An investigation in the remineralization and acid resistant characteristics of nanohydroxyapatite produced from eggshell waste via mechanochemistry

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Abstract
Objectives: This study focuses on the properties of nanohydroxyapatite (nHAp) in terms of remineralization and acid resistance. The nHAp were produced from waste eggshells via the mechanochemistry process.

Materials and methods: The characterization was based on Fourier Transform Spectroscopy, X-ray diffraction, Field Scanning Electron Microscope (FESEM), and High-Resolution Electron Microscope to determine the surface morphology of the nHAp. The acid and remineralization properties were evaluated using bovine enamel and dentine models (n = 5) while the buffering properties against acids were studied using a pH meter. The biocompatibility of the produce nHAp was assessed in vitro against NIH 3T3.

Results: The XRD and FTIR results confirm that nHAp were successfully produced from eggshell waste after 5 h of milling. The HRTEM reveals a semi-sphere morphology with an average dimension of 9 to 20nm. The buffering test suggests that nHAp were highly effective in neutralizing common dietary acids. Also, the nHAp exhibits outstanding remineralization and occluding properties. The cytotoxicity assay suggests that the nHAp had a low toxicity.

Conclusion: The study concludes that using eggshell waste to produce nHAp will help in waste management and at the same time, provide valuable biomaterial for the treatment of tooth sensitivity.

Keywords
Acid resistance, nanohydroxyapatite, mechanochemistry, remineralization

Introduction
Dentine hypersensitivity [DH] is a notable public health concern with a severe consequence to individual quality of life such as their social life, psychological status, and or discomfort from pain.1 Several theories have been proposed in the literature in understanding the mechanism of DH actions.2 However, the hydrodynamic theory is widely accepted for the plausible explanation for DH by the dental community.1 The hydrodynamic theory was proposed by Brännström and his co-authors over 40 years ago, and suggest that that external stimuli cause the movement of fluid.
along the dentin tubules which subsequently activates the terminals of the pulp, thereby causing pain.\(^4\) It is therefore assumed that the occlusion of dentin tubules would render the movement of intratubular flows through mechanical principle.\(^5\)

While it is acknowledged in the literature that there is yet to be a gold standard material to effectively manage the condition,\(^6\) nanomaterials, however, offer an innovative and effective approach in the treatment of DH. Moreover, the unique property of nanomaterials like surface to volume ratio, antibacterial action, physical, mechanical, biological characteristics, and distinctive particle size rendered it effective vehicles for dental applications.\(^7\) Hence incorporating nanomaterials (NMs) in commercial toothpaste is seen as a game-changer in the treatment of DH.\(^8\) Over the last few years, nano-hydroxyapatite has been universally accepted in medicine and dentistry field, as one of the most biocompatible and bioactive materials.\(^9\) From the perspective of dentistry, it has been stated in the literature that nanohydroxyapatite (nHAp) has the potential to repair dental enamel,\(^10\) thereby posing the possibility of effective treatment of DH.\(^3\)

Most of the commercially available hydroxyapatite is processed under controlled conditions using precipitation techniques with configurable processing parameters.\(^11\) Another process by which nHAp could be produced is from biological waste such as eggshells.\(^12\) Eggshells are an inexpensive and abundant material containing calcium carbonate in the form of calcite, which is the main component of bones and teeth.\(^13,14\) Globally, it was suggested that approximately 7.7 million tons of eggshell waste were generated in 2014 alone.\(^15\) From an environmental point of view, the majority of waste eggshells produce is not properly handled leading to negative impacts on the environment.\(^16\) Given this concern, using eggshells for the production of nHAp will not only add value to bio-waste which is important for a cleaner and environmentally sustainable products\(^17\) but will also pave way for effective waste management.\(^15\)

In this study, nHAp was produced from eggshells using the top-down approach via the mechanochemistry method. Mechanochemistry, a branch of chemistry, deals with chemical and physicochemical transitions of substances in all aggregation states triggered by the effect of mechanical energy.\(^18\) It is noted that mechanically aided procedures are usually quicker and cheaper than conventional techniques.\(^19\) Another benefit of the mechanochemistry approach is its high reproducibility, consistency, cleanliness and flexibility while eliminating the use of any solvent in most cases.\(^18\)

Despite the enormous potential and environmental benefits of mechanochemical produce materials, however, there seems to be little evidence to its application for nHAp production. This study, therefore, reports on remineralization and acid-resistant characteristics of nHAp produce from eggshell waste via the mechanochemistry.

