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BIOMIMETIC HUMAN ENAMEL REMINERALIZATION USING CALCIUM EXTRACT FROM NANO FISH BONE AND EGGSHELL: AN IN VITRO STUDY

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ABSTRACT

AIM: The study aimed to investigate the remineralizing potential of two freshly prepared organic biomimetic waste nanomaterial [fish bone (FB) and chicken eggshell (ES)] solutions on partially demineralized human enamel surface using Energy dispersive X-ray spectroscopy (EDX) for quantitative Ca/P assessment.

The study aimed to investigate remineralizing potential of two organic biomimetic waste nanomaterials [fish bone (FB) and chicken eggshell (ES)] on demineralized enamel using Energy Dispersive X-ray spectroscopy (EDX) for quantitative calcium/ phosphate assessment. A total of 34 freshly extracted human 3rd molars were collected to be used. Thirty teeth were divided into three equal groups (n=10). **Group1:** Teeth represented the control group as a reference for the assessed normal quantitative content of calcium and phosphorus. **Group2:** Teeth were immersed in a freshly prepared demineralizing solution for 96 hours, then investigated by EDX. Afterword, those group 2 teeth were again soaked in a freshly elaborated remineralizing fish bone nanoparticle solution for 189 hours, then reevaluated by EDX. **Group3:** Teeth were demineralized with the same regime as group 2 teeth, then remineralized in a freshly prepared ES nanoparticle solution for seven days to be again reassessed by EDX. In addition, the remaining four human teeth were assessed by a scanning electron microscope (SEM); where 1 sound enamel surface was scanned, a second tooth was SEM investigated following demineralization regime, a third one was SEM assessed after remineralization with FB nanoparticle solution and fourth one scanned after ES nanoparticle remineralization. Paired sample t-test and independent t test statistical analysis were used. Freshly prepared nano FB and ES solutions seem promising for remineralization of incipient enamel lesions.

KEYWORDS: Biomimetic, Human enamel, Demineralization, nanoparticles, Eggshell, Remineralizing Solution, Fish Bone.

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INTRODUCTION

The concept of minimally invasive dentistry recently dictate essential need for effective modalities to clinically remineralize early human enamel demineralized lesions [1, 2]. New non-fluoride remineralization strategies were broadly categorized into certain approaches that repair carious defects by enhancing fluoride anticariogenic efficacy and enamel biomimetic regenerative technologies [3, 4]. Cariology allows prevention of dental caries and controls it in its initial stages that primarily cause demineralization of hard tooth tissues. However caries is actually a multifactorial teeth disease, stopping and even reversing its pathogenesis as early as possible is a main goal for recent studies to reduce risk of tooth cavitation and subsequent operatory clinical tooth intervention [4- 6]. Evidently, calcium phosphate hydroxyapatite (CaPHA) is the inorganic fundamental for formation of human teeth and bones. In addition, mineral tissue concentrations determine elastic moduli and strength of hard tissues. Therefore, high mineral content provides rigidity and stiffness of teeth that are subjected to complex masticatory stresses. Clinically, the first sign of enamel caries develops as a white spot lesion that is characterized by an opaque apparently intact outermost surface covering a partially demineralized subsurface [6- 9].

However fluoride products were effective in enamel remineralization, they lacked the potential to stimulate formation of organized tooth apatite crystals [1,10]. Consequently, biomimetic remineralization was an attempted therapy to shift from tooth reparative concept to regenerative hard tissue biomineralization, where the demineralized dental structures were replaced by biologically similar ones [3, 11]. Unlike dentine or bone, particular enamel regeneration seems however challenging; as the mature acellular enamel never remodels itself or resorbs. Advances in tissue engineering have recently yielded biomimetic materials that have demonstrated potential effect for regenerating the hierarchy of enamel microstructure [12-15].

