

# Effect of Indigenously Developed Nano-Hydroxyapatite Crystals from Chicken Egg Shell on the Surface Hardness of Bleached Human Enamel: An *In Vitro* Study

## Abstract

**Objective:** The objective was to evaluate the effect of nano-hydroxyapatite (nHA) derived from chicken eggshell on bleached human enamel in comparison with commercial casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste using Vickers microhardness test. **Materials and Methods:** nHA powder was prepared from chicken eggshell using combustion method. nHA slurry was prepared by mixing 1.8 g of nHA powder with 0.3 ml of distilled water. Forty intact maxillary anterior teeth were collected and decoronated, and the crowns were embedded in acrylic mold with the labial enamel surfaces exposed. Baseline microhardness evaluation was done ( $T_0$ ). The specimens were randomly divided into the following four groups ( $n = 10$ ) based on the surface treatment of enamel: Group 1: no bleaching treatment; Group 2: bleaching with 30% hydrogen peroxide (HP) solution; Group 3: bleaching followed by the application of CPP-ACP; and Group 4: bleaching followed by the application of nHA. The specimens were stored in artificial saliva at 37°C for 2 weeks, after which they were subjected to Vickers microhardness test ( $T_{14}$ ). One-way ANOVA and Tukey's *post hoc* multiple comparison tests were used for statistical analysis ( $P < 0.05$ ). **Results:** Bleaching with HP significantly decreased the enamel microhardness. CPP-ACP and nHA derived from chicken eggshell increased the enamel microhardness significantly. There was no significant difference in microhardness values among the CPP-ACP and nHA groups. **Conclusion:** Nano-hydroxyapatite sourced from chicken eggshell was as effective as CPP-ACP in remineralizing and restoring the lost microhardness of bleached enamel.

**Keywords:** Casein phosphopeptide-amorphous calcium phosphate, chicken eggshells, hydrogen peroxide, microhardness, nano-hydroxyapatite

## Introduction

There is an increasing awareness among patients regarding dental esthetics. Discoloration of teeth resulting from various reasons poses a major obstacle in achieving desirable esthetics. When compared to veneers and full-coverage crowns, bleaching is considered a conservative option for the management of discolored teeth. It can be done as an at-home or in-office procedure.<sup>[1]</sup> The development of bleaching gels that use hydrogen peroxide (HP) or carbamide peroxide in high concentrations (35%–38%) has made in-office bleaching procedures easier, with immediate favorable results achieved, without a need for further patient cooperation.<sup>[2]</sup> Longer application time and multiple visits are required in order to obtain optimum tooth-whitening

results. This has a negative influence on the integrity of dental hard tissues.<sup>[3]</sup> These changes range from microscopical alterations of enamel surface in the form of surface defects and subsurface pores to significant reduction in enamel microhardness.<sup>[4,5]</sup>

Direct topical application of remineralizing agents or their incorporation into bleaching gels has shown to decrease the unfavorable effects of bleaching agents on enamel.<sup>[6,7]</sup> Remineralizing agents such as fluoride, calcium, amorphous calcium phosphate (ACP), casein phosphopeptide-ACP (CPP-ACP), hydroxyapatite (HA), and nano-HA (nHA) have shown promising results in various studies.<sup>[7-9]</sup>

CPP-ACP has been developed based on calcium phosphate remineralization

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Access this article online

**Website:**  
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**DOI:** 10.4103/ccd.ccd\_810\_18

**Quick Response Code:**



**How to cite this article:** Kunam D, Sampath V, Manimaran S, Sekar M. Effect of indigenously developed nano-hydroxyapatite crystals from chicken egg shell on the surface hardness of bleached human enamel: An *in vitro* study. Contemp Clin Dent 2019;10:489-93.

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technology which can inhibit the demineralization process and enhance the remineralization of enamel and dentin.<sup>[8,10,11]</sup> Studies have proven that the remineralization potential of CPP-ACP is capable of repairing initial enamel caries lesions.<sup>[11]</sup> It has been reported that the application of a CPP-ACP paste either before or after in-office bleaching protocols can prevent HP-induced negative changes of roughness and hardness on enamel.<sup>[12]</sup> Incorporation of CPP-ACP in 10% and 16% carbamide peroxide gels has shown to increase postbleaching enamel hardness.<sup>[13]</sup>

