

Adsorption And Antimicrobial Substantivity Of 0.2% Chitosan On Human Root Tooth Surface

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Abstract: Background and Objective: The presence of amino acid and hydroxyl groups in its molecule, chitosan has emerged to be a promising material in adsorption and as a locally delivered antimicrobial adjunct. Chitosan is derived from naturally occurring exoskeletons of crustaceans including prawns, insects, crabs, shrimps, and cell walls of fungi such as aspergillus and mucor. The aim of this study was to establish the adsorption of chitosan solution as well as assess its antimicrobial substantivity against the growth of *Fusobacterium nucleatum* (Fn). **Methodology:** This was an experimental study that implemented a quantitative exploratory type of research. A total of 72 human tooth root discs were purposively sampled and obtained from triplication of sample groups. Zero point two grams (0.2 g) of chitosan was dissolved in 100ml 1% lactic acid solution to achieve a concentration of 0.2%. Each group was submerged in either 0.2% chitosan, 0.2% chlorhexidine, or 0.9% normal saline solution. The samples in each group were later immersed in artificial saliva for 10 minutes, 6hrs and 24 hours. All samples were subjected to a disk diffusion test and incubated for 48 hours. Different zones of inhibition were measured, recorded and analyzed using the Shapiro-Wilks test and One sample t-test analysis at a level of significance of $p < 0.05$. **Results:** The findings indicated that the antimicrobial substantivity against *F.nucleatum* for 0.2% chitosan was partially active at 10 mins and inactive at 6hrs and 24hrs. On the other hand, CHX shows significant activity at 10minutes, 6hrs, and 24hrs. **Conclusion and recommendations:** Chitosan was found to have minimal antimicrobial substantivity (10 minutes). Future researchers can also investigate the adsorption and antimicrobial substantivity of chitosan using other concentrations of chitosan at shorter time intervals. Perhaps greater zones of inhibition indicating greater antimicrobial activity can be observed

Keywords: Chitosan, Zone of inhibition, Adsorption, Antimicrobial substantivity

Introduction

Periodontitis is defined as a multifactorial chronic inflammatory disease related to plaque biofilm accumulation and is depicted by continuous destruction of the tooth-supporting structure. Key features are the loss of periodontal tissue support, manifested through clinical attachment loss (CAL) and radiographically assessed alveolar bone loss, presence of periodontal pocketing, and gingival bleeding (Papapanou et al., 2018). The European Workshop concluded that antimicrobial adjunctive therapy to scaling and root planing (SRP) can be applicable to circumstances, such as a patient's active or progressive disease, patients with deep pockets, and those with specific microbiologic profiles.

Antimicrobial substantivity is the prolonged association between a material and a substrate (e. g oral proteins, oral mucosa, and dental plaque), sustained with a simple deposition mechanism. It is considered that the delivery of an agent to its site of action, in a biologically active form, and in effective doses, increases its properties for prolonged periods (Greenstein G et al., 1998).

It has been demonstrated that, chlorhexidine binding to oral soft tissues allows its substantivity for up to 12 hours (Lang & amp, Jan, 2015). However, use of chlorhexidine mouth rinse is associated with a number of local adverse effects such as swelling of the parotid glands and formation of extrinsic stains brown stains on the teeth and oral tissues particularly the tongue (Martin Addy, 1994). Due to its known side effects, the search for an alternative biocompatible material becomes of interest. One such agent is chitosan.

Chitosan is derived from naturally occurring exoskeletons of crustaceans including prawns, insects, crabs, shrimps, and

cell walls of fungi such as aspergillus and mucor (Kucukgulmez et al., 2016). It is a non-toxic, stable, and sterilizable biocompatible polymer that has antimicrobial characteristics and can stimulate the immune system, hemostasis, and wound healing (Kean, T., & Thanou, M 2010). Lately, chitosan has significant use as a medium of drug delivery with a wide application in dentistry and medicine (Ahsan et al.,2018; Wieckiewicz et al., 2017).

The purpose of the study was to describe the in-vitro adsorption and antimicrobial substantivity of 0.2% chitosan solution on human tooth root surface. We hypothesized that chitosan exhibits similar antimicrobial substantivity characteristics compared to chlorhexidine at various exposure times.

Justification of the study

Results from this study will inform the dental community on the potential of chitosan as an alternative for treatment of periodontal disease.

