

In vivo remineralising effect of GC Tooth Mousse on early dental enamel lesions: SEM analysis

Gianmaria F. Ferrazzano, Ivana Amato, Tiziana Cantile, Giancarla Sangianantoni and Aniello Ingenito

Department of Paediatric Dentistry, School of Dentistry, University of Naples 'Federico II', Naples, Italy.

Objective: Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) exhibit anticariogenic potential in laboratory, animal and human experiments. The remineralising potential of synthetic CPPs on early enamel caries was investigated. **Design:** *In vivo* study. **Setting:** University of Naples 'Federico II', School of Dentistry, Department of Paediatric Dentistry, 2010, Italy. **Participants:** 40 volunteers (age range 10–16 years) were recruited and divided in two groups of 20 (Group A and B). **Interventions:** In Group A subjects two demineralised enamel specimens were placed on the buccal surfaces of the first molars and subjects were instructed to apply a commercial product containing CPPs (GC Tooth Mousse) only on the right-sided specimen and a placebo mousse on the left, for 1 month. In Group B subjects two enamel specimens were similarly placed into the mouth and used as controls. **Results:** SEM analysis revealed a diffuse and homogeneous mineral coating, reducing the surface alterations only in the demineralised specimens treated with synthetic CPPs into the mouth. **Conclusions:** Results demonstrate that CPPs are able to promote remineralisation of early enamel lesions.

Key words: Bioactive peptides, caries, casein phosphopeptides, prevention, GC Tooth Mousse

Dairy products have been recognised as a food group that is effective in preventing dental caries¹. Using *in vitro*, animal and human *in situ* models, the anticariogenic activity of milk and milk products was attributed to the direct chemical effects of casein phosphopeptides, calcium and phosphate².

Casein phosphopeptides (CPPs) are phosphorylated casein-derived peptides produced by proteolytic digestion of α_{s1} -, α_{s2} -, and β -casein *in vitro* or in the digestive tract. CPPs, containing the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu, stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution³.

Casein phosphopeptides-amorphous calcium phosphate localise ACP in dental plaque, which buffers the free calcium and phosphate ion activities, helping to maintain a state of supersaturation with respect to tooth enamel, depressing demineralisation and enhancing remineralisation⁴.

In liquid milk the majority of the casein and calcium and phosphate ions are bound in micelles and then upon consumption would not necessarily be available to promote enamel remineralisation⁵.

In fact, the calcium in casein micelles is unlikely to be available to diffuse across a relatively intact enamel

surface layer into a subsurface lesion and therefore milk may have limited ability to remineralise enamel lesions *in situ*. Therefore, since milk could not be used as a natural product against dental caries, because it needs the addition of 2.0–5.0 g CPP-ACP to substantially increase its ability to remineralise enamel subsurface lesions, a commercial paste containing CPP-ACP showing a remineralisation effect on early enamel lesions is investigated⁶.

The application of a CPPs toothpaste and sodium fluoride (Colgate Neutrafluor 9,000 ppm) (NaF) can provide significant additional prevention of enamel demineralisation when resin-modified glass ionomer cement (RMGIC) is used for bonding molar tubes for orthodontic patient as preventive actions⁷. An *in vitro* study to evaluate the remineralisation of incipient enamel lesions by the topical application of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) using laser fluorescence and scanning electron microscope showed high scores of remineralisation⁸.

Therefore, the aim of this study was to test *in vivo* the effectiveness of synthetic casein phosphopeptides (CPPs) present in a commercial product to promote remineralisation of early enamel caries.

MATERIALS AND METHODS

Subjects and study design

The study was carried out in the Department of Paediatric Dentistry of the University of Naples 'Federico II', Italy. For this study 100 subjects were recruited but only 40 healthy patients (18 males and 22 females) with an age range from 10 to 16 years were selected with the following criteria: good general health (ASA I-II) and agreement to comply with study procedures. An intra-oral examination confirmed that each member had no periodontal disease or other oral pathology.

Exclusion criteria were: milk protein allergies and conditions that interfered with the examination procedure (non-co-operating subjects).

Participation was voluntary. After parents and patients had been given verbal and written explanations of the experimental protocol and the study aims, written informed consent was signed by them prior to the start of the study. Permission was received from the appropriate authorities. The subjects were divided into two groups of 20 (Groups A and B).

