An *in vitro* evaluation of effect of remineralising potential of marine skeletal specie– meretrixmeretrix extract on human enamel

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Comment [A1]: The title would read better as '**The remineralising potential of *Meretrix meretrix* extract on human enamel *in vitro***'

Running title: Remineralising potential of ~~marine skeletal *specie*~~*Meretrix meretrix*

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ABSTRACT

Background: To assess the remineralisation efficiency of enamel subsurface defect using marine skeletal species – *Meretrix meretrix* (cockle shell) powder extract on human tooth enamel.

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Methods: Recently extracted 5 human 3rd molar teeth were sectioned at cement-enamel junction (CEJ) and included for the study. Later, each tooth coronal portion was equally divided into four parts of enamel blocks ~~in order~~ to obtain twenty blocks which were exposed to demineralization procedure and grouped as: Group I: ~~-~~subsurface demineralization cockle shell powder extract. Group II: subsurface demineralization + clinpro application being kept in artificial saliva for a period of thirty days. The samples were assessed ~~for~~ using X-ray fluorescence spectroscopy evaluation, micro hardness testing and atomic evaluation with Energy dispersive x-ray spectroscopy. The values were statistically calculated with one-way ANOVA and Tukey-~~—~~Kramer multiple comparison test.

Results: X-ray fluorescence spectroscopy displayed ~~Calcium~~ calcium concentration ~~in cockle shell~~ of 98.3% ~~&~~ and ~~concentration of Phosphate~~ phosphate concentration of 0.21% respectively in the cockle shell. Group II (Clinpro) displayed highest potential in accelerating the remineralisation ~~then~~ followed by Group I: CSEP (Cockle shell extract powder). The values of atomic evaluation displayed that quantitative volumes of Ca weight % and P weight % is statistically greater for both the groups. Group II (Clinpro) exhibited higher remineralisation efficiency ~~then~~ than Group A (mussel shell extract).

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Conclusion: Marine shells with greater calcium composition can remineralise enamel subsurface defects.

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

BACKGROUND

Dental caries is a multifactorial disease that arises as a result of a disproportion among pathological and protective aspects in oral cavity. Cariogenic bacteria, fermentable carbohydrates, and salivary dysfunction are scientifically proved as significant pathological aspects. The inequality formed will prevent physiological processes of remineralisation and demineralization, favouring demineralization process. [1]Therefore, dental caries treatment should also be emphasized on ~~an~~ understanding the role of remineralisation in inhibiting caries advancement and promoting healthy balance when demineralization process occurs. [2]Human saliva involves calcium and phosphate ions in supersaturated state and therefore, has efficiency to remineralize enamel. [3]After all, if acid exposures overcome the physiological remineralisation process, alternative therapeutic procedures are needed to promote remineralisation process. Various agents to inhibit or stop the formation of carious lesions have been bottled up or are presently under research process.

Lately, fluoride (F) has been extensively used clinically as remineralising agent. Succeeding to ~~Fluoride~~fluoride, ~~Casein~~ casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) consisting of ~~s~~ 80% ~~of~~ milk proteins has been the highest researched remineralising agent in dentistry. Recently, t~~T~~ricalcium phosphate (TCP) is one more remineralising agent recommended to play a role in elevating the free calcium concentration in saliva and dental plaque. [4]Lately, additional remineralising agent Clinpro Tooth Creme (3M ESPE, Saint Paul, MN, USA) was investigated that contained 0.21% w/w sodium fluoride and new functional tri-calcium phosphate (fTCP) system and ~~-~~producer claimed that

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it showed higher remineralising capacity of initial subsurface lesions than CPP-ACP. [5] Marine skeletal extract powder has been studied in many areas regarding its promising use in medical field. A natural marine species, namely the ~~Cockle~~ cockle (*Meretrix meretrix*) shell, has been extensively noted to have superior value and pure calcium carbonate aragonite polymorph composition. [6] ~~The~~C~~c~~ockle shell possesses ~~of~~ about 97% $CaCO_3$ with other contents which include organic substances and oxides like SiO_2, MgO and SO_3. [6] Besides this, it also possesses almost same mineral components as bone with elevated calcium carbon (CaC) and not containing elements like mercury (Hg) or arsenic (As), which is naturally 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. [8,. 9, 10]

Objectives

The purpose of this~~present~~ study was to assess the likely remineralisation of enamel surface lesion with *Meretrix meretrix* (cockle shell) powder extract on human tooth enamel.