Recently, extraction of CaPHA from sustainable natural primary sources seems reliable, highly productive and feasible alternative for synthetic HA[16]. Those CaP food waste reservoirs were mainly bones (of fishes and mammalians [17-19] and biogenic (BioHA) resources (eggshell [20], seashell and whelk [21]. Fish bones revealed rich content of calcium, phosphate, and carbonate; which advocate it as reliable natural sources for production of a predictable biocompatible HA [21-22]. Chicken eggshell extract represents a marvelous source for a cost effective HA due to munificent content of Ca oxides and carbonates [23, 24]. Evidently, nano-biomaterials reported more abundant Ca, fluoride (F) and P ion release profiles compared to that of micro-particles for remineralization of hard dental structures [25, 26].

Therefore, evaluation for the E remineralization potential of tentative elaborated FB and ES nano-biomimetic solutions was a crucial feasible issue to be studied.

MATERIALS AND METHODS

Ethical Aspects

An unconditional approval (Approval number: # REC-FDBSU/03112022-05/EM, Date: 10/2022) was received from Faculty of Dentistry Beni-Suef University Research Ethics Committee (FDBSU-REC), Egypt.

Study Design

A randomized ex vivo laboratory study was conducted. According to sample size calculation of the study, the calculated study sample size was based on a level of precision of ± 5 [27]. A total of thirty four freshly extracted human third molars were collected from oral surgery clinic, Faculty of Dentistry, Beni-Suef University were used in the

study. Thirty teeth were divided into three equal groups where the number of each sample was ten, i.e. (n=10). **Group1:** Teeth represented the control group as reference for the assessed normal quantitative content of calcium (Ca) and phosphorus (P). **Group2:** Teeth were immersed in a freshly prepared demineralizing solution for 96 hours (i.e. four days), then investigated by EDX. Afterword, those group 2 teeth were again soaked in a freshly elaborated remineralizing fish bone nanoparticle solution for 189 hours (i.e., seven days), then reevaluated by EDX (JEOL Ltd., Japan). **Group3:** Teeth were demineralized with the same regime as group 2 teeth, and then remineralized in a freshly prepared chicken eggshell nanoparticle solution for seven days to be reassessed by EDX.

In addition, the remaining four human teeth were assessed by a scanning electron microscope [SEM; (ZOGEAR, Shanghai, China)]; where one sound enamel (E) tooth surface was scanned without any intervention, a second tooth was SEM investigated following the demineralization regime, a third one was SEM assessed after remineralization with fish bone nanoparticle solution and fourth one scanned after the eggshell nanoparticle remineralization.

Nano FB Powder Remineralizing Solution Preparation

Nile Tilapia fish bones were bought from local market, cleaned from organic debris by dipping it in boiling water and left to completely dry at room temperature. Then, FB were calcined for five hours in an electric furnace at 900 °C and left to cool 15 minutes (Faculty of Dentistry, AI -Azhar University, www.dent.azher.edu.eg/) [28]. The FB ashes were ground, sieved to fine powder (100-250 μm, brown color) and collected in a sterile container to be send to a company (Nano Gate; 25 Ibrahim Abou El Naga St., Abbas El Akkad extension, beside ENPPI, Nasr City) that refined the particles to nanoscale (white-yellowish color).

Obtained FB nanoparticles were analyzed with EDX for percentage of elemental composition, while the particle size and shape of the powder was analyzed by SEM (X 400 magnification). One gram of the elaborated Nano calcined FB powder was dissolved into 33.3 mL of deionized sterile water and the clear fluid on the top was collected to be used as a freshly prepared 3% FB remineralizing solution [29].

Nano ES Powder Remineralizing Solution Preparation

Chicken eggshells were washed with distilled water, soaked in 100 °C boiling water for 10 minutes to remove the membrane [30], dried at room temperature and homogenously ground in a sterile mortar and pestle. Calcined ES organic powder was obtained by Tangboriboon [0] process; by heating the ES particles at 900 °C in a laboratory furnace (Kerr Corporation, USA) for one hour (Faculty of Dentistry, AI -Azhar University, www.dent.azher.edu.eg/). Prepared ES fine powder was transformed to nanoparticles by a company (Nano Gate; 25 Ibrahim Abou El Naga St., Abbas El Akkad extension, beside ENPPI, Nasr City). Elaborated ES particles were analyzed by EDX for percentage of elemental composition and by SEM (X400 magnification) the particle size. A 3% ES remineralizing solution was prepared by blending of one gram ES Nano calcined powder in 33.3 mL of deionized sterile water and on the top of the mix the clear solution was collected for used [29)].

