Original Article

DOI: 10.7860/JCDR/2021/46096.14482

Dentistry Section

Remineralising Potential of Marine Skeletal Species-*Perna viridis* Powder Extract on Human Teeth Enamel: An In-vitro Study

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ABSTRACT

Introduction: Biomineralisation is a dynamic, complex, constant mechanism by which living organisms manage precipitations of inorganic nanocrystals within organic matrices to form unique hybrid biological tissues, for example, enamel, dentin, cementum, and bone. Realising the mechanism of mineral deposition is crucial for the progress of treatments for mineralisation associated diseases and also for the innovation and development of scaffolds.

Aim: To measure the remineralisation capacity of enamel surface lesion using marine skeletal species-*Perna viridis* (mussel shell) powder extract on human tooth enamel.

Materials and Methods: The in vitro quantitative observational research study conducted from June 20th, 2020 to July 20th, 2020, included five freshly extracted un-erupted third molars decoronated at Cemento-Enamel Junction (CEJ). The coronal portion of single tooth was sectioned into 4 samples of enamel blocks in order to receive 20 blocks which were subjected to demineralisation process and divided as: Group A(Experimental group)-subsurface demineralisation + mussel shell powder extract and Group B (Control group)-subsurface demineralisation + clinpro application being placed in artificial saliva solution for thirty days. The specimens were tested for X-ray fluorescence spectroscopy analysis, microhardness testing and atomic analyses using Energy dispersive X-ray spectroscopy. Statistical analysis was done using unpaired t-test to check differences at the level of $p \leq 0.05$ between groups which were considered as statistically significant.

Results: X-ray fluorescence spectroscopy showed calcium concentration in mussel shell of 95% and concentration of Phosphate as 0.31%. Group B (Clinpro) presented statistically significantly higher (p-value 0.028) potential in enhancing the remineralisation than Group A: Mussel Shell Powder Extract (MSPE). The results of atomic analyses exhibited that quantitative amounts of Ca weight % and P weight % is statistically insignificant between both the groups. Group B (Clinpro) showed greatest ability in promoting remineralisation than Group A (mussel shell extract).

Conclusion: Marine shells with 95% of calcium concentration can remineralise enamel surface lesion.

Keywords: Calcium, Minimal invasive dentistry, Phosphate, X-ray flurorescence spectroscopy

INTRODUCTION

Dental caries is a multifactorial disease that occurs due to an inequality between pathological and protective factors. Cariogenic bacteria, fermentable carbohydrates, and salivary dysfunction are scientifically refered as relevant pathological factors. The imbalance produced will break physiological processes of remineralisation and demineralisation, promoting demineralisation process [1].

Hence dental caries management should also be nailed by understanding the role of remineralisation in preventing caries progression and stimulating healthy balance when demineralisation process begins [2]. Human saliva contains calcium and phosphate ions in supersaturated state and hence has ability to remineralise enamel [3]. After all, if acid threats overcome this physiological remineralisation process, other therapeutic methods are required to initiate remineralisation process.

Numerous agents to arrest or decline the production of carious lesions have been restrained or are currently under research process. Till date, fluoride (F) has been widely applied clinically as remineralising agent. Succeeding Fluoride, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) contains 80% of the milk proteins has been the highest researched remineralising agent in dentistry. Recently, Tricalcium Phosphate (TCP) has been another remineralising agent recommended to play a role in elevating the free calcium concentration in saliva and dental plaque [4].

Lately, another remineralising agent Clinpro Tooth Creme (3M ESPE, Saint Paul, MN, USA) was studied that possess 0.21% w/w sodium fluoride and an innovative functional Tri-Calcium Phosphate (fTCP) system and the producer claimed that it displayed superior remineralisation of initial lesions than CPP-ACP [5].

Marine skeletal extract powder has been studied in many areas regarding its promising use in medical field. As a matter of fact, a natural marine species, namely the mussel *(Perna viridis)* shell, has been widely recorded to have superior quality and pure calcium carbonate aragonite polymorph composition [6].

