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Comparative Study on The Remineralizing and Antimicrobial Effect of Naturally and Synthetically based Agents

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Comparative Study on The Remineralizing and Antimicrobial Effect of Naturally and Synthetically based Agents

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Restorative Dentistry
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ABSTRACT

Purpose: This study aimed to evaluate and compare the remineralizing and antimicrobial effect of chitosan-based agent and Flavonoid. **Materials and methods:** This study was carried out in two phases; remineralization phase and antimicrobial phase. Ninety (90) bovine mandibular incisors were used in the study with forty-five (45) teeth in each phase. Teeth were prepared and randomized into three groups according to treatment material (n=15 samples) as follows; group I; control group (being immersed in artificial saliva), group II; treated with flavonoid and finally group III; treated with chitosan-based agent. Each agent was applied for 10 minutes six times/day. In phase (1) remineralizing effect was measured through recording radiodenisty (by using digital radiography) at baseline, after creation of lesion and after treatment. In phase (2) antimicrobial effect was measured (by using colony forming unit test and lactic acid production test) at baseline and after treatment. Data were obtained and the differences between groups were estimated by ANOVA test followed by Tukey's post hoc test. **Results**: Statistical analysis by ANOVA test revealed that there was high statistically significant difference between the study groups. Dentin blocks treated by chitosan-based agent showed greater remineralizing effect than flavonoid. Dentin blocks with 1,4 and 7 days biofilms treated with Flavonoids showed greater inhibition of Lactic acid production and Colony forming unit. **Conclusion**:- Chitosan-based agent can be used as remineralizing agent while flavonoid can be used as antimicrobial agent against S.mutans by inhibiting colony forming unit and lactic acid production..

KEYWORDS

Remineralization,
Chitosan,
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- Paper extracted from Master thesis titled "Comparative Study on The Remineralizing and Antimicrobial Effect of Naturally and Synthetically-based Agents".

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INTRODUCTION

Dental caries is a dietary carbohydrate-modified bacterial communicable disease and it is one of the most common global oral health problems within the world today, It's defined as the destruction of the dental hard acellular tissue by acidic by-products from the bacterial fermentation of dietary carbohydrates, particularly sucrose [1]. It's caused by bacteria fermenting sucrose (a common food carbohydrate), which breaks down and eliminates the hard a cellular tissue in teeth. The pH of plaque fluctuates because of a mismatch in the balance between tooth minerals and dental biofilms. Streptococcus mutans is the most common cause of tooth decay [2].

Human dental caries has been linked to Streptococcus mutans. Bacterial biofilm formation on tooth surfaces, which is known as dental plaque, is an essential pathogenic feature. As a consequence of these factors, dental plaque and dental caries are more likely to occur in people who eat foods high in glycosyltransferase and other glucan-binding proteins produced by this organism[3].

Flavonoids are a class of bioactive compounds that have antibacterial, antioxidant, and anti-inflammatory activities [4]. There are a broad variety of fruits and vegetables that have high concentrations of these substances. In addition to their anti-inflammatory and anti-tumor properties, flavonoids may interact with proteins like collagen and receptor molecules, as well as anion molecules like calcium and iron. Dentists have recently been interested in flavonoids. Hesperidin and green tea extract, as well as genipin and proanthocyanidin, have been found to have proven beneficial in promoting oral health[5]. In addition to preserving dentine collagen and stimulating mineral deposition on root surface caries, in vitro and in situ. Besides flavonoids in cranberry extract was found to inhibit the formation of dental biofilm and reduces plaque formation[6].

Chitosan is a biocompatible and non-toxicity polymer obtained by the deacetylation of chitin, one of the most common polymers in nature, and has been widely used in drug delivery systems and tissue engineering[7]. Its positive charge accumulates on the cell walls of the bacteria, promoting a bactericidal and bacteriostatic property coupled with the ability to form a film and adhere to the tooth, making chitosan an ideal base for sustained drug release positive effect on dental health[8].

Its capacity to attach to the teeth and create a film, as well as its propensity to release medication over time, makes chitosan a perfect base for continuous medicine release. As an antibacterial and plaque-reducing agent, chitosan has been found to be efficient in inhibiting the adherence of S. mutans. There are several long-lasting oral anticaries formulas that employ the chitosan in combination with propolis, nano-silver, and chlorhexidine to provide a synergistic antiplaque action [9-11].

Recent research has shown that chitosan may be utilised in dental toothpastes (Chitodent), mouthwash solutions, and chewing gums to cure dental caries. When tested in vitro, chitosan inhibits the growth and adhesion of cariogenic bacteria as well as the demineralization of tooth enamel, and when tested in vivo, it stimulates salivation[12]. Therefore,Chitosan-based chemicals and flavonoids were tested for their ability to remineralize and kill bacteria in this research.

