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Original Article

Dentistry Section

Effect of Green and White Tea Pretreatment on Remineralization of Demineralized Dentin by CPP-ACFP- An Invitro Microhardness Analysis

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ABSTRACT

Introduction: Mechanical performance of dentine is of major significance for the overall function of the teeth. Remineralization of carious dentine is the ultimate goal in re-establishing the functionality of the affected tissue so as to regain and maintain the mechanical properties of dentine. Functional remineralization of the affected dentin involves stabilization of both inorganic and organic component, but Caesin Phosphopeptide Amorphous Calcium Flurophosphate (CPP-ACFP) stabilizes only inorganic content. Hence to stabilize organic content and to bring in functional remineralization the use of anticollagenolytic and antielastastic agent was considered for this study.

Aim: To assess and compare the remineralization of artificial carious dentin pre treated with white and green tea, before and after application of CPP-ACFP using microhardness test. Null hypothesis was that both teas did not have any effect on remineralization potential of CPP ACFP.

Materials and Methods: Forty specimens were subjected to artificial caries lesions and were randomly divided into 4 groups based on the application of tea extract followed by CPP-ACFP (groups A & B) and CPP-ACFP followed by tea extracts (groups C & D). All the specimens were subjected to two pH cycling regimen. The specimens were subjected to Vickers microhardness test to obtain the microhardness values. The values were statistically analysed using one-way ANOVA and multiple comparisons with Tukey's HSD procedure.

Results: After the 1st and 2nd pH cycling in groups A and B, Group B showed significant increase in microhardness values (35.79± 3.12 VHN). But after the pH cycling regimen in groups C and D, microhardness values increased in 1st pH cycling (50.03± 3.64 VHN); (50.03±3.64 VHN), respectively but decreased during the 2nd pH cycling, (33.94±6.45 VHN); (33.11±6.11 VHN) respectively with the level of significance <0.05.

Conclusion: The results of this study rejects the hypothesis tested and showed that both the tea extracts increased the microharness values when used prior to the application of remineralizing agent. However, 10% white tea showed better microhardness indicating stabilization of collagen in dentine resulting in functional remineralization.

Keywords: Anticollagenolytic agent, Catechins, Collagen, Extracellular HAp, Intracellular HAp, pH cycling

INTRODUCTION

Dental caries is thought to be an irreversible disease, due to progressive demineralization of the tooth structure. But in 2001, Ernest Newbrun stated that "caries is a cyclic process with periods of demineralization due to the metabolism of a fermentable substrate by plaque flora, followed by periods of remineralization" [1]. Only after the paradigm shift in the model of dental caries, has remineralization gained more significance [2].

The demineralization and the remineralization process will remain within the physiological limits as long as the biological equilibrium is maintained. Progression of caries occurs once the tooth is constantly cloaked under a surface of biofilm, hence it is only when the speed and level of demineralization becomes dominant, that actual surface cavitation becomes possible [3,4].

Though several studies have been performed on remineralizing enamel, dentin remineralization becomes more important and significant because it is widely known that hardness and modulus of dentin increases in proportion to mineral concentration, thus influencing the overall property of the tooth [5,6]. Considering dentin from a microstructural perspective, the collagen fibrils in the dentine serve as a scaffold for mineral crystallites that reinforces the matrix. Mineralized dentine matrix plays a major role in preventing crack propagation, thus maintaining the functionality of the tooth. Hence it can be stated that remineralization of carious dentine reestablishes the functionality of the dentine [7].

Several approaches have been reported in an effort to remineralize dentin, using carboxylic acid–containing polyelectrolytes, phosphoproteins, fluoride and amorpous calcium phosphate resins [8,9]. Combination of Amorphous Calcium Phosphate (ACP) & Caesin Phospho Peptides (CPP) which form a complex (CPP – ACP) has been shown to be effective in precipitating calcium and phosphate ions and help in remineralizing teeth [10].

Caesin phospho peptides stabilizes calcium phosphate in nano complexes due to the presence of multi phosphoseryl sequences in amorphous calcium phosphate solutions.