With the advent of biomimetic materials in the field of dentistry, materials with properties similar to that of natural tooth structure and those which can completely replace lost tooth structure have evolved. HA is one such material which is considered the most biocompatible and bioactive.<sup>[14,15]</sup> Compared to micron-sized particles, nano-sized particles have shown to possess morphology and crystallinity comparable to dental hydroxyapatite.<sup>[16]</sup> Studies have proven its potential to remineralize early enamel caries.<sup>[9,17,18]</sup> It is also shown to preserve the enamel morphology and prevent the loss of enamel microhardness.<sup>[19]</sup> HA can be produced using natural or synthetic sources. So far, the beneficiary effects of HA have been proven using the synthetically derived form. There is a paucity in the dental literature regarding the role of HA derived from natural sources.

Corals, cuttle fish shell, bovine bone, and eggshell are some of the natural sources of HA, which are otherwise disposed off as biowastes. Chicken eggshell could serve as a raw material for synthesizing HA in a natural and an economical way.<sup>[20]</sup> Kunam *et al.* reported the efficacy of nHA derived from chicken eggshell in combination with 2% sodium fluoride in dentinal tubule occlusion and demonstrated the effective depth of penetration of this combination into dentinal tubules.<sup>[21]</sup> Khoroushi *et al.*<sup>[22]</sup> concluded that incorporation of HA as a remineralizing agent into bleaching gel is effective in decreasing enamel microhardness changes subsequent to in-office bleaching. Moosavi and Hakimi<sup>[23]</sup> conducted a study in which bleaching followed by the application of MI paste, fractional CO<sub>2</sub> laser, or nHA led to an increase in the elastic modulus and hardness of bleached enamel. Gomes *et al.*<sup>[19]</sup> concluded that treatment of enamel with nHA paste prior to bleaching restores the hardness of enamel by minimizing the loss of Ca and P ions and increasing the uptake of F ions. Till date, there are no studies testing the efficacy of nHA derived from chicken eggshell on the microhardness of enamel following bleaching procedure. Hence, the aim of this *in vitro* study was to evaluate the effect of nHA derived from chicken eggshell on the microhardness of bleached human enamel in comparison with commercial CPP-ACP paste using Vickers microhardness test.

## Materials and Methods

### Synthesis of nano-hydroxyapatite powder from chicken eggshell

A simple combustion technique proposed by Sasikumar and Vijayaraghavan for synthesizing nanocrystalline HA powder using chicken eggshell, diammonium hydrogen phosphate [(NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>], and citric acid was adopted in the current study. Eggshells were collected from a local hatchery and boiled in water for 30 min. The shells were dried for 60 min in a hot air oven and blended into a fine powder. The obtained powder was dissolved in concentrated nitric acid (con.HNO<sub>3</sub>), which resulted in the formation of a yellow eggshell solution. Standardized eggshell solution was added to 1 M citric acid. The pH of the solution was adjusted to 9.5 by adding 1:1 NH<sub>4</sub>OH. Adding 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> solution drop wise at 1 ml/min resulted in a white precipitate, which was dissolved by the addition of con.HNO<sub>3</sub>. The solution was stirred at 70°C for 2 h until the formation of a transparent gel. The gel was subjected to combustion in a preheated muffle furnace at 250°C. This resulted in a black-colored precursor, which when sintered at 900°C for 2 h yielded a white-colored pure nanocrystalline HA powder.<sup>[24]</sup> The nHA slurry was prepared by mixing 1.8 g of nHA powder with 0.3 ml of distilled water.

### Specimen preparation

The study protocol was approved by the Institutional Ethical Committee of SRM Dental College (SRMU/MandHS/SRMDC/2011/M.D.S-PG Student/010). Forty extracted noncarious maxillary incisors were collected and stored in distilled water containing 0.2% thymol until use. The teeth were decoronated at the cemento-enamel junction, and the root portions were discarded. The root canal openings were sealed with utility wax. The teeth were positioned in a plastic mold and embedded using a self-curing acrylic resin with the labial enamel surfaces exposed. The enamel surfaces of the teeth were ground into a flat surface using 80-grit silicon carbide papers and polished using 600-, 1200-, and 2400-grit aluminum oxide abrasive papers.