METHODS

Synthesis of Calcium Carbonate Powder: The ~~Calcination~~ calcination procedure was performed to collect pure powder devoid of impurities and to elevate the alkalinity of cockle shell powder. Commonly, cockle shell extract powder possesses of 98% calcium carbonate, which transforms to basic calcium oxide on calcination, and this promotes the boost in alkalinity (9). The cockle shells were obtained from Malpe beach, Mangalore, India. The calcium carbonate powder was prepared from the cockle shells according to the method explained by Islam et al (10). ~~Specimen of the c~~Cockle shells were dehydrated in an oven at 50°~~c~~ C for 7 days, and then crushed and amalgamated into a refined powder, which was filtered through a 90-m laboratory stainless steel sieve (Jai Instrument & Co, Chennai, India).

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The powder was measured for discrete elemental composition percentage by weight with X-ray fluorescence spectroscopy evaluation (Spectrace 6000 USA).

Synthesis of Cockle Shell Powder Extract Solution: One gram of CSPE was mixed in 20 ml of 4% acetic acid in a test tube. The pure fluid which ~~is~~ was found at the top was shifted to a beaker and the pH of the solution was checked with a pH meter which was ~~&~~ found to be 11.8.

Preparation of Sample: Five recently extracted unerupted third molars were cleaned ~~&~~and decoronated at CEJ, proceeded by sectioning tooth longitudinally in a mesio-distal ~~&~~and bucco-lingual direction with diamond saw such that 4 parts (4mm width, 4mm long, and 2mm thick) divided from a single tooth and inserted in acrylic blocks.

Demineralisation Protocol[11]

Carious lesions displaying early subsurface enamel lesion was achieved by immersing samples in 20ml of demineralization bath for 72 hours ($CaCl_2$ = 2.2 Mm NaH_2PO_4 = 2.2 Mm Lactic acid = 0.05 M, Fluoride = 0.2 ppm, solution is adjusted with 50% NaOH to a pH of 4.5). The samples kept in the demineralization solution (CaCl2, NaH2PO4, Lactic acid and Fluoride) for 72 hours at 37°C led to subsurface demineralization of almost 150 microns' diameter exhibiting an early enamel lesion[12]

Group I (n=10) – subsurface demineralization proceeded by placing the tooth samples in cockle shell extract solution for 24 hours for 30 successive days for remineralisation. For every 24 hours, fresh cockle shell extract solution was ~~processed~~processed, and the samples were purified with distilled water. Group II (n=10) - subsurface demineralization proceeded by topical application of clinpro (3M ESPE, Saint Paul, MN, USA) and then placing the tooth samples in artificial saliva for 30 days. For every 24 hours, fresh artificial saliva was prepared & topical application of clinpro was done.

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Comment [A9]: Materials and methods should be in past tense.

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Microhardness Testing: Vickers microhardness testing machine (Lieca, chu-linh, Japan) was used to test the surface microhardness. A mass of 25g was placed for 5 sec and five cycles made for every sample with a spacing of 100 microns.

Atomic Analysis by EDX

All specimens were tested for both calcium and phosphorus content by Energy dispersive ~~x~~X-ray spectrometry (Quanta 200 FEG). Electron beams kept at 2 x10–10amps were used and X-ray dimension in counts per second were detected.

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Statistical analysis

SPSS 11.0 software was used to analyse the obtained data using One-way ANOVA and Tukey – Kramer multiple comparison test.

RESULTS

Table 1 indicates micro hardness values for group I: Cockle shell Powder Extract (42.94 ± 0.04) and group II: Clinpro (59.25 ± 0.05). Table 2 indicates calcium/Phosphate Ratio Mean ± S.D Value. group I: Cockle shell Powder Extract had Ca/P molar ratio of 2.58 and group B: Clinpro had Ca/P molar ratio of 2.35.