MATERIALS AND METHODS

1- Flavonoid:

In this work, a 6.5 percent (w/v) solution of each flavonoid in phosphate buffer (0.025 M KH_2PO_4, pH 7.4) was utilized.

2- Chitosan based agent:

- Nano Tech's chitosan (middle-viscous with a deacetylation level of 80%).
- Fluka BioChemika's-glycerol phosphate disodium salt pentahydrate (-GP).

- Fluka BioChemika supplied the hydroxyethyl cellulose (HEC).
- Nano Tech provided nano amorphous calcium phosphate (NACP).
- The El-Gomhouria Company in Egypt supplied sodium fluoride (NaF).
- Casein phosphopeptide (CPP) was acquired from the Chinese company Xi'an Lyphar Biotech Co.

Preparation of chitosan-based gel:

Chitosan solution (2 percent w/v) was made by mechanically agitating 1600 mg chitosan flakes in 80 ml 0.15 M acetic acid aqueous solution. The resulting chitosan solution had a pH of 4.8. Before adding the remaining components, the chitosan solution was chilled to 4oC in an ice bath for 15 minutes. An aqueous solution of -glycerol phosphate (-GP) was made by dissolving 16 g -GP in 24 ml distilled water, cooled to 4°C, and dropwise added to the chilled chitosan solution. This specific quantity of -GP was chosen to increase the pH of the chitosan solution to 7.2 and to neutralise the positive charges on the chitosan chains, permitting gelation while retaining bio adhesiveness, as will be detailed later. To create a clear solution, the resulting mixture was rapidly agitated for five minutes. 175mg of hydroxyethyl cellulose (HEC) was dissolved in 16 mL of distilled water to make an aqueous solution. The HEC solution was then cooled and dropped into the ice-cold chitosan/-GP combination, where it was stirred for another five minutes. These concentrations of -GP and HEC were added to the chitosan gel since these ratios have previously been studied in prior author's study.

As fluoride and calcium/phosphate ion sources, sodium fluoride (NaF) (2%) and nano amorphous calcium phosphate (NACP) (20%) were added to the chitosan/-GP/ HEC combination. These NaF and NACP doses were chosen because earlier studies indicated them to be beneficial in inducing enamel remineralization. A 10% concentration of casein phosphopeptide (CPP) was added to the mixture. Carboxymethyl cellulose (CMC) (50 mg) was dissolved in 15 ml deionized water before being progressively added to the mixture as a thickening agent to achieve the desired paste-like consistency. The produced gel was then refrigerated in a firmly sealed container until testing [13].

Collection of teeth:

This study was divided into two phases (remineralization) and (antimicrobial) phase. Ninety bovine mandibular incisors teeth were used for each phase .Ninety bovine mandibular incisor teeth were from an animal that was free from any illnesses and anomalies visible to the human eye. The teeth were cleaned and rinsed before being kept in distilled water (dH_2O) at 4°C until usage.

Research ethics approval was obtained from Research Ethics Committee(REC),Faculty of Oral and Dental medicine for girls Al-Azhar University with approval code

(REC-OP-21-03)

Sample size:

There were ninety bovine incisors utilized in each phase. According to the treatment material, the teeth were divided into three groups (30 samples each).

Group I the chitosan-based agent was used in

Group II. Flavonoids were used in

Group III control group artificial saliva were used in.

Specimens preparation:

A diamond-coated band saw (Struers Minitom; Struers, Copenhagen, Denmark) was used to cut 90 bovine permanent incisor crowns into 5x5x2 mm slices, which were cooled by constant water flow. In order to expose the dentin, a portion of the lingual

and buccal enamel was removed. It was used to insert polymethylmethacrylate dentin blocks. The flattening of these surfaces was accomplished using water-cooled carborundum discs of different grits of waterproof silicon carbide paper (1000, 1200, 2400, 3000 and 4000). To eliminate any leftover abrasives, each polished sample was sonicated for five minutes in distilled water.

1. For testing remineralizing effect:

- ***Lesion formation:***

Following the technique., the progression of lesions and pH-cycling were monitored. At least 96 hours in a demineralizing solution at 37°C produced 12 lesions 70–100 mm in depth. The pieces were thoroughly cleaned with deionized water in order to preserve the original lesion. Acid-resistant nail polish was applied to the opposite half of each specimen's window to maintain a baseline lesion (provided by Revlon Corp., NY, USA) [14].