### Baseline microhardness evaluation (T<sub>0</sub>)

Microhardness of the samples was determined using a Vickers microhardness tester (Wilson Wolpert Instruments, Aachen, Germany) fitted with a 300-g load. The indenter was allowed to sink and rest on the enamel surface for 10 s, and the Vickers hardness number (VHN) was determined. Three indentations were performed on each specimen, with a distance of 100 μm between them, and the measurements were averaged. This was taken as the baseline (T<sub>0</sub>) microhardness value (MHV) of the sample. The teeth were randomly divided into four groups based on the remineralizing agent used. No bleaching was done for samples in Group A, whereas Groups B, C, and

D were bleached with 30% HP solution (Thermo Fischer Scientific, Chennai, Tamil Nadu, India), followed by no further treatment in Group B, application of CPP-ACP (GC Tooth Mousse; GC America Inc., USA) in Group C, and nHA slurry in Group D. In Groups B, C, and D, bleaching was done by allowing the HP solution to remain in contact with the labial enamel surface of the samples for 30 min, following which it was rinsed off with distilled water. In Groups C and D, after bleaching, CPP-ACP paste or nHA slurry was applied using a micro brush and allowed to remain on the enamel surface for 2 min. After remineralizing treatment, all the specimens including those of Group A were stored in artificial saliva (Aqwet, Cipla, Mumbai, Maharashtra, India) at 37°C. This procedure was repeated every day for 14 days.

### Final microhardness assessment ( $T_{14}$ )

At the end of 2 weeks, the microhardness of the samples was measured again. This value was taken as the posttreatment ( $T_{14}$ ) MHV of the sample.

### Statistical analysis

The  $T_0$  and  $T_{14}$  MHV were statistically analyzed by one-way ANOVA and Tukey's *post hoc* multiple comparison tests ( $P < 0.05$ ). Descriptive statistics were analyzed using SPSS Statistics V21.0 (IBM, USA).

### Results

The baseline and posttreatment mean MHV and standard deviation of all the groups are summarized in Table 1 and the graphical representation of the same is given in Figure 1. The baseline MHV ranged from  $301 \pm 7.56$  KHV to  $311 \pm 8.38$  KHN, with no significant difference among the groups ( $P > 0.05$ ), indicating uniform distribution of samples between the groups. *Post hoc* comparisons showed that, among the groups at  $T_{14}$ , Group B had a significantly lesser MHV ( $P < 0.05$ ). Independent sample *t*-test showed that no significant difference was seen between MHV at  $T_0$

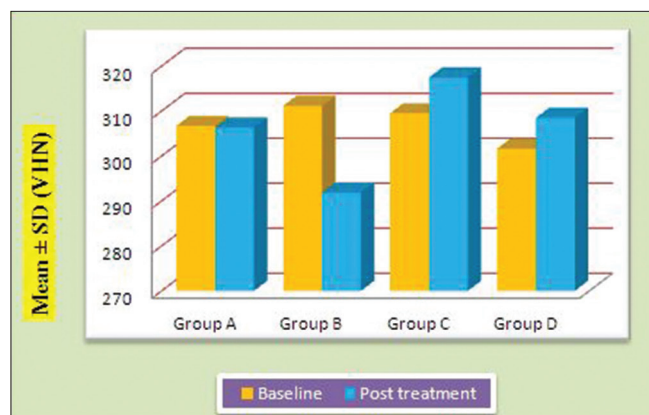


Figure 1: Graphical representation of the mean  $\pm$  standard deviation microhardness value at baseline ( $T_0$ ) and posttreatment ( $T_{14}$ ) of all the groups

Table 1: Mean  $\pm$  standard deviation of baseline ( $T_0$ ) and posttreatment ( $T_{14}$ ) microhardness value of all the study groups

Groups	Baseline MHV ( $T_0$ )	Posttreatment MHV ( $T_{14}$ )
A (no treatment)	306.66 $\pm$ 13.57	306.27 $\pm$ 12.49
B (bleaching only)	311.14 $\pm$ 8.38	291.84 $\pm$ 8.09*
C (bleaching + CPP-ACP)	309.48 $\pm$ 11.41	317.44 $\pm$ 9.66
D (bleaching + nHA)	301.60 $\pm$ 7.56	308.46 $\pm$ 6.67

\*Lowest MHV at  $T_{14}$  and significant difference between  $T_0$  and  $T_{14}$  in Group B ( $P < 0.05$ ). MHV: Microhardness value; CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate; nHA: Nano-hydroxyapatite

and  $T_{14}$  in Groups A, C, and D ( $P > 0.05$ ). A significant decrease in MHV was seen at  $T_{14}$  compared to  $T_0$  in Group B ( $P < 0.05$ ).

