DOI: 10.1111/j.1365-263X.2011.01135.x

INTERNATIONAL JOURNAL OF PAEDIATRIC DENTISTRY

Remineralization effects of casein phosphopeptide-amorphous calcium phosphate crème on artificial early enamel lesions of primary teeth

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International Journal of Paediatric Dentistry 2011; 21: 374–381

Background. Caries in children younger than 72 months is called early childhood caries (ECC). Sixty-six per cent of Chinese children younger than 5 years old have dental decay, and about 97% of them are untreated.

Aims. This *in vitro* study was conducted to evaluate the remineralization effects of the casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) crème on the artificial early enamel lesions of the primary teeth and to assess its caries-prevention efficiency.

Design. Enamel specimens with artificial early lesions were produced and were then randomly divided into Group A: distilled and deionized water, DDW, as negative control; Group B: CPP-ACP crème, test group; Group C: 500 ppm NaF solution, as positive control. The enamel surface microhardness (SMH) was measured before, after demineralization, and 30 days after remineralization. The results were analysed with the SPSS 13.0 software package. The enamel specimens were analysed by the scanning electron microscope.

Results. The CPP-ACP crème increased SMH of the eroded enamel significantly more than 500 ppm NaF solution did. The morphology of the enamel was different in each group.

Conclusions. The CPP-ACP crème is effective in remineralizing early enamel lesions of the primary teeth, a little more effective than 500 ppm NaF and can be used for the prevention of ECC.

Introduction

Early childhood caries (ECC) is the most common oral disease of children. It can rapidly develop and cause many children's health problems. ECC can arouse tooth structure disintegration, as well as chewing and nutrient absorption, and may affect maxillofacial growth and development. Furthermore, caries-related tooth ache, infection, and other morbidities may interfere with children's concentration and school participation and peer interaction, which can negatively influence children's psychological and emotional conditions and can also be very difficult and costly to treat.[1]

The demineralization and remineralization are dynamic processes in the caries initiation, progression, and reversal. Therefore, regulation of the demineralization–remineralization balance is a key to the ECC prevention.[2,3] The ideal method of increasing remineralization is reconstructing the depleted tissues with hydroxyapatite, which is the same inorganic component as the enamel.[4]

Fluoride has a profound effect on the level of caries prevalence, but it is far from a complete cure. Furthermore, fluoride can cause fluorosis through overexposure, especially in young children.[5] Therefore, an appropriate nonfluoride anticaries agent is required. A new technology for remineralization has been developed using phosphopeptides from milk protein casein. Casein phosphopeptides (CPP) containing the sequence of Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu can stabilize the nanoclusters of amorphous calcium phosphate (ACP) in a metastable solution.[6] The multiple phosphoseryl residues of the CPP bind to the nanoclusters of ACP in the supersaturated solutions, preventing the precipitation of calcium and phosphate ions and the growth to

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the critical size required for the phase transformations. The casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) also acts as a reservoir for storing bioavailable calcium and phosphate and maintains the solution supersaturated, hence facilitating remineralization.[7] Many independent studies published till now have demonstrated the efficacy of the CPP-ACP technology in inhibiting demineralization and promoting remineralization of the enamel and dentin *in vivo* and *in vitro*.[8–12]

This *in vitro* study was designed to evaluate the remineralization potentials of the CPP-ACP crème, which was used on the artificial early enamel lesion of the children's primary teeth, through enamel surface microhardness (SMH) analysis and scanning electron microscopy (SEM) examination.

Materials and methods

Sample

The CPP-ACP crème was a GC tooth mousse, a water-based, sugar-free topical crème containing RECALDENT™* (CPP-ACP). The topical crème contained 10% w/w CPP-ACP nanocomplexes with bioavailable calcium and phosphate (GC Corp., Tokyo, Japan).