Preparation of enamel specimens

Forty human teeth, extracted for orthodontic reasons or impaction, were obtained from the Department of Paediatric Dentistry of the University of Naples 'Federico II', Italy.

Teeth were cleaned with sterile gauze imbibed in distilled water and stored at 4 °C in sterile containers with 5% formalin solution. The roots were cut by mean of a diamond disk assembled on a laboratory handpiece and the crowns polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental handpiece and then rinsed in distilled water. From each crown four longitudinal specimens of enamel of about 3 × 2 × 2 mm were obtained by cutting with a diamond disk.

Experiment design-Group A

Eighty specimens were used for this group. Three specimens of the four obtained from each tooth were submitted to a demineralisation procedure by immersion for 96 hours (with one change of solution after 48 hours) in a demineralising solution containing: 50 ml of 0.1 M lactic acid, 0.02% carboxymethylcellulose (Akzo Nobel, Amsterdam, Netherlands) (pH 4.8, T° 37 °C)⁹. The other one specimen from each tooth was left intact and stored in distilled water (with one change of solution after 48 hours).

After 4 days, all specimens were rinsed in distilled water and dried with a jet of warm air for 3 seconds, packaged in individual paper autoclavable bags and

Table 1 Group A: remineralisation protocol with GC Tooth Mousse

20 sound specimens	Subgroup α_1	Outside of oral environment
20 demineralized specimens	Subgroup α_2	Outside of oral environment
20 demineralized specimens	Subgroup α_3	Intra-oral environment and GC Tooth Mousse
20 demineralized specimens	Subgroup α_4	Intra-oral environment and placebo

autoclaved at 121 °C at 15 lbs psi for 30 minutes¹⁰. Two enamel specimens (one sound and one demineralised, respectively) of four obtained from each tooth were stored in distilled water (with one change of solution every 48 hours) and used as control outside the oral environment (Subgroup α_1 and α_2) (Table 1).

In 20 subjects belonging to Group A the remaining two enamel specimens (both demineralised) were placed, with the dentinal face towards the tooth, through an adhesive procedure, on buccal surfaces of the right and the left first or second upper molars for the remineralisation protocol with synthetic casein phosphopeptide; therefore each subject was required to apply a commercial product for professional use containing 10% of casein phosphopeptide (Tooth Mousse GC Corp., Tokyo, Japan) only on the right-side specimen (Subgroup α_3) (Table 1) and a glycerine based (without remineralising substances) placebo gel on the left-side specimen (Subgroup α_4) (Table 1), on a daily basis, three times per day for a month at the following times 7.00, 14.00, 21.00 hours. In addition, subjects were asked to complete a diary documenting their Tooth Mousse application habits.

The GC Tooth Mousse was provided by GC industry as coded products and stored in a secured area at room temperature. No alterations were made to the subjects' diet and oral hygiene procedures during the study.

Experiment design-Group B

Eighty specimens were used for this group. Two of the four specimens obtained from each tooth were submitted to a demineralisation procedure by immersion for 96 hours (with one change of solution after 48 hours) in a demineralising solution containing: 50 ml of 0.1 M lactic acid, 0.02% carboxymethylcellulose (Akzo Nobel, Netherlands) (pH 4.8, T° 37 °C)⁹. The other two specimens from each tooth were left intact and stored in distilled water (with one change of solution after 48 hours). After 4 days, all specimens were rinsed in distilled water, dried with a jet of warm air for 3 seconds, packaged in individual paper autoclavable bags and autoclaved at 121 °C at 15 lbs psi for 30 minutes¹⁰.

Table 2 Group B: control group

20 sound specimens	Subgroup β_1	Outside of oral environment
20 demineralised specimens	Subgroup β_2	Outside of oral environment
20 sound specimens	Subgroup β_3	Intra-oral environment
20 demineralised specimens	Subgroup β_4	Intra-oral environment

Two enamel specimens (one sound and one demineralised, respectively) of four obtained from each tooth were stored in distilled water (with one change of solution every 48 hours) and used as control outside of the oral environment (Subgroup β_1 and β_2) (Table 2).