### Discussion

Microhardness of enamel varies depending on its degree of mineralization, local variations in its structure resulting from the presence of enamel rods, and tufts or porosities near the dentino-enamel junction.<sup>[25]</sup> The morphology of bleached enamel has been extensively studied. It was observed that bleaching agents demineralize enamel to a depth of up to 50  $\mu$ m. This loss of mineral is evidenced as hardness changes. Hence, microhardness tests are considered as appropriate to evaluate the adverse effects of bleaching agents on enamel.<sup>[26]</sup> In the current study, there was a significant reduction in the microhardness ( $T_{14}$ ) of samples that were subjected to bleaching alone (Group B –  $291.84 \pm 8.09$  VHN). These results coincide with those of various other studies<sup>[12,13,19,22,23]</sup> which have shown that bleached enamel is less harder than normal enamel.

A remineralization system should supply stabilized bioavailable calcium, phosphate, and fluoride ions because these minerals are lost after bleaching.<sup>[27]</sup> In the current study, the application of CPP-ACP after bleaching (Group C) increased enamel microhardness ( $317.44 \pm 9.66$  VHN) significantly when compared to samples that received no additional treatment after bleaching. In comparison to the experimental group, where nHA derived from chicken eggshell ( $308.46 \pm 6.67$  VHN) was applied post bleaching (Group D), CPP-ACP increased the enamel microhardness, though not statistically significant.

CPP in CPP-ACP stabilizes calcium and phosphate ions at the tooth surface in a bioavailable state and prevents them from transforming into a crystalline phase. This reservoir of calcium, phosphate, and fluoride ions released from the nanocomplexes of CPP-ACP diffuse down concentration gradients across demineralized zones and deposit themselves into voids in apatite crystals. This promotes crystal growth in the form of fluoride-containing apatite, thereby achieving remineralization.<sup>[27,28]</sup> Yengopal and Mickenautsch conducted a systematic meta-analysis

and concluded that there is sufficient clinical evidence demonstrating enamel remineralization and caries prevention by regular use of products containing CPP-ACP.<sup>[29]</sup> These results are in accordance with other studies,<sup>[12,13,23]</sup> which also found that enamel mineral loss was significantly reduced when CPP-ACP was applied.

In the present study, the nHA-treated group (Group D) showed a significant increase in enamel microhardness post bleaching ( $308.46 \pm 6.67$ ) compared to the bleaching-only group (Group B –  $291.84 \pm 8.09$ ). There was no significant difference between  $T_{14}$  MHV of samples in CPP-ACP group and nHA group. Chicken eggshell has a high percentage of bioavailable calcium (39%); relevant amounts of Mg, P, and Sr; and low levels of toxic metals such as Pb, Al, Cd, and Hg. This composition makes eggshell an attractive source of calcium.<sup>[30]</sup> Eggshell waste helps in reducing the cost of high-quality calcium source and at the same time promoting the recycle of the material.<sup>[20,24]</sup> Synthesis of nHA from chicken eggshell by combustion method is an economical way of obtaining pure, crystalline nanoscale powder.<sup>[24]</sup>

In our previous study, nHA powder synthesized by this technique was characterized using scanning electron microscopy (SEM) and X-ray diffraction (XRD).<sup>[21]</sup> SEM observations confirmed the nanometric size of the particles which were in the range of 19 to 30 nm. The particles were rod shaped and were present in the form of agglomerated clusters. XRD analysis of the synthesized powder showed a sharp and well-defined peak at  $34.12^\circ$  of  $2^\theta$  values and the Ca/P ratio was 1.67, confirming the presence of pure HA.

Crystals of nHA sediment on to the enamel surfaces and act as a template in the mineral precipitation process. The template further attracts large amounts of  $Ca^{2+}$  and  $PO_4^{3-}$  ions continuously from the remineralizing solution to fill up the defects and micropores on the demineralized enamel surface. This facilitates crystal growth and integrity, thereby promoting remineralization. Studies have shown that the effect of remineralization is increased when the size of the HA particle is reduced to nanometric range.<sup>[18]</sup> An *in situ* study concluded that application of nHA paste prior to bleaching with 35% HP was able to restore the hardness of enamel and does not interfere in bleaching effectiveness.<sup>[19]</sup> Studies have also shown that the use of nHA paste was effective at lowering the incidence and intensity of tooth sensitivity after bleaching.<sup>[31,32]</sup>

