ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Evaluation of Remineralisation Potential of Zingiber officinale Roscoe-Apis Mellifera, and Chitosan as compared to control using QLF on white spot lesions: An in-vitro study

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Article History:

Received on: 23 Feb 2020
Revised on: 25 Mar 2020
Accepted on: 27 Mar 2020

Keywords:

Remineralisation,
Demineralisation,
Chitosan,
QLF,
Ginger-Honey,
WSL

ABSTRACT

Early detection and treatment of white spot lesions (WSL) is pivotal in caries control. Several commercially available products are available for WSL reversal. However, the majority of them are either synthetically derived or are not a hundred percent efficient. Thus there is an ever constant need to find newer, more efficacious products for the same. One of the parameters to quantify de and remineralization is by Quantitative Light Induced Fluorescence (QLF). Thus this study aims to evaluate and compare the remineralizing potential of Zingiber officinale Roscoe (Ginger rhizome), Apis Mellifera (Manuka Honey) mixture and chitosan on artificial demineralized human enamel using Quantitative Light Induced Fluorescence. 45 human enamel samples were randomly divided into a control and two test groups. An Area of Interest (AOI) measuring 4x4 mm on the buccal surface of each tooth was formed and all the samples were subjected to demineralization process for a period of 96 hours. Remineralization regime was then carried out with twice daily application of respective interventional agents for a period of 21 days. QLF readings were recorded at the end of demineralization (Baseline), Day 7, Day 14 and Day 21 and fluorescence images were analysed using QLF InpektorTM propriety software. The remineralization action of chitosan was found to be the highest with a statistically significant reduction ($p<0.001$) seen at the end of 7th, 14th and 21st day. Ginger- honey mixture also showed reduction in fluorescence levels but only after the 14th day. The current study showcases the dramatic ability of chitosan to almost completely reverse artificially formed WSL at each subsequent week. Ginger honey on the other hand showed a significant remineralization between 2nd and 3rd week which may be attributed to a possible slower mechanism of action.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i4.3144

Production and Hosted by

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INTRODUCTION

The white spot lesion (WSL) is the first clinically apparent sign of an otherwise silent disease-that is dental caries. Gocmen defined the WSL as "a subsurface enamel porosity from carious demineralisation" that is manifested clinically by a milky white opacity (Gocmen *et al.*, 2016). Caries detection clinically is still largely dependent on visual and radiographic examination. However, these methods can only detect well-advanced lesions, involving at least 300-500μm of enamel. Thus, WSL or non-cavitated

lesions, where a non- surgical reversal is still possible, are difficult to identify using these methods alone. Quantitative Light-Induced Fluorescence or QLF was introduced clinically as a caries detection system in 2004 (QLF-Pro Inspektor, Germany). It works on the principle that enamel will auto fluoresce under certain light conditions. Demineralised enamel will fluoresce less and this loss of fluorescence can be detected, quantified and longitudinally monitored using QLF. Studies have shown QLF to have high sensitivity for quantification and monitoring of de and remineralisation with high correlation to changes in mineral content (Shi *et al.*, 2001; Pretty *et al.*, 2002).

Current paradigms in minimal intervention dentistry especially for WSL treatment show a trend of non-surgical treatment with a greater importance being given to prevention and reversal of disease process over cure; remineralisation of WSL being one such natural repair process.

Several synthetic agents are available to mineralise early enamel carious lesions. However, naturally derived products are known to show lesser toxicity and are considered 'Generally Recognised As Safe' (GRAS) by the US Food and Drug Administration (FDA) (Summitt *et al.*, 2006).

Chitosan, a derivative of chitin, is well known for its use in wound dressings and drug delivery systems (Agnihotri *et al.*, 2004; Kumar *et al.*, 2004).

Its increased drug targeting potential is ascribed to improved drug absorption and stabilisation of the drug components. Enamel WSL remineralisation using phosphorylated Chitosan and chitosan-amelogenin hydrogel have shown promising results (Xu *et al.*, 2011; Ruan *et al.*, 2013).

However, the remineralising potential of deacetylated Chitosan alone in the presence of artificial saliva has not been assessed. Ginger and Manuka Honey are naturally derived products that show exceptional antibacterial activity, especially against oral biofilm (Park *et al.*, 2008; Patel *et al.*, 2011; Azizi *et al.*, 2015). Ginger rhizome also has a high fluoride content making its use in remineralisation therapy hypothetical. Thus, the present study aimed to assess the remineralising potential of 90% deacetylated chitosan and ginger-manuka honey mixture in the presence of artificial saliva on artificially formed incipient enamel lesions.