To 20 subjects belonging to Group B the remaining two enamel specimens (one sound and one demineralised, respectively) (Subgroup β_3 and β_4) (Table 2) were placed, with the dentinal face towards the tooth, through adhesive cementing systems, on buccal surfaces of first or second upper molars for a month. No alterations were made to the subjects' diet and oral hygiene procedures during the study.

SEM analysis

After completion of each treatment period, the enamel specimens in the mouth were removed by orthodontic pliers and were polished with pumice dust and non-fluoride toothpaste, using a circular brush with nylon bristles mounted on a dental handpiece. They were then rinsed in distilled water and compared with the control specimens to assess the morphological changes by SEM. For the ultra-structural examination of the tooth surfaces, the specimens were dehydrated in a graded series of ethanol, critical point dried (SPC-900/EX; The Bomar Co., Tacoma, Washington, USA), mounted in stubs, sputter-coated with gold (E 306; Edwards, Crawley, UK) and observed by SEM (Stereoscan 250 MK3, Cambridge, UK) at 50 \times , 100 \times , 600 \times , 3000 \times , 6000 \times magnifications.

RESULTS

Images that are representative for each group are shown.

Specimens from Group A

Subgroup α_1

SEM images of the enamel specimens from subgroup α_1 are suggestive of sound and smooth surface (Figures 1 and 2).

Subgroup α_2

Characteristic etching patterns are found on the surfaces of the specimens from subgroup α_2 . Acid-etching

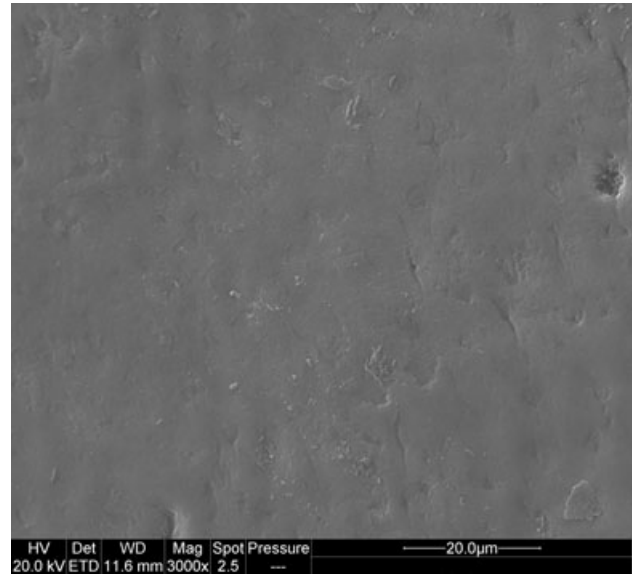


Figure 1. Sound enamel surface subgroup α_1 (3,000 \times).

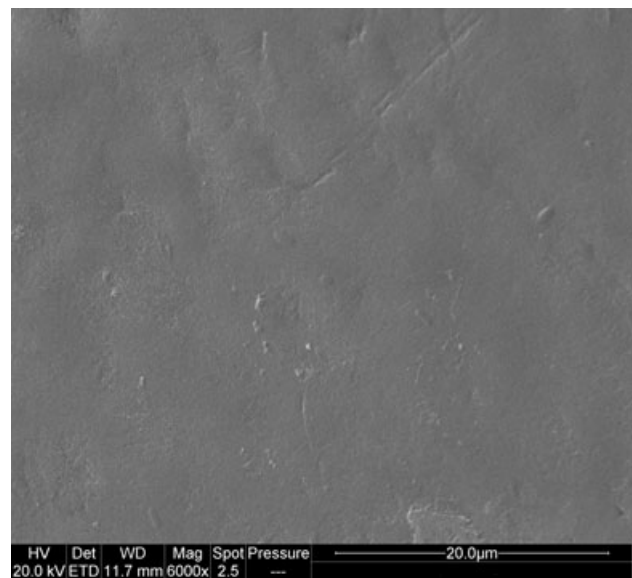


Figure 2. Sound enamel surface subgroup α_1 (6,000 \times).

of sound enamel creates microporosities from 4 to 20 μm in diameter (Figures 3 and 4).