Moreover multiple phosphoseryl sequences helps in binding the CPP to ACP in a metastable solution thus preventing dissolution of calcium and phosphate ions [11].

But CPP-ACP is known to stabilise only the inorganic content of the tooth. On the other hand true functional remineralization involves the stabilisation of both organic and inorganic components. This may be possible by the use of anti-collagenolytic agents during the process of remineralization.

Tea is known to have catechins like epigallocatechin gallate (EGCG), epicatechin gallate, epigallocatechin, epicatechin. Both green and white teas are known to contain these catechins in large volumes [12]. Isolated green tea catechins such as EGCG have already been shown to be inhibitors of collagenase and elastase. In comparison the white tea exhibits significantly higher anti elastase and anti collagenase activities [13]. Recent studies have evaluated that EGCG exhibits profound inhibitory activity on collagenases that degrade the organic matrix during erosion [14,15]. Hence, this study was undertaken to evaluate the effect of two anticollagenolytic agents, green tea and white

tea on the remineralizing effect of CCP-ACP and microhardness of demineralized dentin. Null hypothesis was that green tea and white tea did not have any effect on remineralization potential of CPP-ACP.

AIM

To assess and compare the remineralization of artificial carious dentin pre treated with white and green tea, before and after application of CPP-ACFP using microhardness test. Null hypothesis was that both teas did not have any effect on remineralization potential of CPP-ACFP.

MATERIALS AND METHODS

Microhardness of demineralized and remineralized dentine was evaluated. This study was conducted in the Department of Conservative Dentistry and Endodontics, SRM Dental College & Hospitals, Chennai, Tamil Nadu, India. The study started in January 2009 and was conducted over a time period of eight months. The ethical committee approval was obtained for this study.

Study sample, inclusion and exclusion criteria: 20 freshly extracted human third molars were collected. Erupted teeth, teeth with fractures, cracks, caries, defects, teeth with developmental anomaly and anatomical variations were not included in the present study.

Materials used

Grenn tea; White tea; CPP-ACFP -Casein phosphopeptide amorphous calcium fluoride phosphate {GC Tooth Mousse Plus (Recaldent, GC corporation, Japan)} contains pure water, glycerol, CPP-ACP, D-sorbitol, CMC –Na, propylene glycol, silicon dioxide, titanium dioxide, xylitol, phosphoric acid, sodium fluoride, flavouring agent, sodium saccharin, ethyl P – hydroxybenzoate, propyl P –hydroxybenzoate, butyl P – hydroxybenzoate; artificial caries solution; 40 dentine blocks.

Preparation of the extracts

10% green tea (Tetrahedron Beverages inc., Chennai, India) and white tea extracts (Nutra Max, Changsha Nutramax inc., China) solution were prepared by weighing 50gm of the tea powder and dissolving it in 500ml of distilled water each.

Preparation of Demineralizing solution

$CaCl_2$ (2.2mM), NaH_2PO_4 (2.2mM), lactic acid (0.05 M), fluoride (0.2 ppm), adjusted with 50% NaOH to a pH 4.5.

Preparation of artificial carious lesion

Artificial carious lesions were produced by suspending the specimens into glass tubes containing 20ml of demineralizing solution, for 72 hours, at room temperature.

Preparation of artificial saliva

The composition of artificial saliva used was Na_3PO_4 (3.90mM), NaCl (4.29mM), KCl (17.98mM), $CaCl_2$ (1.10mM), $MgCl_2$ (0.08mM), H_2SO_4 (0.50mM), $NaHCO_3$ (3.27mM) and distilled water with the pH adjusted to 7.2.

Preparation and grouping of the specimens for microhardness evaluation: Specimens were prepared from 20 extracted human third molars free of caries and defects. Radicular part of each tooth was removed and the coronal part was then longitudinally sectioned in the mesio-distal direction into two sections using a diamond tipped disc under high speed and water coolant. Having colour as a criteria to differentiate enamel and dentine, the enamel was removed to expose the dentin, using a coarse grit diamond under water coolant. Dentin specimens were ultrasonicated in a deionized water bath to remove any debris. The dentin specimens were embedded in self cure acrylic resin and allowed to set to create dentin blocks. An acid resistant nail varnish was applied around the exposed dentin surface leaving a window of 3mm X 3mm of dentin exposed at the centre.