Specimen preparation

The Institutional Ethical Committee of West China College of Stomatology, Sichuan University, approved this study. All subjects provided informed written consents. A total of 236 lower incisors from the 6-year-old children were obtained from clinic of Pediatric Dentistry, West China College of Stomatology, Sichuan University, Chengdu, China. Immediately after the extraction of the teeth, the roots of the teeth were removed. The teeth were rinsed with the tap water and then stored at 4 °C in the deionized water (Generic Standard Elix 35 60L Tank, Millipore, USA) that contained 0.05% thymol (AR, Kelong Chemical Factory, Chengdu, China) till the use. Under the stereomicroscope (ACT-1, Nkion, Japan), any teeth with defects, erosions, or microcracks on their enamel surfaces, or visible stains on the lingual and facial surfaces were excluded. Custom-made plastic cylindrical moulds were prepared, and the self-cured acrylic resin (AR, Kelong Chemical Factory) was poured on them. Each enamel block was embedded in the self-cured acrylic resin. The labial surfaces were ground flat and hand-polished with the aqueous slurry of progressively finer grades of silicon carbide, up to 4000 grit (Struers, Copenhagen, Denmark), and about 150-μm thickness of the enamel tissue was removed from the original tooth surface. An acid-resistant nail varnish was applied around the exposed enamel surface, leaving a window (2×2 mm) of the enamel exposed at the centre. Then, the baseline enamel SMH was measured. An enamel SMH tester (Duramin-1/-2; Struers) with a Knoop diamond indenter was used with a 10 g load for 15 s. Five indentations in average were made on each surface of the individual specimens for the enamel SMH determination. A total of 160 enamel blocks were selected for the lesion formation, and the blocks had the baseline Knoop hardness number (KHN) values between 284.20 and 322.30.

Lesion formation

The section specimens of the enamel blocks were immersed in the demineralized solution containing 2.2 mM $Ca(NO_3)_2$ (AR, Beibei Chemical Factory, Chongqing, China), 2.2 mM phosphate as KH_2PO_4 (AR, Kelong Chemical Factory, Chengdu, China), 0.1 ppm NaF (AR, Kelong Chemical Factory), and 50 mM acetic acid (pH 4.5; AR, Kelong Chemical Factory). The solution was stirred at about 0.56 ***g***, and the demineralization was performed at 37 °C for 48 h.[13] Then, the enamel SMH was measured by the same measurement protocol. A total of 90 enamel blocks were selected for the remineralization process, and the blocks had the baseline KHN values between 284.20 and 322.30.

Remineralization

The section specimens were randomly divided into three groups, with 30 specimens in each

group. Group A was a distilled and deionized water group (the DDW group, negative control), Group B was a CPP-ACP crème group (the CPP-ACP group, the test group), Group C was the 500 ppm NaF solution group (the NaF group, positive control). Artificial saliva was used as a remineralizing solution (pH 7.0) according to the research by JM Ten Cate and PP Duijsters.[13] In the CPP-ACP group, a thin layer of the CPP-ACP crème was applied, using a microbrush, left undisturbed for 5 min and then stirred at about 100 rpm in the artificial saliva for 30 min. In the NaF group, the specimens were stirred at about 100 rpm in the 500 ppm NaF solution for 5 min. In the DDW group, no treatment was given to the enamel surfaces of the specimens, and the teeth were kept in the deionized water for 5 min. After the remineralization process, all the specimens were washed with the deionized water and were left in the artificial saliva during the remaining time (approximately 20 h/day). The specimens underwent the remineralization process twice a day (09:00 am, 4:00 pm) for 30 days. Then, the enamel SMH of each specimen was measured by the same measurement protocol.

SEM examination

For the SEM examination, six sample specimens in each group were treated. Air-dried sample specimens were sputtered with gold, resulting in a gold coating. Then, the shapes of the enamel surfaces and the vertical section of the lesion bodies were evaluated with SEM (S5000, Hitachi, Japan). For comparison, the surfaces of the sound and demineralized enamel were also examined.