Teeth Preparation

The 34 selected teeth were cleaned from any hard or soft debris using an ultrasonic scaler (WoodPeaker, Proxy; Ivoclar Vivadent, Schaan, Lichtenstein), then careful enamel (E) examination was performed using a magnifying eyeglass to exclude any cracks, white spot lesion, caries, hypoplasia, or other enamel defects. Throughout the study steps, those specimens were individually

placed in ready-made artificial saliva and stored at 37°C in an incubator (C.B.M. S.r.l. Medical Equipment, Italy). In the middle-middle third of buccal enamel surface of each tooth specimen a square window (3 × 3 mm) was drawn. Furthermore, rest of specimen was double coated by a nail varnish (Florelle Nail polish; Milan Italy, www.florelle.it) and was wrapped by a tin foil after drying [27]. Afterword, prepared specimens were stored in separate test tubes containing a freshly prepared artificial saliva [pH 6.57; prepared by blending of: Sterile deionized water 99.6 %, Potassium chloride 0.12%, Sodium chloride 0.08%, Magnesium chloride 0.01%, Carboxy-methyl cellulose 0.10%, Dibasic potassium phosphate 0.03% and Calcium chloride 0.01%] (Faculty of Pharmacy, Alexandria University, www.pharmacy.alex.edu.eg) for 48 hours at 37°C in the incubator.

Demineralization Regimen

Group 2 and 3 teeth specimens were removed from artificial saliva, and they were individually immersed in test tubes containing a freshly prepared demineralizing (daily changed to prevent saturation, pH= 4.2; 2.2mMol calcium chloride, 0.05 M, acetic acid 2.2 mMol sodium phosphate and 1 M potassium hydroxide) (Faculty of Pharmacy, AI -Azhar University, http://www.pha.azher.edu.eg/)[32] solution at 37°C for 96 hours. Afterword, specimens were removed, rinsed for 5 seconds with distilled water to stop the effect of demineralizing solution and stored again in artificial saliva at 37 °C incubator until subsequent study step.

Remineralization Regimen

Group 2 and 3 demineralized specimens stored in artificial saliva were removed and they were again separately immersed into their respective freshly prepared E remineralizing solution [pH 7; 1.5 mMol calcium chloride, 0.15 mMol potassium chloride and 0.9mMol sodium phosphate, (Faculty of Pharmacy, Tanta University, http://www.pha.tanta.edu.eg/)] (group 2 for FB and group 3 for ES) for 12 min. in an incubator at 37 °C once a day. Afterword, those specimens were removed, rinsed for 5 sec. with distilled water and incubated for the rest of the day in artificial saliva at 37 °C. The remineralization regimen of specimens was daily repeated for 7 consecutive days using freshly prepared FB and ES solution (for group 2 and 3 respectively) [28].

Specimens EDX Analysis

Following the study design and in each group, EDX quantitative evaluation of Ca and P content for teeth specimens was performed.

Specimens SEM Examination

After each specimen EDX analysis, study tooth was washed with distilled water, gently dried, aluminum mounted and enamel structure was qualitatively SEM examined for topography (X400 magnification) at 20 Kv.

Statistical Analysis

Collected EDX data throughout the study steps were tabulated to be statistically analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) at a significant p-value level of ≤ 0.05. Paired Sample t-test statistical analysis was used for the difference between Ca and P level of teeth within group 2 and 3 after remineralization of demineralized enamel surface. An independent t-test was implemented to compare remineralization potential of FB versus ES.