- ***PH cycling regimen:***

The demineralized specimens were divided into three groups (n = 10 per group) based on treatments: (1) flavonoids (2) chitosan based agent (3) control (artificial saliva). All specimens were pH cycled through the treatment solutions (10 minutes), acidic buffer (50 mM acetate; 2.25 mM $CaCl_2.2H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl; pH 5.0; 30 minutes) and neutral buffer (20 mM HEPES; 2.25 mM $CaCl_2.2H_2O$; 1.35 mM KH_2PO_4; 130 mM KCl, pH 7.0; 10 minutes). Six demineralization–remineralization cycles were performed each day and continued for 1,4 and7 days. All solutions were freshly prepared just before use. The specimens were stored in a neutral buffer overnight at 37°C.

- ***Digital radiography records assessment:***

Standardized reproducible periapical radiograph for each treated specimen was obtained using a digital image plate, held in film holder for optimizing parallel technique with standard image. The image plate was exposed by the x-ray machine at 70 Kilovolt and 0.5 Ma dose for 0.04 seconds. Assessment of remineralization was done using measurements of radiodensity (%). Vista scan system (Fig. 1) and DBSWin software were used utilized for performing these measurements. The mineral density mean value was calculated from the equation (average density= average intensity / profile); the profile was determined directly in the utilized software.

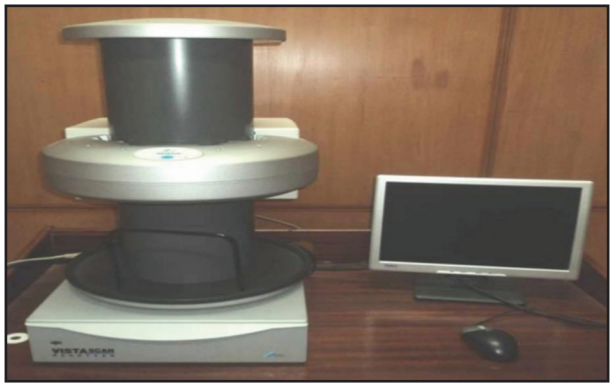

Figure (1): Vista scan system

2. For testing antimicrobial effect:

- ***Inoculation:***

Every tooth was placed in a 24-well plate with the enamel surface facing up. 90 dentin specimens were divided into three groups and treated for 5 minutes prior to injection with the 2 percent gel, the flavonoid, and a control group that was treated with artificial saliva. Thereafter the specimens were placed onto a new 24 well-plate and incubated at 37°C in 5 percent CO_2 with a culture of S. mutans, which contained 1 percent sucrose. For 7 days, each specimen was incubated at 37°C in 5% CO_2 and transferred to a new 24-well plate filled with fresh media, which was then incubated for an additional 8 hours [15].

- ***Colony forming unit counts of adherent biofilms:***

Dentin blocks that had been exposed to biofilms for 1, 4, and 7 days were sonicated and vortexed using a vortex mixer to remove biofilms (Fisher,

Pittsburgh, PA, USA). It took 48 hours at 37oC for the bacterial suspensions, which were serially diluted and disseminated out on BHI plates, to incubate [16].

- ***Lactic acid production by adherent biofilms:***

Biofilms on dentin blocks were washed with CPW before being transferred to 24-well plates (n 14 3) containing 1.5 ml of BPW + 0.2 percent sucrose in order to eliminate any loose bacteria. For the following 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) experiment, the specimens were rinsed with PBS and incubated for 3 hours to allow the biofilms to produce acid. Lactate was extracted from the BPW samples using an enzymatic method. The absorbance of BPW at 340 nm was measured using a microplate reader. Standard curves were generated using a standard lactic acid solution (Supelco Analytical, Bellefonte, PA, USA) [17].

Statistical analysis:

Statistical analysis was conducted using (SPSS 18; SPSS, Chicago, IL, USA). They displayed the mean and standard deviation of the data set (SD). In order to assess the data, the Kolmogorov-Smirnov test of normality was applied. Comparisons were made across groups and between observation periods within the same groups using the One-Way ANOVA test. We were able to compare different groups using Tukey's post hoc test. The significance level was established at a P value of 0.05.

RESULTS

I. For remineralizing effect:

Results obtained from remineralizing test are shown in table (1)

During day 1, the chitosan group had the highest mean value of 183.5710.69, followed by the Flavonoid group, which had the lowest value of 148.438.79. An ANOVA test found a statistically significant difference (P=0.00) in the results. For each of the two groups, Tukey's post hoc test found significant differences.