Statistical analysis

All data were processed by the SPSS 13.0 software package (SPSS Inc., Chicago IL, USA). The surface KHN values were compared at the different time intervals in each group with the Student–Newman–Keuls *post hoc* test at a significance level of 0.05. The effects of the CPP-ACP crème, NaF and DDW on the changes in the enamel SMH (percentage of SMH recovery, %SMHR) were analysed using the two-way ANOVA and the least squares means at a significance level of 0.05. The percentage of SMHR was determined by the following formula:

$$\%\ \text{SMHR} = \text{remineralized enamel SMH} - \text{demineralized enamel SMH} \times 100/\text{sound enamel SMH} - \text{demineralized enamel SMH}.$$

Results

Enamel microhardness

The average KHN values of the surface enamel in each group measured at the different time intervals during the experiment (Table 1) showed that the SMH values of the sound enamel (SMH1, baseline) were not significantly different among the experimental groups ($P = 0.348$). The treatment of the immersion in the demineralized solution for 48 h significantly reduced SMH (SMH2, after erosion) in each group ($P < 0.05$). There was no statistically significant difference among the groups ($P = 0.619$). After remineralization, there was a significant increase in SMH

Table 1. Enamel SMH at baseline, after erosion, and after remineralization and percentage of SMHR (n = 30).

	Enamel treatment*			
Group	SMH1 (baseline)	SMH2 (after erosion)	SMH3 (after remineralization)	% SMHR
A (DDW)	304.96 ± 3.51[a]	154.47 ± 1.51[b]	163.70 ± 4.42[c]	6.13 ± 2.01[f]
B (CPP-ACP)	304.57 ± 3.08[a]	151.75 ± 1.84[b]	225.45 ± 4.53[d]	53.37 ± 5.78[g]
C (NaF)	306.10 ± 2.91[a]	153.85 ± 2.10[b]	216.86 ± 4.38[e]	49.65 ± 2.26[h]

SMH, enamel surface microhardness; SMHR, enamel surface microhardness recovery.
*Means with the same English letters had not a statistically significant difference. Means with different English letters had a statistically significant difference ($P < 0.05$).

(SMH3, after remineralization) in each group ($P < 0.05$) when compared with the data obtained after the erosion treatment. Moreover, the percentage of SMHR increased by 6.13% in the DDW group, 53.37% in the CPP-ACP group, and 49.65% in the NaF group. The results showed a significant difference among groups ($P < 0.05$).

SEM morphological characters

The typical SEM images of the enamel surfaces in the different groups (Fig. 1) showed that the sound enamel had an orderly rod appearance. The enamel crystals were homogeneously arranged with a clear outline. In contrast, the demineralized enamel was disorganized, with variable rod widths and a smaller number of enamel rods. Some of the enamel crystals were even fused together. In the DDW group, numerous spherical crystals could also be observed. The surface was, however, not flat. The enamel crystals were irregularly arranged. Some rod-like crystals were disorderly distributed on the surface of the enamel. In the CPP-ACP group and the