RESULTS

Actually, the pH of used freshly prepared E remineralizing solution was daily rechecked and their measured average values were 9.09 and 9.48 for FB and ES; respectively. That pH showed remarkable alkalinity of both E remineralizing solutions. The mean Ca value in FB and ES was

12.9% and 50.34%; respectively. Furthermore, the mean P value in FB and ES was 16.32% and 0.27%; respectively. In demineralized E, the mean Ca value was 0.33% and that of P was 2.16% (Figure1 c, d, e, f, g and 2).

Obtained EDX data for normal human sound enamel (group1), initially demineralized enamel, nano-FB powder, nanoES particles, FB remineralized enamel (group2) and ES remineralized enamel (group3) was pertained to Ca and P mineral content percentage (Table1, Figure1b, d, f, h, j and l). Statistical EDX analysis showed that mean values ±SD of normal enamel Ca was 40.4% and that of P was 17.7% (group1) and revealed obvious significant differences among all recorded mean values for all groups (p= 0.000).

Paired sample t-test revealed significant difference between Ca and P level of remineralized teeth E after demineralization regime within groups (P-value=0.000); as the ES potential for Ca remineralization was significantly higher than that of FB. However, the efficacy of P remineralization in FB and ES was 17.23% and 20.1%; respectively, (Table1, Figure1b, d, f, h, j and l). Independent sample t-test again showed a significantly higher difference of remineralization efficacy in ES than that in FB (P-value=0.000); (Table2, and Figure1b, d, f, h, j and l).

However, the SEM micrographs of human sound enamel showed a smooth aprismatic layer, those of initially demineralized E crystals revealed prominent variable patterns of E prisms dissolution involving body, tail, and inter-prismatic substance; (Figure1 a, c, e, g, i and k). Furthermore, SEM micrographs of ES showed enamel remineralization patterns more than those achieved by FB extract; (Figure1 e, g, i and k).

TABLE (1) Paired sample t-test of the difference between Ca and P level of remineralized teeth enamel after demineralization regime within groups.

Groups	Normal E (Group1)	Fish Bone (Group2)			Eggshell (Group3)		
	Mean	Mean	±SD	P-value	Mean	±SD	P-value
Ca Level	40.4	38.5	2.26175	0.000*	42.73	1.41805	0.000*
P Level	17.7	17.23	0.59373	0.000*	20.1	0.86474	0.000*

SD; i.e. Standard Deviation ****Significant P≤ 0.05***

TABLE (2) Independent sample t-test of the remineralization efficacy between fish bone versus eggshell.

Groups	Ca Level			P Level		
	Mean Difference	Std. Error Difference	P-value	Mean Difference	Std. Error Difference	P-value
Fish Bone versus Eggshell	5.28000	0.86432	0.000*	5.28000	0.86432	0.000*