Table (1) *Descriptive statistical analysis of mean values and standard deviation of all groups at all time intervals of recorded value and comparison between chitosan based agent and flavonoid groups (ANOVA test)*

		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	F Value	P Value
					Lower Bound	Upper Bound				
	Control	114.70^{c}	6.24	1.97	110.24	119.16	107.00	125.00	196.703	.000*
Day1	**Flavonoid**	148.43^{b}	8.79	1.97	144.32	152.55	133.67	164.00		
	Chitosan	183.57^{a}	10.69	2.39	178.56	188.57	164.33	199.33		
	Control	114.70^{o}	6.24	1.97	110.24	119.16	107.00	125.00	158.265	.000*
Day4	**Flavonoid**	166.08^{n}	27.27	6.10	153.32	178.85	134.67	199.33		
	Chitosan	238.22^{m}	11.08	2.48	233.03	243.40	218.33	250.33		
	Control	114.70^{z}	6.24	1.97	110.24	119.16	107.00	125.00	328.470	.000*
Day7	**Flavonoid**	135.90^{y}	7.99	1.79	132.16	139.64	126.00	155.33		
	Chitosan	221.33^{x}	17.76	3.97	213.02	229.65	179.00	244.00		

*Significance level p≤0.05, *significant*

On day 4, the chitosan group had the greatest mean value (237.22-11.08), followed by the flavonoid group (166.08-27.27) and the control group (114.7-6.24). The lowest mean value was found in the chitosan group. ANOVA test found a statistically significant difference (P=0.00) between the two groups There was a statistically significant difference between the two groups, as shown by Tukey's post hoc test.

Chitosan had the highest mean value at day 7 (221.3317.76), followed by Flavonoid (135.97.99) and the control (114.76.24) groups. An ANOVA test found a statistically significant difference (P=0.00) between the two groups. To assess for statistical significance, Tukey's post hoc test was used.

II. For antimicrobial effect:

1- Colony forming unit(CFU):

CFU counts are shown in table (2) for specimens treated with chitosan-based agent, flavonoid and artificial saliva at 1, 4, and 7 days. The chitosan-based compound reduced the CFU on every day compared to the control. In spite of the fact that CFU dropped, it was still larger in the chitosan-based agent compared to the Flavonoid group. The CFU of all the groups increased, but the CFU of the Flavonoid groups remained lower than the CFU of the other groups. In the samples treated with Flavonoid, there was essentially little antibacterial action, as predicted.

2- Streptococcus mutans lactic acid production*: (m mole/ L)*

Table (3) shows the chitosan-based drug, Flavonoid, and artificial saliva were used to treat the 1-, 4-, and 7-day-old species, respectively. It was the biofilms on the control surfaces that generated the greatest concentrations of acid. The Flavonoid therapy reduced acid generation on a daily base, compared to the chitosan group. Chitosan and contral acid production did not change much when the inoculation duration was doubled, however Flavonoid acid production increased. The acid production of the Flavonoid group and chitosan groups, notably the Flavonoid group, dropped considerably.

Table (2) *Descriptive statistical analysis of mean values and standard deviation of all groups at all time intervals of colony forming unit and comparison between and within groups (ANOVA test)*

		Chitosan		Flavonoids		Control		Between groups	
		Mean	SD	Mean	SD	Mean	SD	F value	P value
CFU of *Streptococcus mutans*	Day 1	5.95	0.02	3.58	0.19	8.89	0.08	14367.6	0.00*
	Day 4	6.44	0.08	5.87	0.08	11.68	0.07	49831.6	0.00*
	Day 7	8.80	0.04	6.40	0.17	12.32	0.12	17234.4	0.00*
Within group	F value	20827.9		2830.7		10888.1			
	P value	0.00*		0.00*		0.00*			

*Significance level p≤0.05, *significant*

Table (3) *Descriptive statistical analysis of mean values and standard deviation of all groups at all time intervals of Streptococcus mutans lactic acid production 1- day (m mole/ L) and comparison between and within groups (ANOVA test)*

		Chitosan		Flavonoids		Control		Between groups	
		Mean	SD	Mean	SD	Mean	SD	F value	P value
m mole/ L	Day 1	3.99	0.46	2.86	0.37	13.61	0.94	1699.4	0.00*
	Day 4	6.63	0.40	5.07	0.59	15.82	0.48	2741.4	0.00*
	Day 7	2.97	0.16	1.69	0.33	11.31	0.57	3564.1	0.00*
Within group	F value	538.9		297.6		212.07			
	P value	0.00*		0.00*		0.00*			

*Significance level p≤0.05, *significant*

DISCUSSION

Microbial glucose metabolism is intimately connected to the progression of tooth decay. With its ability to produce acid and extracellular polysaccharides, Streptococcus mutans has become a major cause of dental caries. Due to a continual decline in pH, teeth begin to lose minerals, leading to tooth hard-tissue demineralization and dental cavities. Keep antibacterial and remineralizing drugs on teeth as long as possible to help prevent or heal early enamel lesions, as long as they are safe. The traditional mouthwash raises a concern of the time interval between applications, which results in a reduced concentration of the agents over time between the doses. A lack of antibacterial or remineralizing chemical therapy may allow pathogenic microorganisms to recolonize on the tooth surface [18].