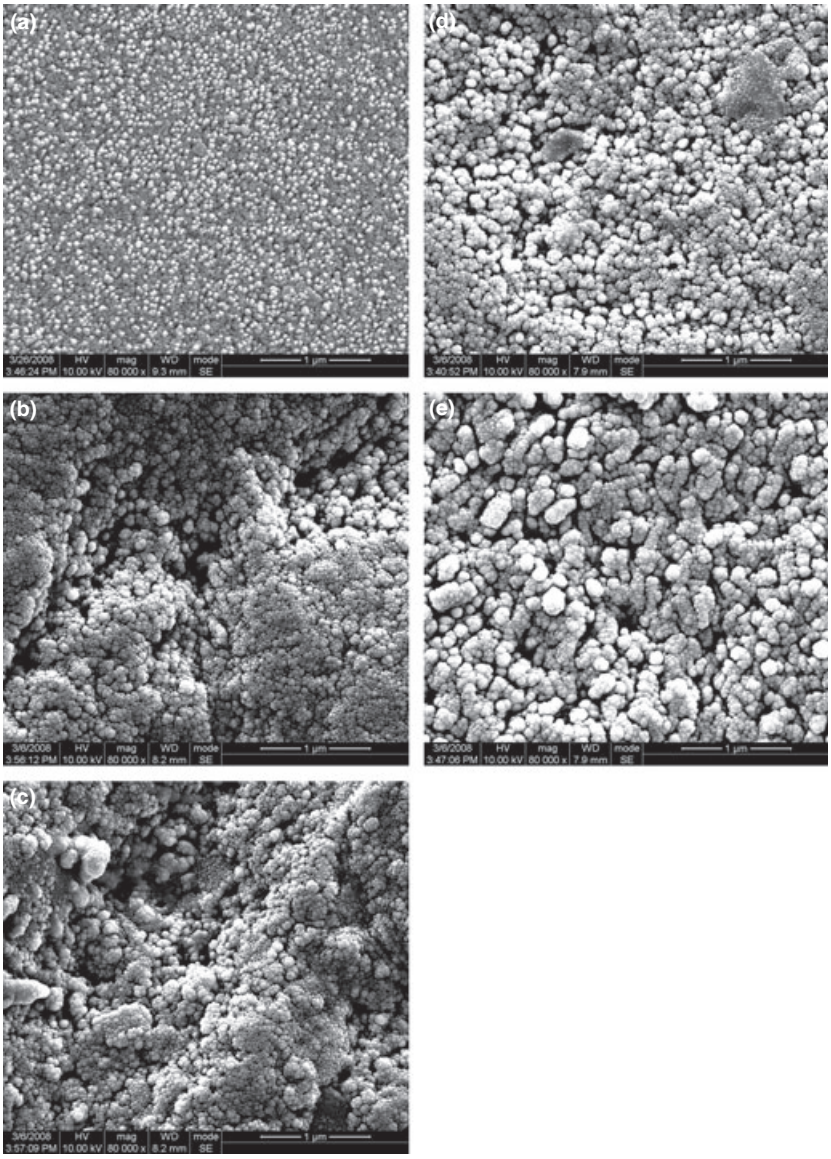

Fig. 1. The SEM images of the enamel surfaces in the different groups, (a) sound enamel (80,000×); (b) after demineralization (80,000×); (c) DDW (80,000×); (d) CPP-ACP (80,000×); (e) NaF (80,000×).

NaF group, numerous particles and amorphous crystals were arranged on the surface, but in the CPP-ACP group, those crystals seemed to be more homogeneous than those in the NaF group, and there was no obvious intercrystalline space.

The images of the vertical section of the specimens in the different groups (Fig. 2) showed that the enamel crystals were regularly sound enamel crystals. In the DDW group, the demineralized enamel had some obvious intercrystalline space and the enamel crystals were even fused together. The enamel crystals were arranged irregularly, quite different from the enamel crystals observed in the CPP-ACP group and the NaF group.

Discussion

The mineral loss or gain in the enamel because of demineralization or remineralization can be measured as changes in the enamel SMH.[14] The indentation hardness test with either the Knoop indenter or the Vicker indenter has been used for measurements of the initial enamel hardness, the enamel