The mussel shell possess of about 95% $CaCO_3$ with other content which include organic substances and oxides like SiO_2, MgO and SO_3 [6]. Besides this, it also possess almost same mineral components as bone with elevated Calcium Carbon (CaC) content and not containing heavy metal elements such as mercury (Hg) or arsenic (As) inside the shell product, which is certainly practical for biomedical purposes [7].

However, no studies have been performed which has measured the outcome of mussel shell extract on remineralisation of early enamel carious lesions. The aim of this in-vitro study, therefore, was to assess the possible remineralisation of enamel surface lesion using-*Perna viridis* (mussel shell) powder extract on human tooth enamel.

MATERIALS AND METHODS

The in vitro quantitative observational research study was carried out in the Department of Conservative Dentistry and Endodontics,

Vinayaka Missions Sankarachariyar Dental College, Salem, Tamil Nadu state, India for one month duration from June 20, 2020 to July 20, 2020. The ethical clearance number was obtained (VMSDC/IEC/Approval No.160).

Synthesis of Calcium Carbonate Powder

The Calcination procedure was performed to collect pure powder free of impurities and to elevate the alkalinity mussel shell powder. Commonly mussel shell extract powder possesses 95% calcium carbonate, which transforms to basic calcium oxide on calcination, and this is responsible for the increase in alkalinity [8]. The mussel shells were obtained from Malpe beach, Mangalore, India. The calcium carbonate powder was prepared from the mussel shells according to the method explained by Islam KN et al., [9]. Specimen of the mussel shells were dehydrated in an oven at 50°C for 7 days, and then crushed and amalgamted into a refined powder, which was filtered through a 90-µm laboratory stainless steel sieve (Jai Instrument and Co, Chennai, India). The powder was measured for its discrete elemental composition percentage by weight using X-ray fluorescence spectroscopy analysis (Spectrace 6000 USA).

Synthesis of Mussel shell Powder Extract Solution

One gram of MSPE dissolved in 20 mL of 4% acetic acid (India Chemicals, Pvt., ltd., Mumbai, India) in a test tube. The clear fluid which is formed at the top was moved to a beaker and the pH of the solution was measured using a pH meter (Deluxe deep vision, model no: 101, California, USA) which was 11.8.

Preparation of sample: Sampling technique applied was purposive sampling technique, power of study not applicable in the study. Five recently extracted unerupted lower third molars were cleaned and decoronated at CEJ, followed by sectioning single tooth longitudinally in a mesio-distal and bucco-lingual direction with diamond saw such that four samples collected from a single tooth. Enamel blocks of four different dimensions (4 mm length, 4 mm width and 2 mm thickness) were taken out of each tooth and then were inserted into the acrylic blocks.

Demineralisation Protocol

Early phase of caries in the form of subsurface enamel lesion was obtained by inserting sectioned tooth samples in 20 mL of artificial carious solution (Demineralisation solution) for 72 hours ($CaCl_2$=2.2 Mm NaH_2PO_4=2.2 Mm Lactic acid=0.05 M, Fluoride=0.2 ppm, solution was regulated with 50% NaOH to a pH of 4.5) [10,11]. The sectioned tooth samples inserted in the demineralisation solution ($CaCl_2$, NaH_2PO_4, Lactic acid and Fluoride) for 72 hours at 37°C formed a subsurface demineralisation of almost 150 microns width with an intact surface exhibiting an early enamel lesion [11,12]. The observations were conducted using SEM (Hitachi SU-1510; Hitachi High-Technologies Corp., Tokyo, Japan) under 20 kV and 50× magnification.

Study contained homogeneous samples and sample size was calculated using Purposive sampling technique. The complete sample of 20 enamel blocks divided into two groups:

Group A (n=10) Experimental Group- subsurface demineralisation followed by inserting the tooth samples in mussel shell extract solution for 24 hours for 30 successive days for remineralisation. For every 24 hours, fresh mussel shell extract solution was prepared and the samples purified twice with distilled water.