Baseline microhardness values were obtained using Vicker's microhardness tester (Zwick Roell Indentec, Zwick Inc, Germany) at a load of 25gm that was applied for five seconds. Vickers Hardness Number of five indentations at spacing of 100 microns were taken and the average value was considered the mean baseline micro-hardness for each specimen.

All the specimens were immersed in the demineralized solution at a specific time period to induce artificial carious lesion. After induction of dentin lesions, all the specimens were evaluated again for surface micro-hardness.

The specimens were then randomly assigned to four groups; in groups A and B, specimens (n= 10 each) were immersed in green and white tea respectively, followed by application of CPP-ACFP. In groups C and D (n = 10 each), CPP-ACFP was applied to the specimens after which they were immersed in green and white teas respectively.

The following protocol was followed for all the groups:

Each group was subjected to two pH cycling regimen. Each pH cycling regimen consisted of demineralization for three hours, followed by experimental application protocol and storage in artificial saliva for 21 hours. This was done for two consecutive days.

Group A: 1st pH cycling regimen with 10% green tea extract.

Ten specimens were subjected to demineralization phase for 3 hours; and for the remaining 21 hours they were immersed in 20 ml of green tea. This was repeated again for another day, after which surface microhardness was measured.

2nd pH cycling regimen with CPP-ACFP:

The same specimens were again subjected to the demineralization phase for 3 hours, after which CPP-ACFP was applied on the surface and left for three minutes. They were then immersed in 20ml of synthetic saliva for 21 hours. This pH cycling was done for a period of two days followed by surface microhardness evaluation.

The same protocol was followed in group B using 10% white tea instead of green tea. In groups C and D, the 1st pH cycling was with CPP-ACFP followed by 10% green tea and 10% white tea extract respectively.

The values were statistically analysed using one-way ANOVA and multiple comparisons were made with Tukey's HSD procedure.

RESULTS

One-way ANOVA and Post-hoc tukey is given in [Table/Fig-1-5]. The microhardness values of all the groups are given in [Table/Fig-6].

Baseline microhardness values of normal dentin (57.23± 9.15 VHN) significantly dropped after demineralization (15.4±4.9 VHN) In groups A and B, after 1st pH cycling, microhardness measured were significantly higher (28.47±8.1VHN); (35.79±3.12 VHN) respectively, than demineralized values. Moreover, it significantly

	Mean±S.D.	DIFFERENCE Mean±S.D.	p–Value
Normal dentin (baseline value)	57.23±9.15	41.83±2.68	<0.05(sig.)
Demineralized dentin	15.4±4.9		

[Table/Fig-1]: Comparison of mean and standard deviation of Vicker's mocrohardness vlaues after production of artificially demineralized lesions.
One-way ANOVA was used to calculate the p-value
Turkey – HSD procedure was employed to identify the significant groups.

	Mean ± S.D.	DIFFERENCE Mean±S.D.	p–value
Demineralized dentin	15.4±4.9		
Group A (GT)	28.47±8.1	13.07±2.68	<0.05(sig.)
Group B (WT)	35.79±3.12	20.39 ±2.68	
Group C & D (CPP-ACFP)	50.03±3.64	34.63±2.68	

[Table/Fig-2]: Comparision of mean and standard deviation of vicker's microhardness values among the groups after 1st pH cycling with demineralized dentin.
One-way ANOVA was used to calculate the p-value
Turkey – HSD procedure was employed to identify the significant groups

	Mean ± S.D.	DIFFERENCE Mean±S.D.	p–value
Normal Dentin (baseline value)	57.23±9.15		
Group A (GT)	28.47±8.1	28.7±2.68	<0.05(sig.)
Group B (WT)	35.79±3.12	21.44±2.68	
Group C & D (CPP-ACFP)	50.03±3.64	7.2±2.68	