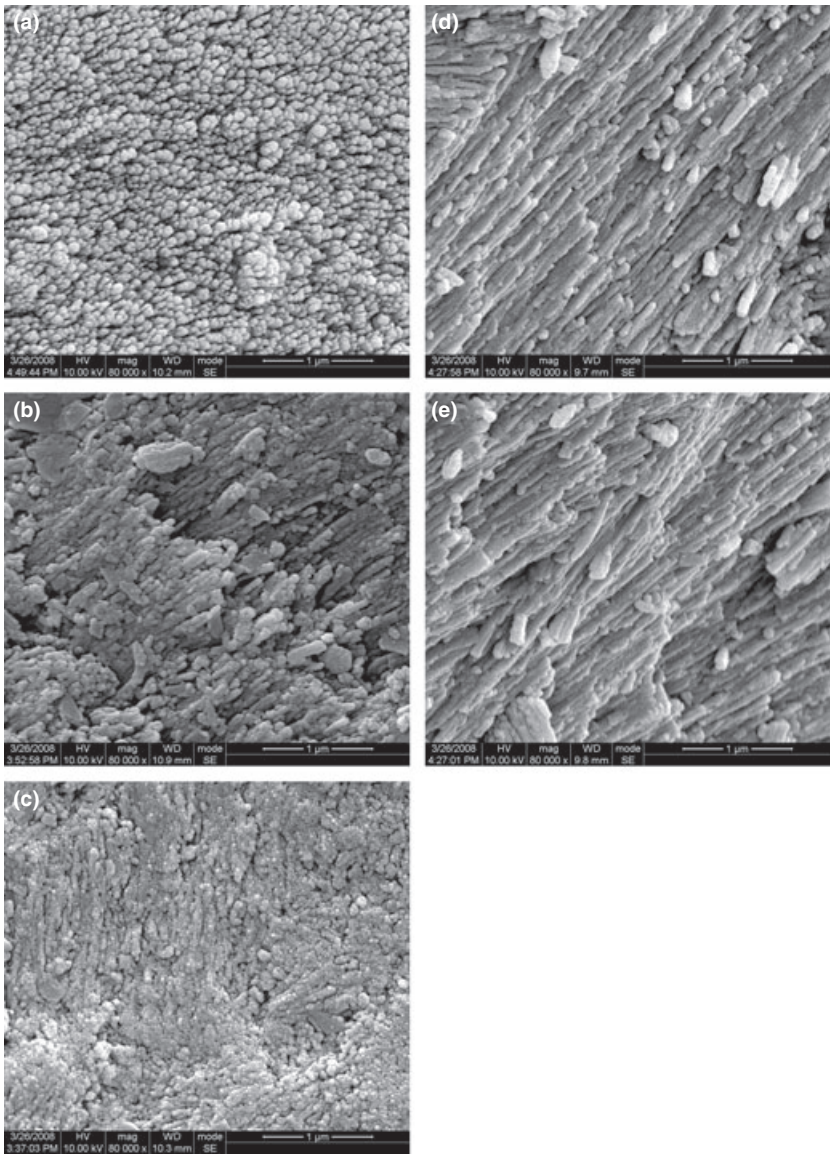

Fig. 2. The SEM images of the lesion bodies in the different groups, (a) sound enamel (80,000×); (b) after demineralization (80,000×); (c) DDW (80,000×); (d) CPP-ACP (80,000×); (e) NaF (80,000×).

softening (early erosion manifestation), and the enamel hardening (after remineralization).[14,15] Both the indenters are suitable for the hardness testing of nonmetallic materials. The measurement of the Knoop long diagonal is less affected by the elastic recovery than the short diagonal or the equal diagonals of the 136° diamond pyramid of the Vicker indenter. The Knoop hardness number has been correlated with the volume percentage of the enamel mineral.[14] The Knoop indenter with 10 g load for 15 s was selected because it provided an appropriate size of the indentations for an accurate measurement with the available equipment and the present experimental design.

According to the clinical trial by Winter, *et al.*,[16] the experimental toothpaste with 550 ppm fluoride would have a similar anticaries efficacy to that of the control toothpaste with 1055 ppm fluoride. Using the prevented fraction as the primary measure of the anticaries effect, in the placebo-controlled trials, the benefits from the increased fluoride concentration to prevent caries had only a statistically significant difference at the fluoride concentrations of 1000/1055/1100/1250 ppm or above; the fluoride concentrations of 440/500/550 ppm or below had no statistically significant anticaries effect when compared to the placebo.[17] When the 1000 ppm or higher fluoride toothpaste is used in children under 6 years old, the risk of fluorosis should be taken into account. Therefore, it was appropriate to select 500 ppm fluoride in our study.

The baseline enamel SMH values for the enamel in our study ranged from 284.20 to 322.30 KHN (Table 1). These values were similar to those in the previous studies by Lussi, *et al.*[18] The study design required a sufficiently flat enamel area to allow the enamel SMH measurements; thus, the area subjected to the erosion treatment was not the original surface of the enamel. Moreover, a decrease in enamel SMH because of erosion and an increase in enamel SMH caused by remineralization in the polished enamel could be different from those obtained in the uncut enamel. Removal of the outer layer of the enamel (the hypermineralized layer often containing fluorapatite) made the enamel more susceptible to the softening process.

The sharp decrease in enamel SMH (139.38–162.64 KHN) occurred after the demineralization process (Table 1). The test material was applied to the enamel blocks twice a day to simulate the normal recommended daily oral prophylaxis. After the remineralization process, the mean SMH and the percentage of SMHR increased in each groups. All the values were significantly different among the groups. Therefore, 500 ppm NaF could promote the remineralization process of the primary teeth, but not as effectively as the CPP-ACP crème. Lata, *et al.*[12] compared fluoride varnish (Fluorprotector Intro pack; Ivaclar Vivadent, containing 1000 ppm NaF) with the CPP-ACP crème in the remineralization ability. The percentage of SMHR was calculated, which showed the greatest recovery rate of 35% in the fluoride plus CPP-ACP group, followed by 32% in the fluoride group, and 17% in the CPP-ACP group. There was, however, no statistically significant difference in the percentage of SMHR between the fluoride group and the fluoride plus CPP-ACP group. This comparison showed that the CPP-ACP crème had smaller effectiveness than 1000 ppm NaF, although it had a little greater effectiveness than the 500 ppm NaF, in the remineralization for the early enamel caries at the surface level.