SD; i.e. Standard Deviation ****significant P≤ 0.05***

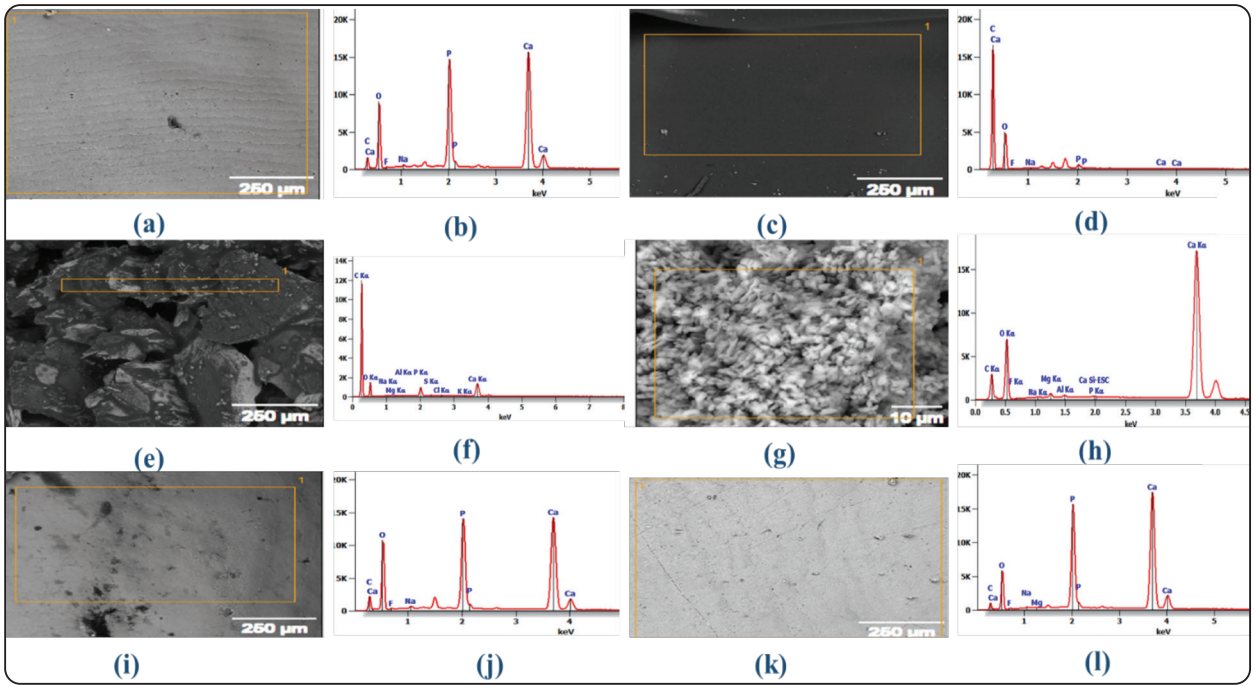

Fig. (1) SEM and EDX of Study Groups: a) SEM of Normal Human Sound E, b) EDX of Human Sound E, c) SEM of Human Demineralized E, d) EDX of Human Demineralized E, e) SEM of Nano-FB, f) EDX of Nano-FB, g) SEM of Nano-ES, h) EDX of ES, i) SEM of FB Remineralized E, j) EDX of FB Remineralized E, k) SEM of ES Remineralized E and l) EDX of ES Remineralized E.