### Materials and methods

#### Preparation of nanohydroxyapatite

The collected eggshells were prepared following the steps described by Onwubu et al.\(^13\) The prepared eggshells were calcined in a furnace at 900°C for 3 h at a heating rate of 3°C/min to obtain a snow-white powder. In the preparation of nHAp, 20 g of the white powder formed after calcination was mixed 13.4 g of sodium tripolyphosphate according to the stoichiometric ratio of 1:5. The mixed were wet-milled in a 100 mL deionization water using a planetary milling ball (Retsch® PM 100) at 500 rpm for 5 h. The milling parameters include 30 stainless steel balls of 10 mm diameter in a 250 mL bowl. After milling, the mixture was centrifuged at speed of 1000 rpm for 30 min. The resulting powder was oven dried for 5 days at 40°C. The white powder obtained was subsequently characterized to establish the formation of nHAp.

#### Characterization

**Fourier transform infrared spectroscopy analysis.** The functional group present in the prepared nHAp were identified using FTIR (Perkin Elmer Universal ATR). An initial background check was carried out before scanning. Thereafter, a few quantities of the nHAp were dropped in the sample holder and scanned at a resolution of 4 cm\(^{-1}\). The range of scan was 400 to 4500 cm\(^{-1}\).

**X-ray diffraction analysis.** The crystallinity of the prepared nHAp was studied using X-ray diffraction (XRD). The XRD patterns were documented using a diffractometer (D8 Advance BRUKER AXS instrument Germany instrument; Cu-Ka radiation (\(lKa1 = 1.5406\) Å). The operating condition includes a pass time of 0.5 s, a voltage of 40 Kv, and current of 40 mA. The range of analysis was 0 to 90 (2\(^\theta\)).

**High-resolution transmission electron microscopic analysis.** The morphology of the prepared nHAp was studied using a high-resolution transmission electron microscope (HRTEM; Philips CM 120 model) operating at 120kV. Small amounts of nHAp were scattered in 10 mL ethanol and sonicated at 10 K v for 10 min before HRTEM observation. A cryo-microtomed samples were subsequently formed with a Leica microtome (South Africa), positioned on 100 mm by 100 mm carbon copper grids.

**pH and buffering test**

The anti-acid properties of the eggshell, calcined eggshells, and nHAp powder were studied using citric and maleic acid, respectively. A stock solution of citric acid (2.1022 g) and maleic (1.1607 g) was prepared by dissolving in a 100 mL volumetric flask. The solution was constantly agitated at a low speed of 600 rpm for 5 min. The
changes in pH were recorded using a pH meter. For the pH, 1 g of the powders were dissolved in 30 mL deionized water. The pH was read after 1 min.

**Acid resistance test and remineralization test**

The acid resistance and remineralization properties of the eggshells, calcined eggshells, and nHAp were studied using bovine tooth enamel (Table 1). The tooth enamel was placed in a 4% citric acid solution containing each of the respective powders for 2 min and thereafter rinsed with deionized water and blot dried. For the remineralization test, the dentine specimens used were prepared according to Onwubu et al., and then assigned randomly into a group of five (n = 5). Using 1 g of the sample powder and 40 mL deionized water at a time interval of 5 min, the specimens were agitated following the protocol described by Onwubu et al. The image of the specimens before and after treatment and exposure were studied using SEM.

**Field-scanning electron microscope observation.** The surface morphology and elemental composition of the prepared nHAp were studied using a scanning electron microscope (FESEM) equipped with energy-dispersive X-ray spectroscopy (Field Emission-Carl Zeiss). The instrument was operated at controlled atmospheric conditions at 20 kV. The surface of the sample was gold for 30 min prior to observation FESEM observation to prevent the build-up of electrostatic charge.

**Cytotoxicity of nHAp**

Using the MTS endpoint assay (3-(4,5-dimethylthiazol-2-yi)-5-(3-carboxymethoxy-phenyl)-2(4-sulfophenyl)-2H-tetrazolium) the cytotoxicity of nHAp was assessed in vitro against NIH 3T3 (mouse fibroblast). The cells were incubated and tested in accordance with the procedure described by Onwubu et al. In this procedure, the NIH 3T3 cells were left to incubate for 4 days, whereupon MTS (5 µL) was added to the cells. The absorbance values were measured at 490 nm after 1, 2, and 4 h incubation periods, averaged and the viability curves are drawn up. The cells (1 × 105 cells/mL) were incubated in 96 well plates at 37°C overnight, with the subsequent addition of the supplied compounds, in concentrations of (100, 50.0, 25.0, 12.5, 6.25, 3.125, and 0 µg/mL. Auranofin was used as a negative control. The samples were tested across three plates in duplicate (n = 6) and the average value reported.