MATERIALS AND METHODS

Ethical Clearance & Study design

The procedure protocol was approved by the Institutional Research Ethics Committee (JSS/DCH/IEC/MD-26/2016-17(2)) before the commencement of the study. This experimental in-vitro study was conducted in the research unit of our institution. The demineralising solutions and test solutions were prepared in the College of Pharmacy.

Preparation of Demineralising Solution and artificial saliva

2 Litres of the demineralising solution was freshly prepared every day according to the composition given by Featherstone and Zero (1992). 4 Litres of artificial saliva was made once in every two days, according to the compositional structure given by Sato *et al.* (2006).

Preparation of interventional solutions

Ginger-Honey

Collection and Identification of Ginger

The ginger rhizomes were collected from the northern part of Haryana in Jhajjhar district. The ginger rhizomes were identified and classified in the Department of Biological sciences.

The rhizomes were washed with clean water and allowed to air dry to reduce the microbial load of the plant material due to handling and transportation.

The outer covering of ginger was peeled, and the rhizomes were allowed to sun dry for two weeks. The dried ginger rhizomes were cut and pulverised into powder using an electronic blender.

Collection of Honey

The honey was harvested in Taranaki, in the west-central part of the North Island of New Zealand in the summer-early spring of 2016 and consisted mainly of nectar gathered from the blossoms of Manuka trees.

The extracted honey from combs neither contain any preservatives nor went through any preservative processing. Extraction, storage and transportation of Manuka honey was done in glass containers.

Preparation of ginger honey mixture

The Ginger powder was mixed with Manuka honey (MGO activity of 580) in a ratio of 8mg/ml (w/v) (Bilgin *et al.*, 2016).

Chitosan solution

Chitosan solution of 2.5mg/ml concentration was prepared by dissolving 25mg of 90% deacetylated chitosan (SR Chemicals, India) in 10ml of 2% acetic acid (Arnaud *et al.*, 2010).

All the interventional agents were freshly prepared daily.

Table 1: Mean Difference values of ΔF for Control, Ginger-Honey and Chitosan Groups at different time intervals of remineralization cycles

Day	Control Group Mean Difference	Z Value	P Value	Ginger Honey Group Mean Difference	Z Value	P Value	Chitosan Group Mean Difference	Z Value	P Value
Baseline to Day 7	-0.87±16.70	-1.14	0.26	3.28±4.59	-2.22	0.03*	-6.38±5.54	-3.01	0.001***
Day 7 to Day 14	-2.91±10.05	-1.20	0.23	-2.22±5.72	-1.60	0.11	-2.02±1.98	-3.41	0.001***
Day 14 to Day 21	-4.75±6.26	-2.49	0.01*	-3.70±3.77	-3.42	0.001***	-2.38±2.72	-2.90	0.001***
Baseline to Day 21	-8.52±10.17	-2.61	0.009*	-2.63±4.90	-1.71	0.87	-10.76±8.48	-3.24	0.001***

Wilcoxon Signed Ranks Test (significance level $p \leq 0.05$)

Table 2: Comparison between Control and Ginger-Honey across time

Time Interval		ΔF			ΔF Max			ΔQ		
		Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.
Baseline	7th Day	1.21	2.35	0.956	-6.06	4.02	0.436	8519.65	15157.10	0.943
	14th Day	-1.36	2.35	0.939	-12.75*	4.02	0.01**	-11114.43	15157.10	0.884
	21st Day	-5.58	2.35	0.087	-20.25*	4.17	0.001***	-33294.56	15157.10	0.130
7th Day	14th Day	-2.56	2.35	0.696	-6.69	4.02	0.347	-19634.08	15157.10	0.568
	21st Day	-6.78	2.34	0.023*	-14.19	4.17	0.005**	-41814.20	15157.10	0.034*
14th day	21st Day	-4.22	2.35	0.279	-7.50	4.17	0.28	-22180.13	15157.10	0.463

One Way ANOVA with Tukey's Post hoc Test (Significance level $p \leq 0.05$)