Group B (n=10) Control Group- Topical application of Clinpro (3M ESPE, Saint Paul, MN, USA) was performed after subsurface demineralisation and then placing the tooth samples in artificial saliva for 24 hours for 30 successive days for remineralisation. For every 24 hours, fresh artificial saliva was prepared and topical application of clinpro done.

Microhardness Testing

Samples were collected by single trained examiner. Training and calibration was done before the start of the study and it was found to be 0.83 which indicates excellent agreement for single examiner on two different occasions. Unit for Microhardness test is micrometre (µm). Vickers microhardness testing machine (Lieca, chu-linh, Japan) was used to test the surface microhardness. A load of 25 g applied for 5 seconds and five indentations was applied for single sample with a spacing of 100 microns [11]. Microhardness testing was done in Alpha omega Hitech Bio Research centre, Salem, India.

Atomic Analysis by Energy Dispersive X-ray Spectrometry

Energy dispersive X-ray spectrometry (Quanta 200 FEG) was employed in all samples to analyse both calcium and phosphorus ions. Electronic beams sustained at 2 x10-10 amp and X-ray magnitude in counts per second was calculated.

STATISTICAL ANALYSIS

Data were computerised and analysed using Statistical Package for the Social Sciences (SPSS) version 22.0. Unpaired t-test was used to compare mean scores of both groups.

RESULTS

Statistical analysis using unpaired t-test was used to check differences for Microhardness (Vickers microhardness testing machine) and atomic analyses (Energy Dispersive X-ray spectrometry) between groups at the level of p≤0.05 which was considered statistically significant.

Clinpro exhibited statistically higher microhardness value than MSPE as shown in [Table/Fig-1].

[Table/Fig-2] depicts no statistical difference in calcium/phosphate ratio value for both groups.

S. No.	Group	Vickers hardness number (Mean±SD)	p-value*
1.	Group A-Mussel Shell Powder Extract (MSPE)	40.93±0.03	0.028
2.	Group B-Clinpro	58.20±0.06	

[Table/Fig-1]: Vickers microhardness testing values (µm)
*unpaired t-test, level of significance ≤0.05

S. No.	Group	Calcium	Phosphate	Ca/P molar ratio	p-value
1.	Group A-Mussel Shell Powder Extract (MSPE)	38.21	14.44	2.36	0.225
2.	Group B-Clinpro	36.02	16.12	2.23	

[Table/Fig-2]: Calcium/Phosphate Ratio analysis by EDX (au) between groups.
Unpaired t-test, level of significance ≤0.05; Ca: Calcium; P: Phosphate; EDX: Energy dispersive X-ray spectrometry

X-ray Fluorescence Spectroscopy Analysis

Chemical analysis of Mussel shell powder extract using X-ray fluorescence spectroscopy demonstrates highest calcium concentration of 95% and 0.46% of phosphate. It also shows 0.53% of Magnesium, 0.18% of Strontium, 0.11% of Sulfur and 0.03% of Potassium. The results exhibited no statistical difference in quantitative amounts of Ca weight % and P weight % for both groups as shown in [Table/Fig-2].

DISCUSSION

Remineralisation process of enamel is reinforced by the amount of calcium content that exist on tooth surface. MSPE has a

good percentage of bio-available calcium. In late years, mussel shell powder extract has accomplished importance in various fields. The chemical analysis of MSPE performed with X-ray fluorescence spectroscopy assessed that it comprises 95% calcium. Lately, many studies have stated the uses of mussel shell like calcium oral supplements [13-16]. Calcination was carried out in this study to increase the alkaline nature of the powder and make it free from pathogens [11]. Additionly, 10% acetic acid was added so that the mussel shell powder is nearly devoid of pathogens [8]. This was an in-vitro research study and the first of its type to assess the remineralisation potential of early enamel carious lesions by MSPE solution. MSPE solution was prepared by dissolving one gram of MSPE in 20 mL of 4% acetic acid (India Chemicals, Pvt., ltd., Mumbai, India) in a test tube. The clear fluid which is formed at the top was moved to a beaker and demineralised tooth samples were suspended in it. The MSPE solution can also be applied to the tooth surface with help of different vehicles such as glycerin solution or methylcellulose gel in an application.