**Results**

**Characterization of the nHAp**

The FTIR spectra of the prepared nHAp, eggshell powder, and calcined eggshell powder are given in Figure 1. For the eggshells spectrum (Figure 1(a), the wide absorption band, which appeared around 1411 cm⁻¹, is attributed to the asymmetrical and symmetrical stretching of calcium carbonate (CO₃²⁻) vibration in eggshell powder. In addition, the spectra show the absorption bands that appeared around 711 and 873 cm⁻¹ correspond to calcite. While the carbonate band was present in the eggshell calcined (Figure 1(b), the intensity was however lowered. Added to this, a visible absorption band at 3450 cm⁻¹ corresponding to the O–H bending found in the calcined eggshell is indicative of the presence of calcium hydroxide. By contrast, and as shown in Figure 1(c), the hydroxyapatite characteristics bands were observed for the nHAp. For instance, the absorption band observed around ~1000 cm⁻¹ is attributed to the P–O asymmetrical stretching of a PO₄³⁻ group. The hydroxyl O–H bending identified by the broad band around ~3450 and 3000 cm⁻¹ and sharp band at 1600 cm⁻¹ was attributed to water molecules, and the sharp band around 3750 cm⁻¹ is attributed to the OH stretching. In addition, the bands at 1450 cm⁻¹ suggest that the presence of the CO₃²⁻ ions in the structure of the produced nHAp. The plausible explanation for the carbonate peak found in the nHAp may be from the substitution of the PO₄³⁻ ions in the nHAp structure.

Figure 2 displays the XRD models of nHAp packed, eggshell powder and calcinated eggshell powder. Figure 2(a)
confirms the presence of calcite in the ovarian powder at characteristic peaks indicated around 35° (2θ). On the other hand, and as seen in Figure 2(b), the structure of calcined eggshells changes after calcination at 900°C. The calcium carbonate intensity decreased, but then another new peak, identified at around 40° (2-degree), was generated. The new peak might have resulted from the breakdown of the carbon carbonate constituents of eggshell powder into calcium oxide. For the XRD pattern of nHAp observed in Figure 2(c), the diffraction angles marked at 19.3°, 27.0°, 29.7°, 34.0°, and 48.8°, suggest the formation of nHAp, and it is confirmed by the international standard (JCP2-76-0694). Moreover, the crystalline peak observed at 35° correlates to the calcium carbonate calcite phase. Overall, the synthesized nHAp has a crystallinity value of 82.19%. The peak area was determined from the Gaussian plot and the crystallinity calculated using the formula below.

\[
\text{Crystallinity} = \frac{\text{Area of crystalline peak}}{\text{Area of all peaks}} \times 100
\]

The HRTEM image of the prepared nHAp is shown in Figure 3. A semi-sphere like morphology was observed for the nHAp particles. It was also seen that the particles of the prepared nHAp assembled into short chains that bind into clusters with a dimension of 9 to 21 nm. This is also in agreement with Nuamsrinuan, Kaewwiset, Limsuwan and Naemchanthara. An irregular-red like structure with a slightly higher particle size of 80 to 100 nm, were, however, observed in another study. The difference in the morphology observed in both studies may be attributed to the preparation technique.

**Acid resistance and remineralization assessment**

The mean pH of the sample groups is given in Figure 4. The pH results suggest that nHAp (13.94) had the highest mean pH value, while the lowest was measured for eggshells (10.07). All the sample group tested have strong alkaline characteristics which could be ascribed to the elevated calcium content in the samples.

The buffering characteristics of eggshells, calcined eggshells, sodium triphosphate, and nHAp against citric and maleic acid are given in Figure 5. The results suggest that nHAp had the best buffering characteristics as it effectively neutralizes the pH of both citric and maleic acids.