Table 3: Comparison between Control and Chitosan across time

Time Interval		ΔF			ΔF Max			ΔQ		
		Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.
Baseline	7th Day	-3.62	2.27	0.384	-9.40	4.45	0.155	8519.65	15157.10	0.943
	14th Day	-6.08	2.27	0.041*	-16.69*	4.45	0.002***	-11114.43	15157.10	0.884
	21st Day	-9.64*	2.27	0.001***	-25.60	4.61	0.001***	-33294.56	15157.10	0.130
7th Day	14th Day	-2.46	2.27	0.698	-7.29	4.45	0.36	-19634.08	15157.10	0.568
	21st Day	-6.02	2.26	0.044*	-16.20	4.61	0.004**	-41814.20	15157.10	0.034*
14th day	21st Day	-3.56	2.27	0.398	-8.91	4.61	0.221	-22180.13	15157.10	0.463

One Way ANOVA with Tukey's Post hoc Test (Significance level $p \leq 0.05$)

Table 4: Comparison between Ginger-Honey and Chitosan across time

Time Interval		ΔF			ΔF Max			ΔQ		
		Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.	Mean Difference	Std. Error	Sig.
Baseline	7th Day	-1.55	1.89	0.846	-9.71	4.20	0.101	-2614.89	11513.70	0.996
	14th Day	-3.67	1.89	0.219	-11.23*	4.20	0.042*	-11646.60	11513.70	0.743
	21st Day	-6.70	1.89	0.003**	-23.40	4.36	0.001***	-26746.95	11513.70	0.099
7th Day	14th Day	-2.12	1.89	0.679	-1.52	4.20	0.984	-9031.71	11513.70	0.861
	21st Day	-5.15	1.89	0.037*	-13.69	4.35897	0.011*	-24132.06	11513.70	0.161
14th day	21st Day	-3.04	1.89	0.38*	-12.17	4.36	0.031	-15100.35	11513.70	0.558

One Way ANOVA with Tukey's Post hoc Test (Significance level $p \leq 0.05$)

Collection, storage & Preparation of human permanent enamel samples

For the present study, enamel samples were obtained from 45 human premolars freshly extracted for orthodontic reasons; free from dental caries, fracture, hypoplastic lesions, intrinsic stains, wasting diseases like attrition, abrasion, erosion, developmental anomalies and restorations.

Immediately after extraction, the teeth were stored in 10% formalin and were thoroughly cleaned of debris, calculus and soft tissues.

They were washed in 0.1M Phosphate buffer (pH 7.4), rinsed with de-ionised water and were stored in distilled water at a temperature of 4º C until further use (Shanbhog *et al.*, 2016).

The premolars were embedded in clear acrylic blocks measuring 2 x 2.5 x 1 cm using a customised jig. The middle third of the buccal surface of each tooth was covered in a polyvinyl stencil measuring 4 x 4 mm to form an Area of Interest (AOI).

Each sample was then painted with two coatings of colourless acid-resistant nail varnish (Colour Plus,

MaybellineTM) barring the 4 x 4 mm window.

AOI was considered for the analysis of mineralisation and lesion progression (Mehta *et al.*, 2013).

Demineralisation of enamel samples

Each sample was placed in sterile containers containing 30ml of the demineralising solution and placed in an orbital shaking incubator at 37°C at 50 RPM for 96 hours. Immediately after the demineralisation cycle, baseline QLF readings were done. Only teeth with an ICDAS score of 1 or 2 after demineralisation were included in the study.

The demineralised samples were then randomly assigned to either Control, Ginger-Honey or Chitosan groups and subjected for remineralisation cycle.

Remineralisation Cycle

The demineralised samples were subjected for 21 days of remineralisation cycle. All the samples from three groups were kept immersed in 30ml of artificial saliva for the entire duration of the study. The control group was treated with distilled water, and the two experimental groups were treated with ginger honey mixture and Chitosan, respectively. All samples were subjected to the 1-minute application of respective interventional agents twice a day for 21days.

QLF analysis

QLF analysis was carried out by a single examiner using QLF-D Biluminator™ device (Inspektor). QLF and white light digital images were captured from buccal aspects of the specimens under class 1 ASA darkroom conditions (3) at baseline, 7th day, 14th day and 21st day of remineralisation. Lesion depth (ΔF), Maximum fluorescence loss (ΔF Max) and lesion volume (ΔQ) were recorded and analysed using QA2 v 1.26, Inspektor Research Systems software.