To take use of chitosan's unique biological properties, a chitosan-based enamel remineralizing gel was developed and compared to flavonoid. Although chitosan is the main component of the experimental gel, other materials inserted at much lower percentages play a vital role in changing the properties of the chitosan gel in order to properly transport and distribute the minerals necessary for successful remineralization. As a consequence rephase the quantities of these compounds has a considerable influence on the performance of the gel. In aqueous acidic circumstances, for example, the amine groups on the chitosan chains get protonated and develop positive charges, resulting in repulsion between the chains and the chitosan in solution.

The addition of -GP to the solution partially neutralizes the positive charges on the chitosan chains, allowing them to migrate closer together. This chain approximation enables the production of numerous inter-chain interactions, such as hydrogen bonding and hydrophobic interactions, which eventually culminate in gelation[19]. The positive charges on the chitosan chains, on the other hand, are the fundamental reason of the chitosan's beneficial bioadhesive properties, as they allow the chitosan to be electrostatically attracted to negatively charged biological surfaces[20].

Demineralization and degradation of dentine's organic phase, which is composed of 90% type I collagen and 10% non-collagenous proteins, results in dentin caries. Because mineral crystallites interact with the dentine collagen matrix, dentine remineralization is facilitated by the organic matrix of dentine. Dentine remineralization necessitates the preservation of the dentine matrix, which serves as

a model for mineral deposition. Flavonoid-treated groups may be protected against mineral loss due to the stability of the collagen matrix [21].

All flavonoids have hydroxyl groups, which make them susceptible to calcium ion complexes. Dentine that has been demineralized is covered with minerals. Hydroxyapatite crystal nucleation sites may be created by mineral deposition in the subsurface layer. Phosphorylated serine and threonine residues are also found in the organic matrix of negatively charged non-collagenous proteins. Because of this, the formation of hydroxyapatite may also be facilitated by these residues [22].

In this study, antibacterial activity was measured using CFU counts and lactic acid generation. Dentin that had been demineralized and treated to different treatments had biofilms of bacteria growing on its surfaces. After seven days, the biofilms treated with Chitosan had much lower CFU counts and lactic acid generation than the control groups, despite this. When used for a longer period of time, chitosan-based treatment may have reduced the biofilm's cariogenic activity while minimizing its unfavorable consequences. S. mutans' biofilm biomass, lactate generation, and metabolic activity may be hindered by the hydrogel's contact with the bacterial cell wall, according to the literature. Direct or indirect effects on metabolic activity are possible[23].

As a result of its antimicrobial properties, chitosan may also develop its own film that functions as an efficient barrier on the teeth. An exopolysaccharide-, protein-, and nucleic acid-rich extracellular matrix known as a biofilm is a highly active and well-organized colony of microbial cells. Proliferation and clustering of microbes are stimulated by S. mutans' synthesis of EPS on a given surface. In addition, as the biofilm grows, the spatial heterogeneities caused by EPS production result in the formation of a complex 3D matrix architecture[24].

While curcumin has been proven to have an effect on EPS structure, this effect is short-lived and does not have long-term consequences. Significantly reduced EPS structure and biofilm thickening were seen after curcumin treatment, as well as decreased bacterium counts. An extract of Thymol seed has been shown to influence the expression of several virulence genes in the pathogen S. mutans, causing disruptions in S. mutans cariogenicity, S. mutans bioflm may be prevented by the tea catechin epigallocatechin gallate, according to Xu et al [25].

S. mutans biofilms are commonly affected by alterations in cariogenic genes when natural chemicals are applied to the biofilm, according to these findings. While the flavonoid beat the chitosan and control in terms of remineralization, it outperformed both the flavonoid and control in terms of S.mutans antibacterial activity.

CONCLUSION

Chitosan-based agent can be used as remineralizing agent while flavonoid can be used as antimicrobial agent against S.mutans by inhibiting colony forming unit and lactic acid production..

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Conflict Of Interest

It has been made clear by the authors that there is not any conflict of interest that may skew the findings.

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