As depicted in Figure 6, there was a noticeable difference in the enamel surface of the tooth specimens exposed to citric acid alone (Figure 6(b)), and in the presence of nHAp (Figure 6(c)). In Figure 6(b), for example, enamel dissolution and destruction of the prismatic structure were evident. In contrast, the FESEM image in Figure 6(c) suggest that nHAp offers effective protection against the acid dissolution. It was observed that citric acid had minimal effect on the enamel surface integrity in the presence of nHAp when compared with the image of the unexposed specimen in Figure 6(a).

To help establish the remineralization properties of the prepared nHAp against the calcined eggshells, eggshells, and
sodium triphosphate, the morphological changes of the dentine tubule occlusion after agitation treatment were studied by FESEM. While partial occlusion was observed for the samples treated with eggshells (Figure 7(b)), and sodium triphosphate (Figure 7(d)), the samples treated with calcined eggshell powder (Figure 7(c)), showed no occluding characteristics. In contrast, the samples treated with nHAp shows complete remineralization of the dentine tubules (Figure 7(e)).

**Biocompatibility assessment**

The cytotoxicity of the prepared nHAp is given in Figure 8. The result revealed that the NIH 3T3 mouse fibroblast cells displayed 100% cell viability against all the different nHAp concentration (µg/mL). This suggests that nHAp exhibits little or no cytotoxicity on the cell nucleus of the mouse fibroblast that was tested.

**Discussion**

Nanohydroxyapatite is widely incorporated in toothpaste to promote enamel remineralization as well as treat dentine hypersensitivity. This study reports on remineralization and acid-resistant characteristics of nHAp produce from eggshell waste via the mechanochemistry. The results suggest that the nHAp had a good buffering characteristic against acids (Figure 5). This is highly important given that enamel demineralization from acidic substances has become a significant concern in the public health sector with the increased intake of erosive beverages that contain citric acids. Moreover, citric and maleic acids are the most common ingredients in soft and fruit juice that is consumed in our daily lives. Hence, the buffering characteristics of nHAp against citric and maleic acids suggest that it could effectively neutralize both citric and maleic acids which are responsible for tooth demineralization, and, thus, offer high bioavailable calcium essential for remineralization.

Furthermore, and given the negative impact of dietary acids on tooth enamel structural integrity, citric acids of pH 2 was used to simulate and evaluate the acidic resistance of nHAp on dietary acid conditions. In support of the buffering characteristics of nHAp in Figure 5, the FESEM image in Figure 6(c) suggest that nHAp offers effective protection against the acid dissolution. It was observed that citric acid had minimal effect on the enamel surface integrity in the presence of nHAp. Further to this, the image in Figure 6(c) suggests that there was a form of enamel remineralization. This may likely have resulted from the buffering abilities of nHAp thereby hampering the dissolution of the enamel. This confirms the speculation made by Tschoppe et al. that the higher pH values of the nHAp slurries increased remineralization.

Equally essential, the dentine tubule occluding properties observed for the samples treated with nHAp, for example, was highly superior to the calcined eggshells (Figure 7(c)). This may likely be attributed to the solubility of the calcined eggshell in the deionized water, and or the absence phosphate group. On the contrary, the samples treated with nHAp shows complete remineralization of the dentine tubules (Figure 7(e)). This is in agreement with several randomized clinical trials that confirmed the remineralization characteristics of nHAp.

Two possible scenarios could be used to explain the remineralization potential of nHAp. First of all, it is assumed that the nHAp serves as the reservoir in this remineralization phase to preserve the topical condition of the supersaturation continuously, at the attraction of large amounts of calcium (Ca²⁺) and phosphate ions (PO₄³⁻). According to Yu et al., this process promote crystal growth and deposition. The second plausible explanation for the outstanding tubule occlusion observed for the nHAp samples may be attributed to the small size of the particles (Figure 3).

It is reported in the literature that the small size of nHAp combined with the large surface proportion of their atomic number may allow them to readily combine with other atoms.
Figure 6. FESEM image of tooth enamel. (a) Unexposed tooth. (b) Exposed in citric acid. (c) Exposed in citric acid + nHAp.