X- Ray Fluorescence Spectroscopy Analysis

Cockle shell powder extract, chemical analysis done by means of X-ray fluorescence spectroscopy exhibited maximum calcium concentration of 98% and 0.21% of phosphate. It also demonstrated 0.54% of Magnesium, 0.17% of Strontium, 0.13% of Sulfur and 0.04% of Potassium. The results of atomic testing exhibited that quantitative amounts of Ca weight % and P weight % ~~is~~ was statistically higher for both groups.

DISCUSSION

Remineralisation process of enamel is reinforced by calcium content exist on tooth surface. CSPE has a good percentage of bio-available calcium. In late years, cockle shell powder extract has accomplished wide use in different specialities. The chemical evaluation of CSPE

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performed with X-ray fluorescence spectroscopy assessed that it contains 98%_calcium. Lately, various ~~researches~~research have stated the uses of cockle shell like calcium oral supplements. In ~~this~~ the present research, calcination process was performed to collect pure powder devoid of pathogens and to enhance the alkalinity of powder. Additionally, 10% acetic acid was added so that the cockle shell powder is nearly devoid of pathogens. [9]This study is first one to assess the remineralisation potential of early enamel carious lesions by CSPE solution. On the surface of all samples with help of glycerine solution, CSPE was administered.

Subsurface demineralisation of relatively 150 microns' width with intact enamel mimicking subsurface enamel lesion, according to Lata et al. The demineralising solution exhibited only 50% of saturation level of calcium and phosphates leading to dissolution of only enamel subsurface area. Formation of fluorapatite at the surface occurs by addition of Fluoride. [12]

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Tri calcium phosphate with 950 ppm fluoride, as assured by the manufacturer in Clinpro Tooth Creme shows good remineralization efficacy on surface and sub-surface lesions of enamel. During manufacturing process calcium fluoride, coexist in a protective barrier. [13]This will aid in transfer of tricalcium phosphate on to the surface of tooth. When it combines with saliva during brushing, the barrier breaks and lead to calcium, ~~phosphate~~phosphate, and fluoride easily accessible to the tooth. This will stop the demineralization and promotes remineralization process. [9]This research exhibited that clinpro showed statistically better remineralisation property than CSPE [Table-1]. When linked with another natural calcium sources, CSPE has decreased levels of toxic metals like Pb, Al, Cd, and Hg. The N-terminal sequence of cockle shell matrix proteins will promote in higher calcium transport and regarded as a potential significance of cockle shell when consumed as calcium supplements.[14]Hence, CSPE solution was administered in this research. The pH

meter was used to test pH of a CSPE solution, suggested to be 11.8. ~~The raised~~ An increase in 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. Thus, there will be higher availability of these ions for remineralisation. There will be higher levels ~~of H~~of H+ ions at low pH, which will join with these anions making them less available for remineralization. In addition, the basic form of phosphate anion exist in hydroxyapatite is PO_4^{3-} and these anions are present in larger concentrations only at a high pH of 11-12. For remineralization to occur, bioavailable phosphates and calcium are beneficial. [15]Hence, the higher bioavailability of calcium together with the increased concentration of phosphates present in CSPE solution [Table-2] in coordination with its greater pH may be responsible for remineralization.

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CONCLUSION

Within limitations of the study, it was found that, clinpro exhibited better remineralisation than CSPE. Advanced, clinical ~~researches~~research ~~are~~ is required regarding ideal vehicle for CSPE as that might ~~raise~~ increase the remineralisation potential of CSPE equivalent to the commercially accessible remineralising agents.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Legends for illustrations

Tables

Table 1: Microhardness test Mean ± S.D Value

S.NO	GROUP	Mean ± S.D
1	Group I (Cockle shell Powder Extract)	42.94 ± 0.04
2	Group II (Clinpro)	59.25 ± 0.05

Table 2: Calcium/Phosphate Ratio Mean ± S.D Value

S.NO	GROUP	Calcium	Phosphate	Ca/P molar ratio
1	Group I (Cockle shell Powder Extract)	37.31	14.26	2.58
2	Group II (Clinpro)	35.02	16.24	2.35