The current study results are contradictory to the *in vitro* study conducted by Comar *et al.* where different concentrations of nHA with and without fluoride were used in comparison with a commercial CPP-ACP paste by evaluating cross-sectional hardness on bovine enamel and dentin. There was no significant improvement in microhardness in samples treated with nHA.<sup>[33]</sup> Santos *et al.*<sup>[34]</sup> showed that the application of the nHA paste after the bleaching procedure did not significantly reduce

the enamel loss compared to the unmodified HP group. This may be attributed to the high pH of the paste (8.6). It was shown that an increase of pH from 4.0 to 7.0 resulted in a decrease of calcium and phosphate released from nHA.<sup>[18]</sup> However, the eggshell-derived nHA used in the present study is acidic in nature; hence, an enhanced remineralization effect is seen. Studies have also quoted that low solubility associated with pure HA might not provide enough available Ca and  $PO_4^{3-}$  ions to increase the stability of the HA in the enamel.<sup>[35]</sup> Further studies using nHA derived from chicken eggshell are needed in this regard. Although this study could not simulate the complex oral environment completely, these results could pave way for further research on the use of this naturally sourced nHA as a potential remineralizing agent.

## Conclusion

From the results of this *in vitro* study, it can be concluded that:

1. Bleaching with 30% HP significantly reduces the microhardness of enamel
2. The use of CPP-ACP or nHA restores the lost microhardness of bleached enamel
3. nHA was as effective as CPP-ACP in remineralizing bleached enamel.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

## References

1. Haywood VB. History, safety, and effectiveness of current bleaching techniques and applications of the Nightguard vital bleaching technique. *Quintessence Int* 1992;23:471-88.
2. Borges AB, Yui KC, D'Avila TC, Takahashi CL, Torres CR, Borges AL, *et al.* Influence of remineralizing gels on bleached enamel microhardness in different time intervals. *Oper Dent* 2010;35:180-6.
3. Hegedüs C, Bistey T, Flóra-Nagy E, Keszthelyi G, Jenei A. An atomic force microscopy study on the effect of bleaching agents on enamel surface. *J Dent* 1999;27:509-15.
4. Bitter NC. A scanning electron microscopy study of the effect of bleaching agents on enamel: A preliminary report. *J Prosthet Dent* 1992;67:852-5.
5. Akal N, Over H, Olmez A, Bodur H. Effects of carbamide peroxide containing bleaching agents on the morphology and subsurface hardness of enamel. *J Clin Pediatr Dent* 2001;25:293-6.
6. Sun L, Liang S, Sa Y, Wang Z, Ma X, Jiang T. Surface alteration of human tooth enamel subjected to acidic and neutral 30% hydrogen peroxide. *J Dent* 2011;39:686-92.
7. Borges AB, Yui KC, D'Avila TC, Takahashi CL, Torres CR, Borges AL. Influence of remineralizing gels on bleached enamel microhardness in different time intervals. *Oper Dent* 2010;35:180-6.
8. Reynolds EC. The prevention of sub-surface demineralization of