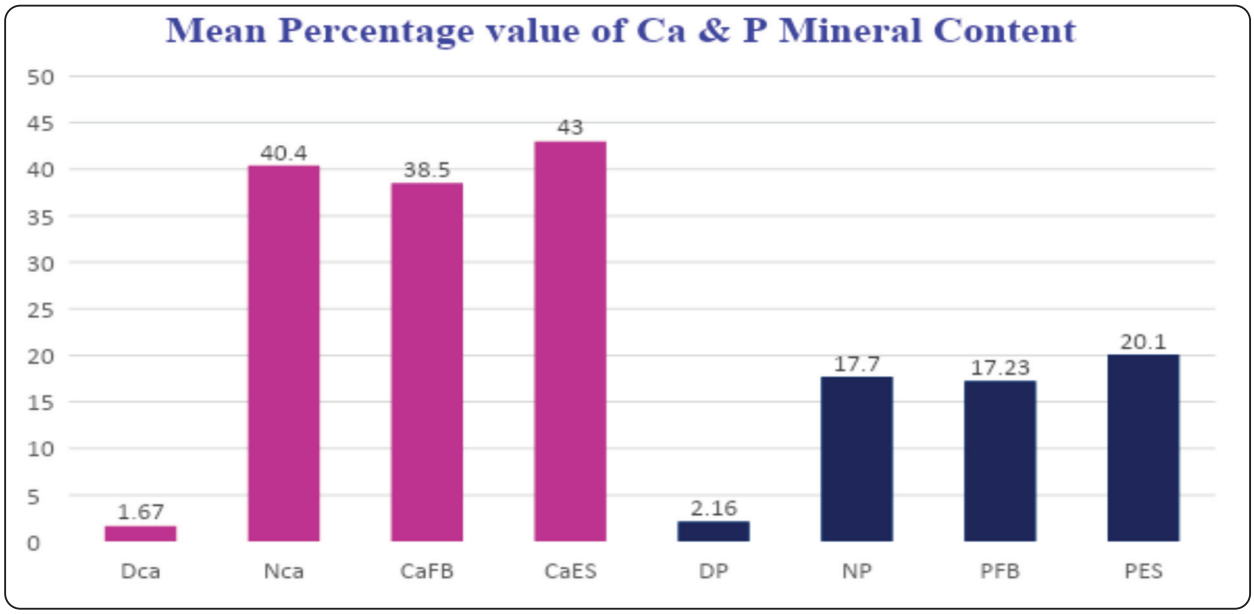

Fig. (2) EDX Percentage Level of Ca and P Mineral Content in Demineralized E, Normal E, Nano-FB Remineralized E and Nano-ES Remineralized E.

DISCUSSION

Structurally, human hard E is a nanocomposite bioceramic characterized by significant specific resilience that protects the tooth hydroxyapatite (HA) from chemical, physical and mechanical oral environment damage. The wonderful mechanical properties of enamel are associated to its hierarchical HA organization as well as its amazing thorough connection with the viscoelastic underlying dentin. Accordingly, that E dynamic, still obscure mineralized system offers a wealth of information about basic principles for organic matrix-mediated biomineralization. Those principles are potentially applied in engineering and biomaterial science fields for smart designing and development of biomimetic materials[16, 33].

The implemented demineralization regime for 96 hours stressed changing the freshly prepared solution daily to keep its pH constant at 4.2 and to prevent its saturation [33, 34]. The average pH mean values of both elaborated remineralizing solutions (FB and ES) were 9.09 and 9.48 for FB and ES; respectively; which confirmed their alkalinity. That remarkable alkalinity especially with ES (higher value than FB) was essential for the achieved E remineralization (again higher in ES than that of FB); (Table1, and Figure1 i, j, k and l). Those findings were in agreement with Hamdi K, et al.; 2022 [6]. Elaborated nano-fish bone powder were yellowish-white, which not only eliminated the brown color of the micro-particles but also might had improved their dissolution in distilled water as well as had improved its penetration and precipitation in the demineralized enamel [26, 36].

Statistical EDX analysis revealed significant differences between the two-nano biomimetic remineralizing materials (FB and ES) of the study. In addition, ES mean value for Ca remineralization (group 3) was significantly higher than that of FB (group 2), (Table1, and Figure2); which might be due to higher percentage of Ca in nano-ES than in nano-FB. Those findings were incongruent with Duta L, et al. 2021; [6] reporting that CaP obtained from biogenic (BioHA) resources were more biocompatible than synthetic HA, possessed a disordered nanostructure and a nonstoichiometric composition and they proved a metabolic activity better than that of stoichiometric forms. Furthermore, biomimetic HA differs from synthetic HA form in terms of stoichiometry, composition, degree of crystallinity [37, 38]. Therefore, biomimetic HA demonstrated a smart biodegradation rate and an overall marvelous biological performance that rendered it appropriate to repair the skeletal calcified tissues [38-40].

However, the P content of nano-ES was (0.27%), obtained statistical data showed that mean P values was higher in ES (group3) than FB (group2); (Table1 and Figure2); which might be due to the critical Ca/P molar ratio in mineralized hard structures [6]. Therefore, in comparison to synthetic HA studies confirmed that BioHA extracted from low-cost production resources; like: eggshell, fish bone and animal bones, might potentially remineralize the demineralized hard tissues [41-44].

CONCLUSIONS

With limitations of this ex-vivo study, the following may be concluded:

1. Demineralization regimes seem applicable for evaluation of remineralization potential of certain biomaterials.
2. Nanotechnology alters some biological, physical and mechanical biomaterial properties.
3. Biomimetic Ca/P of prepared nano-FB and nano-ES remineralizing solution seem promising, effective and low-cost remineralizing materials for enamel.
4. Biogenic HA resources deserve further studies to be clinically useful.

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