[Table/Fig-3]: Comparision of mean and standard deviation of vicker's microhardness values among the groups after 1st pH cycling with normal dentin.
One-way ANOVA was used to calculate the p-value
Turkey – HSD procedure was employed to identify the significant groups

	Mean ± S.D.	DIFFERENCE Mean±S.D.	p–value
Demineralized dentin	15.4±4.9		
Group A (GT+CPP-ACFP)	46.96±3.72	31.56±2.68	<0.05(sig.)
Group B (WT+CPP-ACFP)	58.65±5.85	43.25±2.68	
Group C (CPP-ACFP+GT)	33.94±6.45	18.84±2.68	
Group D (CPP-ACFP+WT)	33.11±6.11	17.71±2.68	

[Table/Fig-4]: Comparision of mean and standard deviation of vicker's microhardness values among the groups after 2nd pH cycling with demineralized dentin.
One-way ANOVA was used to calculate the p-value
Turkey – HSD procedure was employed to identify the significant groups

	Mean ± S.D.	DIFFERENCE Mean±S.D.	p–value
Normal dentin (baseline value)	57.23±9.15		
Group A (GT+CPP-ACFP)	46.96 ±3.72	10.27±2.68	<0.05(sig.)
Group B (WT+CPP-ACFP)	58.6±5.85	-1.42 ±2.68	
Group C (CPP-ACFP+GT)	33.94 ±6.45	23.29±2.68	
Group D (CPP-ACFP+WT)	33.11±6.11	24.12±2.68	

[Table/Fig-5]: Comparision of mean and standard deviation of vicker's microhardness values among the groups after 2nd pH cycling with normal dentin.
One-way ANOVA was used to calculate the p-value
Turkey – HSD procedure was employed to identify the significant groups

		Mean±S.D.	p–value
	Normal dentin (baseline value)	57.23±9.15	<0.05(sig.)
	Demineralized dentin	15.4±4.9	
Group A	GT (1ST pH Cycling)	28.47±8.1	
	GT + CPP –ACFP (2ND pH Cycling)	46.96±3.72	
Group B	WT (1ST pH Cycling)	35.79±3.12	
	WT + CPP –ACFP (2ND pH Cycling)	58.65±5.85	
Group C	CPP - ACFP (1ST pH Cycling)	50.03±3.64	
	CPP –ACFP + GT (2ND pH Cycling)	33.94±6.45	
Group D	CPP - ACFP (1ST pH Cycling)	50.03±3.64	
	CPP –ACFP + WT (2ND pH Cycling)	33.11±6.11	

[Table/Fig-6]: Comparison of mean and standard deviation of vicker's microhardness values in group a after 1st and 2nd pH cycling with normal dentin and demineralized dentin.

increased after 2nd pH cycling in both the groups (46.96±3.72 VHN); (58.65±5.85 VHN) respectively. Among both the teas, white tea showed significantly higher values than green tea.

After 1st pH cycling in groups C and D, the microhardness significantly increased (50.03±3.64 VHN); (50.03±3.64 VHN) respectively, compared to both demineralized samples and groups A & B values. However, this decreased significantly after the 2nd pH cycling (33.94±6.45 VHN); (33.11±6.11VHN) respectively, though the values were still greater than those of demineralized dentine.

Among the groups, the 2nd pH cycling values of groups C and D were significantly lower than those of groups A and B.

DISCUSSION

Identification of early caries lesions and treatment with non surgical methods such as remineralization, is a contemporary approach that has been well received and adopted. The concept of minimally invasive dentistry through remineralization of diseased tooth is of great significance for retaining remaining tooth structure [16,17].

Enamel caries and dentin caries are two independent entities due to the remarkable differences between both tissues. Unlike enamel, of which the organic matrix makes up only about 0.4 – 0.6% volume, dentin possesses 30% vol organic matrix comprising 90% collagen and 10% non-collagenase protein providing itself as a substrate for degradation by either bacteria or host proteinases [16].