Data Presentation and statistical analysis

The collected data were coded in EXCEL and analysed using SPSS Version 23. For data presentation, mean values and standard deviations of ΔF, ΔF Max, ΔQ were calculated. Data analysis was performed using Wilcoxon signed ranks test and repeated measures of ANOVA followed by Tukey's post hoc test.

RESULTS

Mean difference values of lesion depth (ΔF), Maximum fluorescence loss (F Max) and Lesion volume (ΔQ) for Control, Ginger-Honey and Chitosan groups at different time intervals of remineralisation cycles were obtained. Table 1 depicting mean lesion depth (ΔF), showed a reduction in all the three groups across different time intervals. However, the chitosan group showed a statistically significant fluorescence gain at 7th, 14thand 21stday of remineralisation cycle as compared to baseline (p-0.001). Ginger-Honey showed statistically significant fluorescence gain at 21stday of remineralisation cycle as compared to 14th day (p-0.001). Control also showed statistically substantial fluorescence gain after 14th day (p-0.01).

Mean Maximum fluorescence (F Max) loss also reduced in the three groups across different time intervals. Control, Ginger-Honey and Chitosan Groups showed statistically significant fluorescence gain at 21st day of remineralisation cycle as compared to baseline (p- 0.001, 0.004 and 0.003 respectively). Mean Lesion volume (ΔQ) reduced in the three groups across different time intervals. Control and Chitosan Groups showed statistically significant fluorescence gain at 21stday of remineralisation cycle as compared to baseline (p-0.031 and respectively).

Comparison of remineralisation potential of Ginger-Honey with Control across the period showed a statistically non-significant difference. Table 2 Comparison of remineralisation potential of Chitosan with Control across the period showed a statistically significant difference between baseline to 14thand 21stday (p-0.041 and 0.001). Table 3 Comparison of remineralisation potential of Chitosan with Ginger-Honey across the period showed a statistically significant difference between baselines to 21st day (p-0.003). Table 4

DISCUSSION

Remineralisation treatment protocols are based on the physiological phenomenon of mineral loss and gain and changing the balance between the two. Various preventive therapies have been studied to enhance remineralisation, reduce demineralisation and to arrest active carious lesions- fluoride being the most commonly used amongst them (Pulido *et al.*, 2008). Fluoride levels of about three parts per million (ppm) in the enamel are required to shift the balance from net demineralisation to net remineralisation (Pretty *et al.*, 2002). However, normal remineralisation by fluoride is found to be a self-limiting surface phenomenon that prevents the penetration of ions into the depth of the lesion (Cate, 1990). Rapid deposition of a surface layer of fluorapatite is not only observed to resist demineralisation but also prevents any further penetration of calcium and phosphate ions. Thus 100% reversal of the incipient lesions is

not possible with fluoride alone. Several materials like Stannous Fluoride, Casein Phosphopeptide, Casein Phosphopeptide-Amorphous Calcium Phosphate, Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride etc. were brought into the market with varied results (Reynolds *et al.*, 1995; Cochrane *et al.*, 2008) to overcome these shortcomings. Natural products have been used with dental formulations like mouthwashes, irrigating agents, intra-canal medicaments, anti-inflammatory etc. However, only an exiguous number of the commercially available remineralising agents are naturally derived. The current study used 90% deacetylated Chitosan and an experimental formulation of ginger and Manuka honey to test their remineralisation potential in vitro against control. Chitosan and its derivatives have well documented biological activity and are used in medicine mainly as a drug delivery system. Majority of the remineralisation studies have used Chitosan in combination with various interventional agents. Some of the interventional agents are phosphorylated Chitosan, chitosan pre-treatment with bioglass and chitosan-amelogenin hydrogel (Ruan *et al.*, 2013; Zhang *et al.*, 2018; Xu *et al.*, 2011). Various mechanisms of action have been proposed for the same (Hayashi *et al.*, 2007; Decker *et al.*, 2005). Chitosan has been found to have the ability to bind calcium ions to form nucleating sites, and it also gets adsorbed to the surface of hydroxyapatite crystals which helps in the formation of nano- complexes thereby leading to remineralisation (Xu *et al.*, 2011; Lee *et al.*, 2012). The present study made use of Chitosan alone and tested its ability to successfully deliver calcium and phosphate ions from artificial saliva to the inner enamel layers. Study results showed statistically significant fluorescence gain across week 1, 2 and 3 with 90% deacetylated chitosan intervention in the presence of artificial saliva ($p<0.001$) leading to substantial remineralisation of lesion across the study period.