Figure 7. Showing SEM images of dentin. (a) Before treatment. (b) Treatment with eggshells. (c) Treatment with calcined eggshells. (d) Treatment with sodium triphosphate. (e) Treatment nHAp.
Although nHAp had enormous and enviable properties, their biocompatibility with natural tissues should be of utmost interest for the acceptability of its use in consumer products. Accordingly, the cytotoxicity of the prepared nHAp was studied using MTT cell-culture. Fotakis and Timbrell\textsuperscript{34} note that in in vitro toxicologist research, cytotoxicity assays are commonly used. As highlighted in the ISO 10993-5 standard,\textsuperscript{35} it is considered toxic when the cells’ viability is below 70%. In this paper, prepared nHAp dispersed into water and seemed to have a marginal effect even at the maximum concentration of 100 $\mu$g/mL on the cell nucleus (Figure 8). Similar findings were also observed reported by Coelho and his team who found no toxicity effects in their study on commercially available nHAp using living/dead staining assay.\textsuperscript{36} Corroborating with Opris et al.,\textsuperscript{37} it can, therefore, be assumed that the prepared nHAp will be suitable for use in commercial products given its low toxicity.

**Conclusion**

In summary, the finding from this study suggests nHAp was successfully produced via an environmentally friendly technique. The XRD and FTIR characterization of nHAp confirmed the presence of hydroxyapatite structure. Drawing from the study outcome, it is reasonable to speculate that the produce nHAp will be highly effective as an ingredient in toothpaste formulation for enamel remineralization as well as the potential treatment of tooth sensitivity. Although the produce nHAp had low toxicity making it viable for oral health use, it is, however, important to note that intra-oral environment is complex and may present a different reaction compared to the static in vitro assessment used in the present study. Hence and Vivo experiment is proposed to further assess the cytotoxicity profile of the produce nHAp. More so, the present study did not take into account other oral conditions such as the role of saliva and tooth pellicle in the demineralization and remineralization cycle. Future studies will, therefore, aim to mimic these conditions to approximate clinical setting.

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**Author contributions**

S.C.O. developed the research idea and analyzed the data. D.N., S.C.M., and N.L.N.M. were involved conducting the experimentation and literature review search. P.S.M. and S.T. supervised the research and helped in data analysis. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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**References**

1. Bekes K and Hirsch C. What is known about the influence of dentine hypersensitivity on oral health-related quality of life? *Clin Oral Investig* 2013; 17: 45–51.
2. Mantzourani M and Sharma D. Dentine sensitivity: past, present and future. *J Dent* 2013; 41: S3–S17.
3. de Melo Alencar C, de Paula BLF, Ortiz MIG, et al. Clinical efficacy of nano-hydroxyapatite in dentin hypersensitivity: a systematic review and meta-analysis. *J Dent* 2019; 82: 11–21.
4. Brännström M, Lindén L and Åström A. The hydrodynamics of the dental tubule and of pulp fluid. *Caries Res* 1967; 1: 310–317.
5. Sykes L. Dentine hypersensitivity: a review of its aetiology, pathogenesis and management: clinical. *S Afr Dent J* 2007; 62: 66–71.
6. Schmidlin PR and Sahrmann P. Current management of dentin hypersensitivity. *Clin Oral Investig* 2013; 17: 55–59.
7. Bapat RA, Joshi CP, Bapat P, et al. The use of nanoparticles as biomaterials in dentistry. *Drug Discov Today* 2019; 24: 85–98.
8. Yu J, Yang H, Li K, et al. A novel application of nano-hydroxyapatite/mesoporous silica bio composite on treating dentin hypersensitivity: an in vitro study. *J Dent* 2016; 50: 21–29.
9. Hannig M and Hannig C. Nanomaterials in preventive dentistry. *Nat Nanotechnol* 2010; 5: 565.
10. Gjorgievska ES, Nicholson JW, Slipper IJ, et al. Remineralization of demineralized enamel by toothpastes: a scanning electron microscopy, energy dispersive X-ray analysis, and three-dimensional stereo-micrographic study. *Microsc Microanal* 2013; 19: 587–595.

11. Zhou H and Lee J. Nanoscale hydroxyapatite particles for bone tissue engineering. *Acta Biomater* 2011; 7: 2769–2781.

12. Nuamsrinuan N, Kaewwiset W, Limsuwan P, et al. Hydroxyapatite synthesized from waste eggshell via ball milling. *Appl Mech. Mater* 2017; 12–16.

13. Onwubu SC, Vahed A, Singh S, et al. Physicochemical characterization of a dental eggshell powder abrasive material. *J Appl Biomater Funct Mater* 2017; 15: 341–346.

14. Cree D and Rutter A. Sustainable bio-inspired limestone eggshell powder for potential industrialized applications. *ACS Sustain Chem Eng* 2015; 3: 941–949.