Bacterial proteases have always been considered the major culprit in the degradation of dentin extracellular matrix during the caries process [18]. However, several studies have suggested that host derived matrix metalloproteinases (MMPs) also participate in dentin destruction following demineralisation by bacterial acids [19-21].

Microstructure, mineral density and location of the minerals with respective organic structures of the tissue which influence the competence of dentine, dictates the functionality of dentine. Hence functional remineralization was considered for this study [7].

Several studies have proven that CPP binds to the pellicle and plaque on the tooth surface and stabilizes high concentrations of calcium and phosphate along with the fluoride ions present. These ions effectively promote remineralization in, invivo conditions, as they diffuse down concentration gradients into enamel subsurface lesions due to their free bioavailability [11,22-26].

During remineralization process of enamel, along with calcium and phosphate, fluoride ions play an important role in the formation of FA $\{Ca_{10}(PO_4)6F_2\}$.

Even though CPP-ACFP is known to re-mineralize dentin, functional remineralisation of the affected dentin by the agent on its own remains doubtful [7,9].

The anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants were compared and observed that the highest activity was seen in white tea (-87%), green tea (-47%), rose tincture (-41%) and lavender (-31%). Both green and white teas are known to have catechins like epigallocatechingallate (EGCG), epicatechingallate, epigallocatechin, epicatechin in large volume [12,13,26-29].

Hence in this study green tea and white tea pretreatment were comparatively evaluated for their effect on re-mineralization of artificial carious dentin, before and after application of (CPP-ACFP) using microhardness test.

The preparation of artificial carious lesions was in accordance with the protocol followed by Christos-Rahiotis et al., [30]. According to Rehder-Neto et al., this procedure produces a lesion to a depth of around 150µm and the micro hardness at this depth is similar to a natural carious lesion at the same depth [31].

Among invitro protocols commonly employed in cariology research, pH cycling models involve exposure of dental substrates

that is enamel or dentin, to combinations of demineralization and remineralization [32]. These experiments are designed to mimic the dynamics of mineral loss and gain involved in caries formation. Thus, the pH cycling model was preferred in this study [33].

Properties measured under hydrated conditions will be more relevant and provide more realistic estimates of those found invivo since hydration and dehydration affect the mechanical properties of the dentin, especially demineralized dentin. Hence this protocol was observed in the present study [9,34].

According to the results of this study, the mean baseline microhardness values of normal dentin samples ranges from 50.67 to 63.78 ± 9.15VHN (baseline value) [Table/Fig-1]. These Vicker's microhardness values are in accordance with other microhardness studies done by Victoria Fuentes et al., and Fernanda Brandao Mollica et al., [35,36].

The artificial demineralised lesions showed a significant decrease (15.4 ± 4.94 VHN) in the Vicker's microhardness compared to the baseline value [Table/Fig-1]. These values were comparable to those obtained by Marcella Marquezan et al., [37].

1st pH cycling: [Table/Fig-2,3] In groups A and B, there was an increase in the hardness values compared to demineralised samples after the first pH cycling regimen, with green tea (28.47 ± 8.1) and white tea (35.79 ± 3.12). Though green tea and white teas are non-mineralising agents, the marginal increase in the hardness values could be due to their anti-collagenolytic, anti-elastase and anti-oxidant activity on collagen structure. MT Kato et al., attributed the inhibitory activity of green tea against MMPs to EGCG, that exhibits a hydrogen bonding and hydrophobic interaction with collagenases which may be responsible for the change in secondary structure of collagenases and consequently their inhibition [38]. After 1st pH cycling, groups C & D (CPP-ACFP without pre-treatment) showed a significant increase in the hardness values (50.03 ± 3.64 VHN) compared to the demineralised values (15.4 ± 4.94 VHN). This increase could be attributed to the remineralizing potential of CPP-ACFP [30].