Manoj (2007) and Nigus and Chandravanshi (2016) have shown dried ginger rhizome to have a fluoride concentration ranging from 2.0-2.8 mg/kg. Ginger-honey group in the present study showed minimal fluorescence gain from baseline to week one and from week 1 to week 2. However, between week two and week 3, a highly significant fluorescence gain was noticed ($p<0.002$). This may be attributed to a possible slower mechanism of action. Further studies with a more extended study period and remineralisation cycle are advocated for the same.

This result was found conflicting with that of previous studies wherein a significant amount of remineralisation was noticed on the usage of ginger-honey mixture (Gocmen *et al.*, 2016; Korkut *et al.*, 2017). This could be attributed to mineral variations in the soil, irrigation water, and the atmosphere, and differences in the agrochemicals used during cultivation, such as fertilisers, pesticides, and herbicides.

CONCLUSION

It can be concluded taking into consideration the limitation of the present in vitro study, that: Compared to ginger honey mixture, Chitosan produced significant amounts of remineralisation of the artificial white spot lesions. Chitosan produced significant amounts of remineralisation at 7th, 14th and 21st day as compared to only 21st day for artificial saliva and ginger honey groups. Ginger honey showed significant remineralisation between 2nd and 3rd week , possibly due to a slower mechanism of action. Study with an increased remineralisation period is advocated for identification of the same.

ACKNOWLEDGEMENTS

The authors acknowledge the help and assistance rendered by Dr. B. Nandlal of Department of Pedodontics and Preventive Dentistry, JSS Dental College as well as Mr. Tanmoy Das from the Department of Pharmaceutics, JSS College of Pharmacy.

Funding Support

The authors declare that they have no funding support for this study.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

Ethics

The procedure protocol was approved by the Institutional Research Ethics Committee (JSS/DCH/IEC/MD-26/2016-17(2))

REFERENCES

Agnihotri, S. A., Mallikarjuna, N. N., Aminabhavi, T. M. 2004. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of Controlled Release*, 100(1):5–28.

Arnaud, T. M. S., de Barros Neto, B., Diniz, F. B. 2010. Chitosan effect on dental enamel de-remineralization: An in vitro evaluation. *Journal of Dentistry*, 38(11):848–852.

Azizi, A., Aghayan, S., Zaker, S., Shakeri, M., Entezari, N., Lawaf, S. 2015. In Vitro Effect of Zingiber officinale Extract on Growth of Streptococcus mutans and Streptococcus sanguinis. *International Journal of Dentistry*, 2015:1–5.

Bilgin, G., Yanikoglu, F., D, T. 2016. Remineralization Potential of Herbal Mixtures: An In Situ Study. *Paripex-Indian Journal of Research*, 5(2):264–268.

Cate, J. T. 1990. In vitro Studies on the Effects of Fluoride on De- and Remineralization. *Journal of Dental Research*, 69(2_suppl):614–619.

Cochrane, N. J., Saranathan, S., Cai, F., Cross, K. J., Reynolds, E. C. 2008. Enamel Subsurface Lesion Remineralisation with Casein Phosphopeptide Stabilised Solutions of Calcium, Phosphate and Fluoride. *Caries Research*, 42(2):88–97.

Decker, E. M., von Ohle, C., Weiger, R., Wiech, I., Brecx, M. 2005. A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies. *Journal of Periodontal Research*, 40(5):373–377.

Featherstone, J. D. B., Zero, D. T. 1992. An in sita Model for Simultaneous Assessment of Inhibition of Demineralization and Enhancement of Remineralization. *Journal of Dental Research*, 71(3_suppl):804–810.

Gocmen, G. B., Yanikoglu, F., Tagtekin, D., Stookey, G. K., Schemehorn, B. R., Hayran, O. 2016. Effectiveness of some herbals on initial enamel caries lesion. *Asian Pacific Journal of Tropical Biomedicine*, 6(10):846–850.

Hayashi, Y., Ohara, N., Ganno, T., Yamaguchi, K., Ishizaki, T., Nakamura, T., Sato, M. 2007. Chewing chitosan-containing gum effectively inhibits the growth of cariogenic bacteria. *Archives of Oral Biology*, 52(3):290–294.

Korkut, B., Korkut, D., Yanikoglu, F., Tagtekin, D. 2017. Clinical assessment of demineralization and remineralization surrounding orthodontic brackets with FluoreCam. *Asian Pacific Journal of Tropical Biomedicine*, 7(4):373–377.

Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., Domb, A. J. 2004. Chitosan Chemistry and Pharmaceutical Perspectives. *Chemical Reviews*, 104(12):6017–6084.

Lee, H.-S., Tsai, S., Kuo, C.-C., Bassani, A. W., Pepe-Mooney, B., Miksa, D., Masters, J., Sullivan, R., Composto, R. J. 2012. Chitosan adsorption on hydroxyapatite and its role in preventing acid erosion. *Journal of Colloid and Interface Science*, 385(1):235–243.

Manoj, K. M. 2007. Fluoride menace in Orissa. Bhubaneswar: RCDC Centre for Water for Life. *RCDC INDIA*.

Mehta, R., Nandlal, B., Prashanth, S. 2013. Comparative evaluation of remineralization potential of casein phosphopeptide-amorphous calcium phosphate and casein phosphopeptide-amorphous calcium phosphate fluoride on artificial enamel white spot lesion: An in vitro light fluorescence study. *Indian Journal of Dental Research*, 24(6):681.

Nigus, K., Chandravanshi, B. S. 2016. Levels of fluoride in widely used traditional Ethiopian spices. *Fluoride*, 49(2):165–77.

Park, M., Bae, J., Lee, D. S. 2008. Antibacterial activity of [10]-gingerol and [12]-gingerol isolated from ginger rhizome against periodontal bacteria. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 22(11):1446–1449.

Patel, R. V., Thaker, V. T., Patel, V. K. 2011. Antimicrobial activity of ginger and honey on isolates of extracted carious teeth during orthodontic treatment. *Asian Pacific Journal of Tropical Biomedicine*, 1(1):S58–S61.

Pretty, I. A., Edgar, W. M., Higham, S. M. 2002. Detection of in vitro demineralization of primary teeth using quantitative light-induced fluorescence (QLF). *International Journal of Paediatric Dentistry*, 12(3):158–167.

Pulido, M. T., Wefel, J. S., Hernandez, M. M., Denehy, G. E., Guzman-Armstrong, S., Chalmers, J. M., Qian, F. 2008. The Inhibitory Effect of MI Paste, Fluoride and a Combination of Both on the Progression of Artificial Caries-like Lesions in Enamel. *Operative Dentistry*, 33(5):550–555.

Reynolds, E. C., Cain, C. J., Webber, E. L., Black, C. L., Riley, P. F., Johnson, I. H., Perich, J. W. 1995. Anticariogenicity of Calcium Phosphate Complexes of Tryptic Casein Phosphopeptides in the Rat. *Journal of Dental Research*, 74(6):1272–1279.

Ruan, Q., Zhang, Y., Yang, X., Nutt, S., Moradian-Oldak, J. 2013. An amelogenin–chitosan matrix promotes assembly of an enamel-like layer with a dense interface. *Acta Biomaterialia*, 9(7):7289–7297.

Sato, Y., Sato, T., Niwa, M., Aoki, H. 2006. Precipitation of octacalcium phosphates on artificial enamel in artificial saliva. *Journal of Materials Science: Materials in Medicine*, 17(11):1173–1177.

Shanbhog, R., Nikitha, B. S., Nandlal, B., Thippeswamy, M. 2016. Effect of dentifrice of varying fluoride concentration on surface microhardness of fluorosed enamel: an in vitro study. *European Archives of Paediatric Dentistry*, 17(4):257–264.

Shi, X. Q., Tranæus, S., Angmar-Månsson, B. 2001. Comparison of QLF and DIAGNO dent for Quantification of Smooth Surface Caries. *Caries Research*, 35(1):21–26.

Summitt, J. B., Robbins, J. W., Hilton, T. J., Schwartz,

R. S., Santos, J. 2006. Fundamentals of operative dentistry: a contemporary approach. pages 68–80. Quintessence Pub.

Xu, Z., Neoh, K. G., Lin, C. C., Kishen, A. 2011. Biomimetic deposition of calcium phosphate minerals on the surface of partially demineralized dentine modified with phosphorylated chitosan. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 98B(1):150–159.

Zhang, J., Lynch, R. J., Watson, T. F., Banerjee, A. 2018. Remineralisation of enamel white spot lesions pre-treated with chitosan in the presence of salivary pellicle. *Journal of Dentistry*, 72:21–28.