15. Ronan K and Kannan MB. Novel sustainable route for synthesis of hydroxyapatite biomaterial from biowastes. *ACS Sustain Chem Eng* 2017; 5: 2237–2245.

16. Abdulrahman I, Tijani HI, Mohammed BA, et al. From garbage to biomaterials: an overview on egg shell based hydroxyapatite. *J Mater* 2014; 2014: 1–7.

17. Faridi H and Arabhosseini A. Application of eggshell wastes as valuable and utilizable products: a review. *Res Agric Eng* 2018; 64: 104–114.

18. Baláž M. Ball milling of eggshell waste as a green and sustainable approach: a review. *Adv Colloid Interface Sci* 2018; 256: 256–275.

19. Cova CM and Luque R. Advances in mechanochemical processes for biomass valorization. *BM Chem Eng* 2019; 1: 1–16.

20. Onwubu SC, Mhlungu S and Mdluli PS. In vitro evaluation of nanohydroxyapatite synthesized from eggshell waste in occluding dentin tubules. *J Appl Biomater Funct Mater* 2019; 17: 1–6.

21. Onwubu SC, Mdluli PS, Singh S, et al. A novel application of nano eggshell/titanium dioxide composite on occluding dentine tubules: an in vitro study. *Braz Oral Res* 2019; 33: 1–9.

22. Farzadi A, Bakhshi F, Solati-Hashjin M, et al. Magnesium incorporated hydroxyapatite: synthesis and structural properties characterization. *Ceram Int* 2014; 40: 6021–6029.

23. Kamalanathan P, Ramesh S, Bang LT, et al. Synthesis and sintering of hydroxyapatite derived from eggshells as a calcium precursor. *Ceram Int* 2014; 40: 16349–16359.

24. West N, Seong J, Hellin N, et al. A clinical study to measure anti-erosion properties of a stabilized stannous fluoride dentifrice relative to a sodium fluoride/triclosan dentifrice. *Int J Dent Hyg* 2017; 15: 113–119.

25. Macri DV. Implementing a minimally invasive approach. *Dimens Dent Hyg* 2016; 14: 32–37.

26. Jain P, Hall-May E, Golabek K, et al. A comparison of sports and energy drinks–physiochemical properties and enamel dissolution. *Gen Dent* 2012; 60: 190–197.

27. Tschoppe P, Zandim DL, Martus P, et al. Enamel and dentine remineralization by nano-hydroxyapatite toothpastes. *J Dent* 2011; 39: 430–437.

28. Browning WD, Cho SD and Deschepper EJ. Effect of a nano-hydroxyapatite paste on bleaching-related tooth sensitivity. *J Esth Restor Dent* 2012; 24: 268–276.

29. Vano M, Derchi G, Barone A, et al. Effectiveness of nano-hydroxyapatite toothpaste in reducing dentin hypersensitivity: a double-blind randomized controlled trial. *Quintessence Int* 2014; 45: 1–9.

30. Vano M, Derchi G, Barone A, et al. Reducing dentine hypersensitivity with nano-hydroxyapatite toothpaste: a double-blind randomized controlled trial. *Clin Oral Investig* 2018; 22: 313–320.

31. Huang S, Gao S, Cheng L, et al. Remineralization potential of nano-hydroxyapatite on initial enamel lesions: an in vitro study. *Caries Res* 2011; 45: 460–468.

32. Hannig M and Hannig C. Nanotechnology and its role in caries therapy. *Adv Dent Res* 2012; 24: 53–57.

33. Besinis A, van Noort R and Martin N. Infiltration of demineralized dentin with silica and hydroxyapatite nanoparticles. *Dent Mater* 2012; 28: 1012–1023.

34. Fotakis G and Timbrell JA. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 2006; 160: 171–177.

35. International Standard Organization. *Biological evaluation of medical devices – part 5: tests for in vitro cytotoxicity*. 2017. ISO Geneva, Switzerland.

36. Coelho CC, Grenho L, Gomes PS, et al. Nano-hydroxyapatite in oral care cosmetics: characterization and cytotoxicity assessment. *Sci Rep* 2019; 9: 1–10.

37. Opris H, Bran S, Dinu C, et al. Clinical applications of avian eggshell-derived hydroxyapatite. *Bosn J Basic Med Sci* 2020; 20(4): 430–437.