At the enamel surface, when fluoride ions come into contact with free calcium and phosphate ions, fluorapatite will rapidly form in the surface layer. The presence of CPP can, however, prevent the rapid transformation of the calcium phosphate phase; thus, the ions will be stabilized and maintained in a form that will drive the diffusion down activity gradients into the subsurface lesions. Hence, the ability to deliver calcium, phosphate, and fluoride ions in the correct molar ratio deep into the subsurface lesions may be attributable to the ability of CPP to localize and stabilize the ions at the tooth surface in the correct molar ratio ($Ca:PO_4:F = 5 : 3 : 1$).[19] The mineral formed in the surface and subsurface lesions is consistent with hydroxyapatite and fluorapatite for remineralization with CPP-ACP and CPP-ACP plus fluoride,

respectively. The activity gradient of the neutral ion pair $CaHPO_4^0$ into the lesion is closely correlated with remineralization, which, together with HF^0, can be identified as important species for diffusion.[20] That can probably explain why, in our present research, numerous nano-size particles and amorphous crystals were arranged on the enamel surface and the lesion bodies after the CPP-ACP application and those crystals in the CPP-ACP group were more homogeneous than those in the NaF group, and the intercrystalline spaces were not as obvious as those in the other groups.

Remineralization of the eroded lesions may occur as a result of the deposition of the mineral into the porous zone rather than the emergence of the eroded crystals.[21] In the oral environment, hydroxyapatite crystals are formed from the supersaturated calcium and phosphates ions, and the presence of fluoride ions is likely to further promote the remineralization process by forming fluorapatite crystals that are more resistant to future demineralization than hydroxyapatite.[22] In our study, the artificial saliva resulted in slight remineralization of the enamel surface (6.13%), which was much lower when compared with the remineralization resulting from the CPP-ACP crème (Table 1). The SEM observation showed that some new crystals formed on the remineralized enamel surface and even some enamel cracks existed (Fig. 2). Several previous studies have reported the remineralization potential because of the saliva in the presence of an erosion,[23,24] but this kind of the remineralization potential seems limited. So, an efficient remineralizing agent should be used shortly after an erosive challenge appears, so that mechanical injuries to the softened dentin caused by the mastication and friction from the oral soft tissues can be prevented. In the remineralization phase, artificial saliva and acquired pellicle can favourably influence the outcome only if CPP-ACP crème is better retained on the enamel surface. The effectiveness of the CPP-ACP crème can be enhanced in the oral cavity if a biofilm exists, which can bind to CPP and act as a reservoir for the calcium and phosphate ions. Although our study could not completely simulate the complex oral environment, the study results still demonstrated the remineralization effectiveness of the CPP-ACP crème on the artificial early enamel lesions of primary teeth.

In conclusion, the CPP-ACP crème is effective in remineralizing early enamel lesions of the primary teeth, a little more effective than 500ppm NaF. The remineralization *in vitro* may, however, have some differences when compared to the remineralization *in vivo* because of the dynamic complex biological system in the oral cavity. Thus, further studies *in vivo* are still required for a proper clinical use of the CPP-ACP crème.

What this paper adds?

- The CPP-ACP crème, as an appropriate bioavailable anticaries agent, has been shown to remineralize enamel surface lesions *in vitro*, furthermore, is a little more effective than 500 ppm NaF solution.

Why this paper is important for paediatric dentistry?

- Evidence from our study exists to support the clinical use of the CPP-ACP crème superior than the fluoride treatment in the preventive management of ECC in young children.

Acknowledgements

We would like to thank Prof. Yuqing Hao at the State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Chengdu, China, for her providing us with the Knoop hardness testing machine for this study.

This investigation was supported by the Key Technology R & D Program of Science & Technology department of Sichuan Province, China (Grant No. 2010SZ0102).

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