Subgroup α_3

The enamel specimens from subgroup α_3 do not show any erosion or demineralisation of the enamel surface. A mineral sediment with a trend of wavy lines is clearly visible at low magnification (600 \times) (Figure 5). At higher magnification (6,000 \times) it can be clearly seen that the surfaces are covered with an amorphous deposit, which completely obscures the underlying prism structure. In all the specimens this mineral

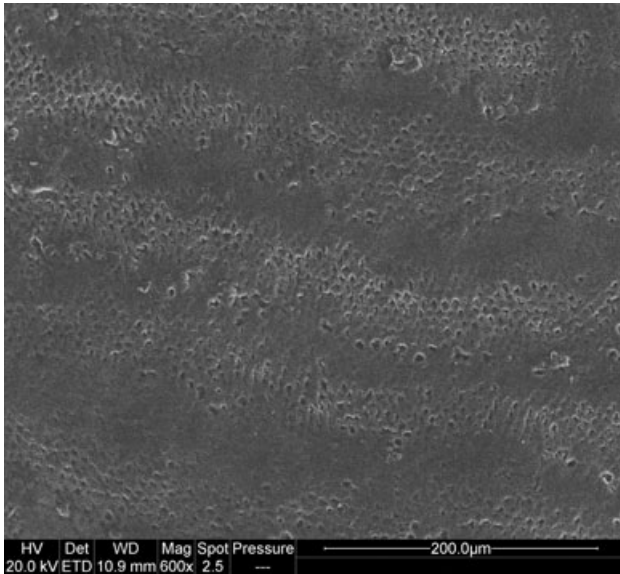


Figure 3. Demineralised enamel surface subgroup α_2 (600 \times).

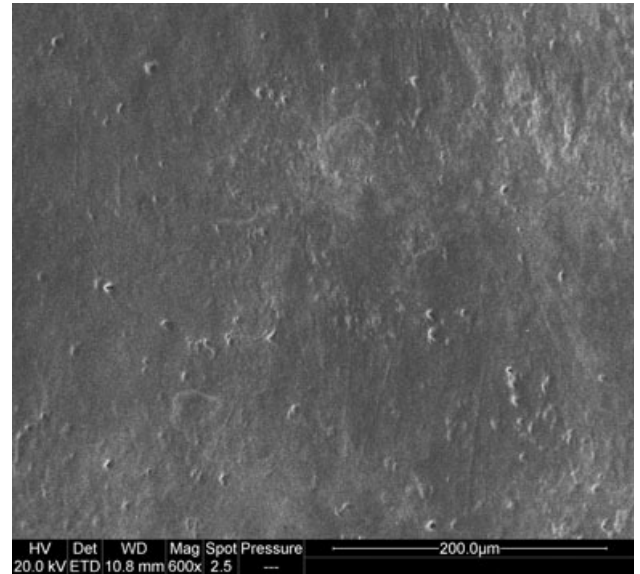


Figure 5. Remineralised enamel surface with GC Tooth Mousse subgroup α_3 (600 \times).

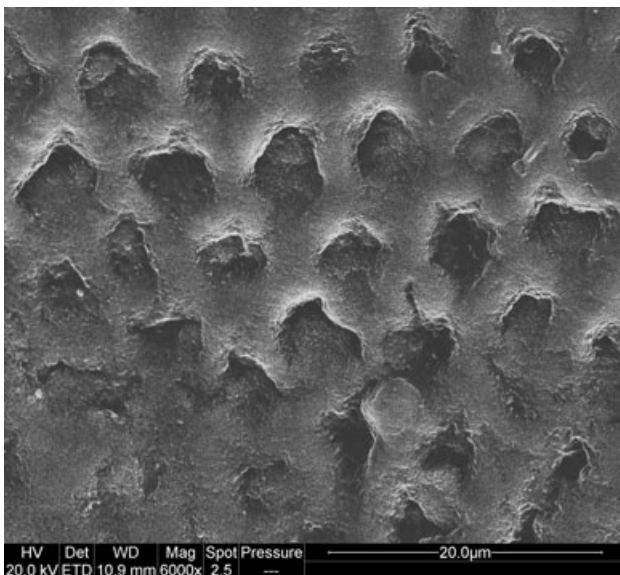


Figure 4. Demineralised enamel surface subgroup α_2 (6,000 \times).