In a study done by Lata S et al., demineralisation solution produced a subsurface deminerlisation of width of 150 microns and the surface was intact like an early enamel lesion. The calcium and phosphate (50% saturation) present in the solution caused dissolution of enamel subsurface only [12].

Surface demineralisation was blocked by deposition of fluoride and forming fluorapatite at the surface [12]. Superior remineralising ability on surface and subsurface lesions of enamel was exhibited by Clinpro Tooth Creme involving Tri Calcium Phospate (TCP) with 950 ppm fluoride, as proved by the manufacturer. Calcium fluoride co-exist in a protective barrier at the time of manufacturing process. This will help in movement of TCP to the teeth. When it combines with saliva during brushing, the barrier breaks and lead to calcium, phosphate and fluoride easily accessible to the tooth. This arrests the demineralisation and promotes remineralisation [8].

In this study, Clinpro displayed statistically better remineralisation property by exhibiting more hardness value than MSPE. When linked with another natural calcium sources, MSPE has low levels of toxic metals like Pb, Al, Cd, and Hg. The N-terminal sequence of mussel shell matrix proteins will promote in increased calcium transport and regarded as a potential significance of mussel shell when consumed as calcium supplements [16]. Hence, MSPE solution was used in this study.

The pH of a MSPE solution was tested by pH meter which was 11.8. The raised pH of a remineralising solution is indicated, as it upgrades the ion activity of anions such as phosphate and hydroxyl ions in the solution. The ionic activity relates to the concentrations of these ions in the solution. Asian green mussel (*Perna viridis*) shells most commonly found and they are most often discarded after consumption and can therefore be acquired with minimal economic incentives. Since, there's increased availability of these shells, it will be easy to evaluate enamel remineralisation potential effectively.

Since, this study is an in vitro quantitative observational research study, Experimental group samples were immersed in MSPE solution to evaluate effectiveness to remineralisation and control group samples were immersed in artificial saliva solution.

At low pH, there will be increased H+ ions, which will bind with these anions making them less accessible for remineralisation. In addition, the basic form of phosphate anion present in hydroxyapatite is PO_4^{-3} and these anions are present in higher concentrations only at a high pH of 11-12. For remineralisation to occur, bioavailable calcium and phosphates are beneficial [17]. Therefore, the increased bioavailability of calcium along with the raised concentration of phosphates present in MSPE solution in conjunction with its higher pH may be responsible for remineralisation.

Limitation(s)

The lacunae in the area of study are some difficulties in sterility and removing all contaminants from the surface of marine skeletal species.

CONCLUSION(S)

Within the limitations of this study, it can be concluded that the raised pH of the of mussel shell powder extract solution in addition to rich bioavailable calcium content of mussel shell has the efficiency to favour remineralisation. Even though Clinpro showed more remineralisation than MSPE, the latter due to its easy bioavailability and natural source of calcium and phosphate can be the future in remineralising enamel carious lesions. Further, clinical studies regarding suitable vehicle for MSPE is required as this might raise the remineralisation potential of MSPE comparable to the commercially available agents.

Financial aid: Authors are extremely thankful for supporting this work by Vinayaka Mission's Research Foundation - (Deemed to be University) Unique ID: VMRF/Seed Money/2020/VMSDC-Salem/10.

Authors contribution: VDR and RS designed project and computational framework, VDR performed the experiments and derived the models, YCT and EMI assisted with collected measurements and helped to carry out the collected simulations, VDR wrote the manuscript in consultation with other authors.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Aug 01, 2020
- Manual Googling: Dec 15, 2020
- iThenticate Software: Jan 07, 2021 (22%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: As declared above
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jul 28, 2020**
Date of Peer Review: **Aug 27, 2020**
Date of Acceptance: **Dec 15, 2020**
Date of Publishing: **Feb 01, 2021**