitably, certain limitations are associated with *in vitro* studies. In this study too, there was a lack of human saliva which would vary in quantity in each individual and contribute to a certain amount of remineralization. Another limitation is that though the samples were randomly divided into treatment groups, some teeth might have greater susceptibility than others to demineralization due to the age of the patient and exposure to environmental factors such as fluorides.[32] In future, *in vivo* studies must be carried out using these commercially available remineralizing toothpastes to minimize the lack of clinical extrapolation and a long-term evaluation to authenticate the efficacy of these agents for the prevention of erosion.

CONCLUSION

Within the limitations of this study, it can be concluded that the regular consumption of acidic beverages can lead to tooth erosion which can be prevented to a certain extent by a regular remineralizing regimen, though not completely. Curodont Protect demonstrated improved mineral content quantitatively and qualitatively when compared to Biomin-F and GC Tooth Mousse Plus.

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Conflicts of interest
There are no conflicts of interest.

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