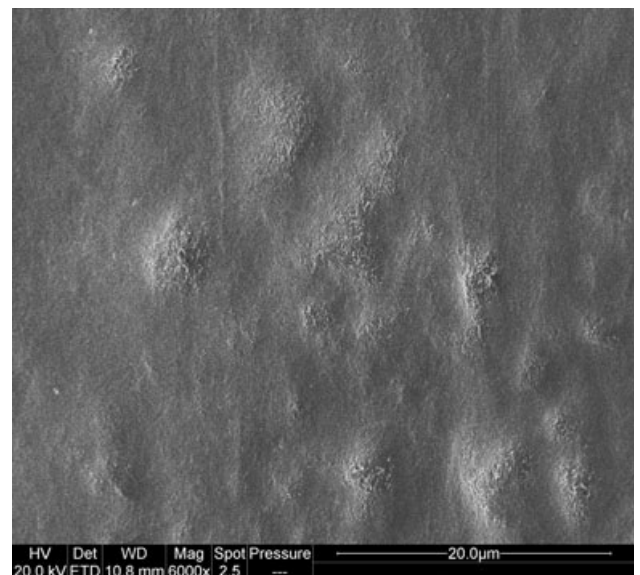


Figure 6. Remineralised enamel surface with GC Tooth Mousse subgroup α_3 (6,000 \times).

sediment entirely fills the previously created lesions (Figure 6).

Subgroup α_4

The enamel specimens from subgroup α_4 show strong superficial alterations, loss of enamel and pronounced holes from 5 to 25 μm in diameter, higher than the typical lesions created by the acid solution used for the demineralisation protocol. In particular, the surfaces appear disorganised, without any distinct etching pattern (Figures 7 and 8).

Specimens from Group B

Subgroup β_1

SEM evaluation of the enamel specimens from subgroup β_1 reveals that they are like a typical sound enamel surface at low and high magnification (Figures 9 and 10).

Subgroup β_2

The specimens from subgroup β_2 present roughening surfaces, in which tissue loss gives the enamel a porous

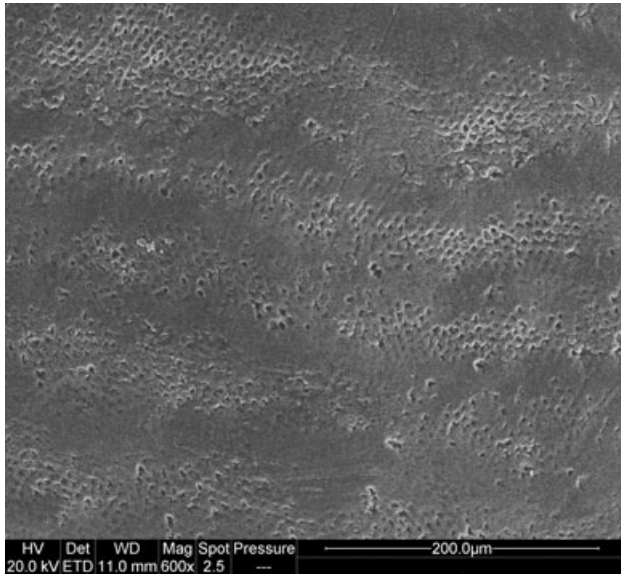


Figure 7. Demineralised enamel surface with placebo subgroup α_4 (600 \times).

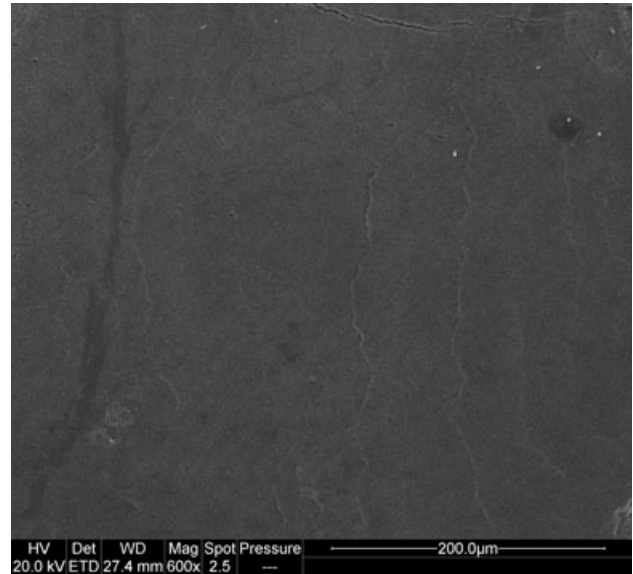


Figure 9. Sound enamel surface subgroup β_1 (600 \times).