CPP- ACFP exhibits a synergistic effect by formation of CPP stabilised amorphous calcium fluoride phosphate, resulting in the increased incorporation of fluoride ions in to plaque [39]. The substitution of F for OH in the lattice brings about a reduction in the volume of the unit cell so that the chemical stability of the apatite lattice is enhanced due to the electro static bond between fluoride and the adjacent ions. Fluoride can impede the rate of demineralisation by reducing the solubility of precipitating fluoridated apatites and thereby increasing the driving force for apatite formation [4].

2nd pH cycling: [Table/Fig-4,5] When the specimens of group A and B were treated with CPP – ACFP following application of the respective teas (2nd pH cycling), both group A (46.96±3.72 VHN) and group B (58.65±5.85 VHN) showed a significant increase in microhardness compared to demineralised samples. This may be due to the stabilization of collagen prior to application of the remineralizing agent that could have resulted in functional remineralization of dentin.

Also, white tea showed a significant increase in microhardness value compared to green tea. This could be due to the superior anti-collagenolytic action of white tea [12,13].

It was further noted that during the 2nd pH cycling regimen in group C and group D with green tea and white teas respectively, there was a significant decrease in the hardness values compared to the values obtained after the 1st pH cycling with CPP-ACFP. This indicates that the minerals deposited during remineralization with CPP-ACFP during the first pH cycling were in poor association with the organic collagen structures. Hence, on subsequent exposure to green tea and white tea there is a probable leaching of minerals into the surrounding medium.

Hypothesis of functional remineralization: The highlight of this study is the significant increase in the microhardness values of white tea after 2nd pH cycling, compared to CPP-ACFP alone (1st pH cycling) [Table/Fig-6]. This clearly substantiates collagen stabilisation of dentine by the tea extracts.

Dentin matrix is mainly composed of type I collagen fibrils with associated non-collagenous proteins, forming a three dimensional matrix that is reinforced by the carbonated nano crystalline mineral (HAp). The hydroxyapatite that is located in the spaces separating the collagen fibrils are termed extrafibrillar minerals, and those present in the gap regions of the fibrils extending between tropocollagen molecules are termed intrafibrillar minerals.

In normal dentine, the extrafibrillar HAp is highly compliant due to moisture and attached proteins, whearas the intrafibrillar mineral resists demineralization and dominates the elastic behaviour of collagen fibrils during loading, influencing the stiffness of the collagen fibrils. In a partially demineralized dentine, there can be presence of intact collagen fibrils with remnant minerals along with non collagenous proteins. This could play an important role during remineralization, as these could act as sites for regrowth of the lost minerals [9].

Simple precipitation of minerals into the demineralised dentin matrix provides an increased mineral content but may not necessarily provide an optimal interaction with the organic components of the dentin matrix [9]. This could be the reason for the decreased hardness values after remineralization with CPP-ACFP after 2nd pH cycling.

In the absence of the intra-fibrillar minerals and their optimal interaction within the collagen fibrils, dentin matrix tend to incorporate water and swell, more than the sound dentine. As a result, the compressive stresses that consolidated the extrafibrillar minerals no longer exist. The elastic constants become largely dependent on the highly deformable organic network and therefore are quite low. The net effect may be high mineral content, but very low mechanical properties [9].

According to the results of the study, the use of white tea and green tea increased microhardness of dentine indicating functional remineralization. This could be due to the action of tea on the collagen network which may have stabilized the collagen and maintained the collagen network in an expanded state so that the intrafibrillar spaces are left open for remineralization [9]. Hence, the results of this study negates the hypothesis tested.

The actual interaction of the white tea with organic and inorganic constituents of the dentine needs to be evaluated at the nanolevel, hence further studies are required to interpret at molecular level.

LIMITATION

The actual functional remineralization needs to be evaluated at the molecular level.

CONCLUSION

Under the limitations of this study, it can be concluded that, prior to the application of remineralizing agents, pretreatment with white tea can be considered as a viable option to functionally remineralize the demineralized dentine. Stability of the collagen improves the mechanical properties of the tooth and can influence the bond strength of the adhesive restoration and their durability.

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