- bovine enamel and change in plaque composition by casein in an intra-oral model. *J Dent Res* 1987;66:1120-7.
9. Huang SB, Gao SS, Yu HY. Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion *in vitro*. *Biomed Mater* 2009;4:034104.
  10. Reynolds EC, Cai F, Shen P, Walker GD. Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. *J Dent Res* 2003;82:206-11.
  11. Reynolds EC. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: A review. *Spec Care Dentist* 1998;18:8-16.
  12. Poggio C, Grasso N, Ceci M, Beltrami R, Colombo M, Chiesa M. Ultrastructural evaluation of enamel surface morphology after tooth bleaching followed by the application of protective pastes. *Scanning* 2016;38:221-6.
  13. Borges BC, Borges JS, de Melo CD, Pinheiro IV, Santos AJ, Braz R, *et al.* Efficacy of a novel at-home bleaching technique with carbamide peroxides modified by CPP-ACP and its effect on the microhardness of bleached enamel. *Oper Dent* 2011;36:521-8.
  14. Hannig M, Hannig C. Nanomaterials in preventive dentistry. *Nat Nanotechnol* 2010;5:565-9.
  15. Tschoppe P, Zandim DL, Martus P, Kielbassa AM. Enamel and dentine remineralization by nano-hydroxyapatite toothpastes. *J Dent* 2011;39:430-7.
  16. Vandiver J, Dean D, Patel N, Bonfield W, Ortiz C. Nanoscale variation in surface charge of synthetic hydroxyapatite detected by chemically and spatially specific high-resolution force spectroscopy. *Biomaterials* 2005;26:271-83.
  17. Huang S, Gao S, Cheng L, Yu H. Combined effects of nano-hydroxyapatite and *Galla Chinensis* on remineralisation of initial enamel lesion *in vitro*. *J Dent* 2010;38:811-9.
  18. Huang S, Gao S, Cheng L, Yu H. Remineralization potential of nano-hydroxyapatite on initial enamel lesions: An *in vitro* study. *Caries Res* 2011;45:460-8.
  19. Gomes YS, Alexandrino LD, Alencar CM, Alves EB, Faial KC, Silva CM. *In situ* effect of nanohydroxyapatite paste in enamel teeth bleaching. *J Contemp Dent Pract* 2017;18:996-1003.
  20. Sanosh KP, Chu MC, Balakrishnan A, Kim TN, Cho SJ. Utilization of biowaste eggshells to synthesize nanocrystalline hydroxyapatite powders. *Materials* 2009;63:2100-210.
  21. Kunam D, Manimaran S, Sampath V, Sekar M. Evaluation of dentinal tubule occlusion and depth of penetration of nano-hydroxyapatite derived from chicken eggshell powder with and without addition of sodium fluoride: An *in vitro* study. *J Conserv Dent* 2016;19:239-44.
  22. Khoroushi M, Shirban F, Kaveh S, Doustfateme S. Effect of three nanobiomaterials on microhardness of bleached enamel. *Restor Dent Endod* 2016;41:196-201.
  23. Moosavi H, Hakimi N. The effects of fractional CO<sub>2</sub> laser, Nano-hydroxyapatite and MI paste on mechanical properties of bovine enamel after bleaching. *J Clin Exp Dent* 2017;9:e1390-6.
  24. Sasikumar S, Vijayaraghavan R. Low temperature synthesis of nanocrystalline hydroxyapatite from egg shells by combustion method. *Trends Biomater Artif Organs* 2006;19:70-3.
  25. Cuy JL, Mann AB, Livi KJ, Teaford MF, Weihs TP. Nanoindentation mapping of the mechanical properties of human molar tooth enamel. *Arch Oral Biol* 2002;47:281-91.
  26. Featherstone JD, ten Cate JM, Shariati M, Arends J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. *Caries Res* 1983;17:385-91.
  27. Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. *J Dent Res* 2010;89:1187-97.
  28. Reynolds EC. Calcium phosphate-based remineralization systems: Scientific evidence? *Aust Dent J* 2008;53:268-73.
  29. Yengopal V, Mickenautsch S. Caries preventive effect of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP): A meta-analysis. *Acta Odontol Scand* 2009;67:321-32.
  30. Schaafsma A, Pagan I, Hofstede GJ, Muskiet FA, Van Der Veer E, De Vries PJ. Mineral, amino acid, and hormonal composition of chicken eggshell powder and the evaluation of its use in human nutrition. *Poult Sci* 2000;79:1833-8.
  31. Browning WD, Cho SD, Deschepper EJ. Effect of a nano-hydroxyapatite paste on bleaching-related tooth sensitivity. *J Esthet Restor Dent* 2012;24:268-76.
  32. Vano M, Derchi G, Barone A, Genovesi A, Covani U. Tooth bleaching with hydrogen peroxide and nano-hydroxyapatite: A 9-month follow-up randomized clinical trial. *Int J Dent Hyg* 2015;13:301-7.
  33. Comar LP, Souza BM, Gracindo LF, Buzalaf MA, Magalhães AC. Impact of experimental nano-HAP pastes on bovine enamel and dentin submitted to a pH cycling model. *Braz Dent J* 2013;24:273-8.
  34. Santos LF, Torres CR, Caneppele TM, Magalhães AC, Borges AB. Effect of home-bleaching gels modified by calcium and/or fluoride and the application of nano-hydroxyapatite paste on *in vitro* enamel erosion susceptibility. *Acta Odontol Scand* 2016;74:121-6.
  35. Ghiasi B, Sefidbakht Y, Rezaei M. Hydroxyapatite for biomedicine and drug delivery. In: *Nanomaterials for advanced biological applications*. Advanced structured materials, vol.104. Rahmandoust M, Ayatollahi M, editors. Cham, Switzerland: Springer Nature; 2019. p. 85-120.