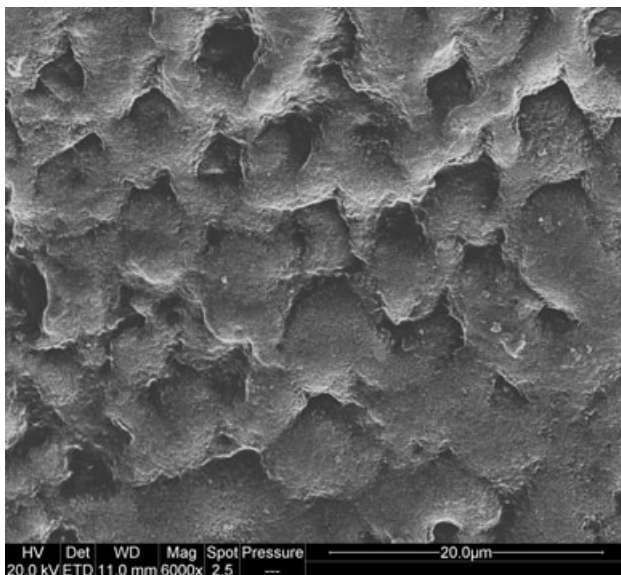


Figure 8. Demineralised enamel surface with placebo subgroup α_4 (6,000 \times).

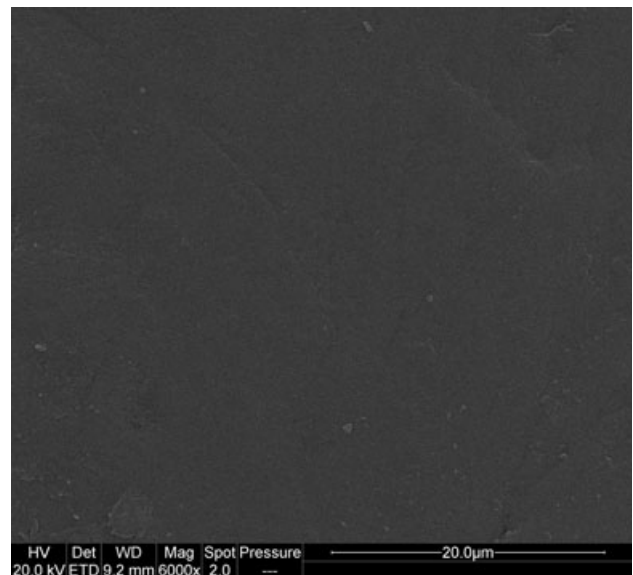


Figure 10. Sound enamel surface subgroup β_1 (6,000 \times).

appearance. The entire prism core is dissolved. In all the samples, the characteristic honeycomb structure of demineralised enamel is clearly evident with relatively deep, but tapered etched pits varied in size from 4 to 20 μm in diameter (Figure 11).

Subgroup β_3

The images of the subgroup β_3 specimens are similar to the images of the sound specimens stored in distilled water outside of the oral environment (Figure 12).

Subgroup β_4

The enamel specimens from subgroup β_4 show loss of enamel and evident holes from 5 to 25 μm in diameter, deeper than the typical damages caused by the acid solution used for the demineralisation protocol (Figure 13).

DISCUSSION

SEM analysis provided useful information about structural changes occurring on the enamel surfaces during

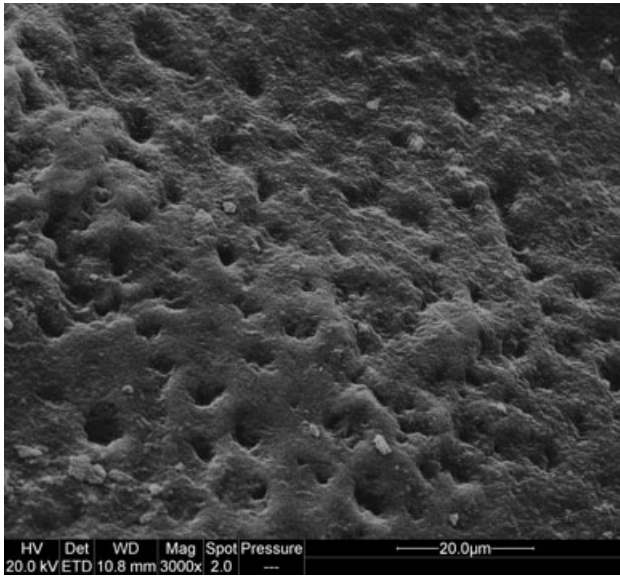


Figure 11. Demineralised enamel surface subgroup β_2 (3,000 \times).

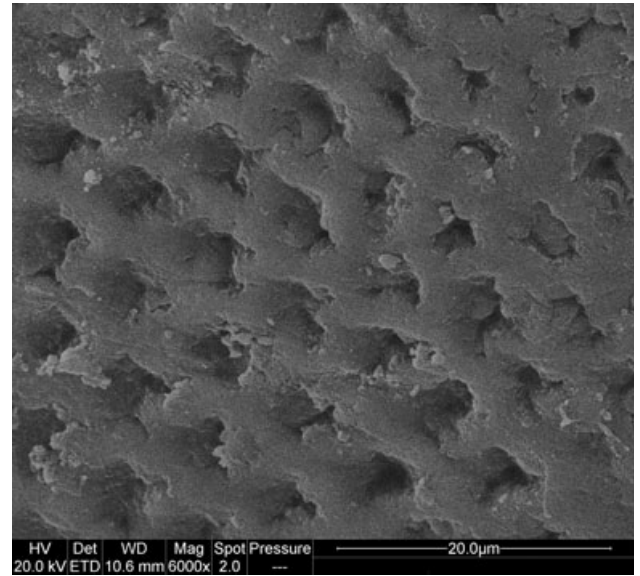


Figure 13. Demineralised enamel surface subgroup β_4 (6,000 \times).

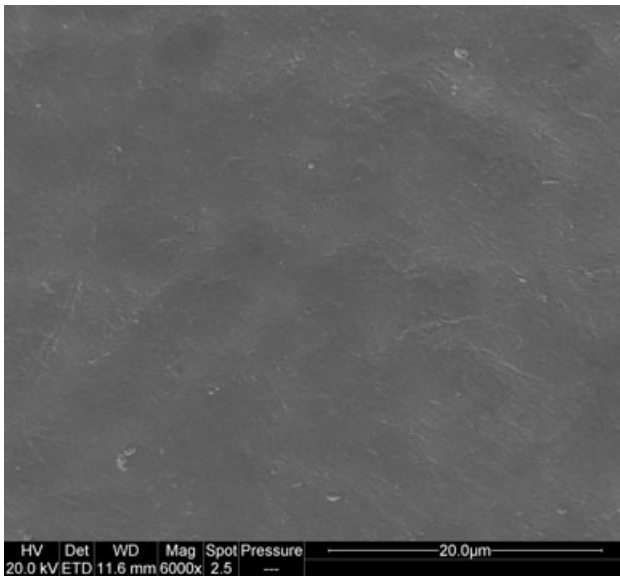


Figure 12. Sound enamel surface subgroup β_3 (6,000 \times).

the test period. In particular, the effectiveness of synthetic CPPs was clearly visible from the images of the specimens demineralised for 4 days at pH 4.8 and then subjected to the treatment with CPPs in the mouth, in which the previously created microporosities were covered and filled by an amorphous mineral sediment. In fact, the inorganic components contained in high concentrations in CPP-ACP acted to enhance remineralisation of the enamel structure.

The increased enamel remineralisation found *in vivo* in this study by synthetic CPPs was consistent with previous studies showing the anti-cariogenic and remineralisation potential of CPP-ACP in solution and the proposed mechanism of the localisation of amorphous

calcium phosphate on the tooth surface by the CPPs, depressing enamel demineralisation and enhancing remineralisation¹¹⁻¹⁶. GC Tooth Mousse can be considered a valid product with anti-caries effect. Furthermore, the intent of this *in vivo* model was also to mimic what occurred in the natural caries process, and to give clinically relevant information about the remineralisation mechanism over a short period of time without causing irreversible tissue changes in the natural dentition.

It should be emphasised that this *in vivo* system could be considered, as suggested by Zero, as a bridge between the natural uncontrolled clinical situation and the highly controlled laboratory situation¹⁷. The main advantages of this model are: use of a 'natural' environment consisting of tooth substrate; formation or presence of dental plaque with cariogenic potential; carbohydrate challenge, provided by the subject's normal diet, oral microflora, host factors such as saliva. Enamel specimens fixed in the mouth also had the advantage that subject compliance with wearing an appliance is not a factor, although model systems that use the subjects' normally worn partial dentures ensure a level of certainty of compliance with the study requirements. Furthermore, the enamel specimens, mounted on the buccal sites, were well tolerated by subjects.

CONCLUSION

Casein phosphopeptides exhibit physiological functions such as minerals carrier activity that is one of the main mechanisms in dental caries prevention³. The remineralisation potential of CPP-ACP highlighted the importance of using milk-derived peptides against dental caries in daily oral care (delivered by commercial toothpaste).

Future studies should, then, be focused on the evaluation of the potential effects of synthetic CPPs, in order to create a new preventive strategy based on utilising these bio-active principles in personal hygiene products to reduce cariogenicity.

REFERENCES

1. Aimutis WR. Bioactive properties of milk proteins with particular focus on anticariogenesis. *J Nutr* 2004 134: 989S–995S.
2. McDougall WA. Effect of milk on enamel demineralization and remineralization *in vitro*. *Caries Res* 1977 11: 166–172.
3. Reynolds EC. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. *Spec Care Dentist* 1998 18: 8–16.
4. Cai F, Shen P, Morgan MV *et al.* Remineralization of enamel subsurface lesions *in situ* by sugar-free lozenges containing casein phosphopeptide-amorphous calcium phosphate. *Aust Dent J* 2003 48: 240–243.
5. Walker G, Cai F, Shen P *et al.* Increased remineralization of tooth enamel by milk containing added casein phosphopeptide-amorphous calcium phosphate. *J Dairy Res* 2006 73: 74–78.
6. Kumar VL, Itthagarun A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an *in vitro* study. *Aust Dent J* 2008 53: 34–40.
7. Sudjalim TR, Woods MG, Manton DJ *et al.* Prevention of demineralization around orthodontic brackets *in vitro*. *Am J Orthod Dentofacial Orthop* 2007 131: 1–9.
8. Pai D, Bhat SS, Taranath A *et al.* Use of laser fluorescence and scanning electron microscope to evaluate remineralization of incipient enamel lesions remineralized by topical application of casein phosphopeptide amorphous calcium phosphate (CPP-ACP) containing cream. *J Clin Pediatr Dent* 2008 32: 201–206.
9. Iijima Y, Takagi O, Ruben J *et al.* *In vitro* remineralization of *in vivo* and *in vitro* formed enamel lesions. *Caries Res* 1999 33: 206–213.
10. Kumar M, Sequeira PS, Peter S *et al.* Sterilisation of extracted human teeth for educational use. *Indian J Med Microbiol* 2005 23: 256–258.
11. Reynolds EC, Cain CJ, Webber FL *et al.* Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat. *J Dent Res* 1995 74: 1272–1279.
12. Reynolds EC. Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *J Dent Res* 1997 76: 1587–1595.
13. Iijima Y, Cai F, Shen P *et al.* Acid resistance of enamel subsurface lesions remineralized by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *Caries Res* 2004 38: 551–556.
14. Yamaguchi K, Miyazaki M, Takamizawa T *et al.* Effect of CPP-ACP paste on mechanical properties of bovine enamel as determined by an ultrasonic device. *J Dent* 2006 34: 230–236.
15. Rahiotis C, Vougiouklakis G. Effect of a CPP-ACP agent on the demineralization and remineralization of dentine *in vitro*. *J Dent* 2007 35: 695–698.
16. Reynolds EC. Calcium phosphate-based remineralization systems: scientific evidence? *Aust Dent J* 2008 53: 268–273.
17. Zero DT. *In situ* caries models. *Adv Dent Res* 1995 9: 214–230.

Correspondence to:
Dr Gianmaria Fabrizio Ferrazzano,
Via Cuoco n° 5,
80100 Naples, Italy.
Email: gianmariafabrizio@yahoo.it