Specific aim

To evaluate the adsorption and antimicrobial substantivity of 0.2% chitosan on human tooth root surface

Methods

This in vitro experimental study was done in the Postgraduate Prosthodontics Laboratory, University of the East as well as the Department of Medical Microbiology, University of the Philippines, Ermita, Manila. A total of 25 recently extracted premolars (due to orthodontic purposes) with intact, well-developed roots without root caries were included. All the extracted teeth were cleansed of blood and saliva using the ultrasonic scaler, and distilled water. Teeth were then stored in 0.9% NaCl, prior to sectioning them into

tooth discs. To obtain tooth discs, extracted premolars were cleansed and then sectioned at the mid-root level using a high-speed water-cooled diamond disk. The tooth discs were measured using a caliper to standardize them to 1 mm thickness and 6 mm diameter. Convenient sampling technique was employed. A total of 72 tooth discs were selected and stored in normal saline at room temperature. There were six samples of the dentin discs in three groups in 0.2% chitosan solution, 0.2% chlorhexidine as a positive control and normal saline as a negative control

Preparation of Chitosan solution

For the preparation of the chitosan solution, 0.2g of chitosan powder were dissolved in 100ml 1% lactic acid solution and stirred continuously using a magnetic mixer for 24 hours until a homogenous appearance of 0.2% chitosan concentration was achieved

Adsorption procedure

The adsorption procedure was conducted. The tooth discs were submerged in 0.2% chitosan for three minutes. The non-adsorbed solution was removed by washing the discs for 30 seconds using distilled water. The discs were then observed under the scanning electron microscope JOEL JSM 5310 with SEM Afore digitizer system.

Culturing of the *Fusobacterium nucleatum*

F. nucleatum ATCC # 25586 was prepared following the manufacturer's instructions in Trypticase Soy Broth (TSB) and incubated at 37°C for 48 hours. After preparation, 10µl of the standardized inoculum of the culture was compared with that of 0.5 McFarland standard (approximately 1.5 x 10⁸ CFU/ml). The density of the turbidity was checked by comparing the visual clarity of the lines on Wickerham card.

Disk Diffusion Test

The 72 tooth discs were randomly divided into three groups, each comprising of 24 discs. The three groups were assigned to a) 0.2% chitosan, b) chlorhexidine and c) normal saline. Each group was divided further into three sub-groups containing eight discs. The discs in the subgroups were later transferred to beakers containing artificial saliva stored at 37°C (to simulate the oral cavity *in vitro*) for 10 minutes, 6 hours, and 24 hours. Using of a cotton swab, the streak method was done to inoculate *F. nucleatum* on Tryptic Soy agar (TSA) plates then the root discs were placed on agar plates using sterile forceps. All the plates were prepared in triplicate and incubated at 37°C for 48 hours. The zones of inhibition (ZOI) at the widest diameter were used to describe the observations made in the experiment

Data analysis

Data from all the samples were recorded in the data collection sheet and later subjected to computerized statistical analysis using SPSS software computer program (Version 25.0). Descriptive analysis (e.g., mean, standard deviation, and minimum and maximum values) was conducted to describe the overall results. Normality of the data was assessed using Shapiro Wilk's test. One sample T-test was used to compare the cut off ranges against mean observations of chitosan at different immersion times in artificial saliva. The level of significance for all tests was $p \leq 0.05$ for both tests

Standard Zones of inhibition and corresponding inferences (Guevara et al., 2015)

Zone of inhibition	Inferences
<10mm	Maybe expressed as inactive
10-13mm	Partially active
14-19mm	Active
>19mm	Very active

The resulting data was compared with the standard table of inferences as described above.

Results

Initially five human tooth root discs were coated with 0.2% chitosan and examined under the Scanning Electron Microscope. This was done to evaluate adsorption of the chitosan.

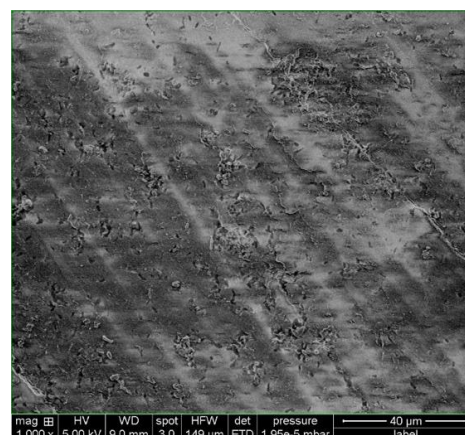


Figure 1: SEM image of a control sample (without 0.2% Chitosan) at 1000X magnification

The figure above showed an irregular surface without a chitosan coating which appears as a non-porous membranous whitish coating on human tooth root surface.

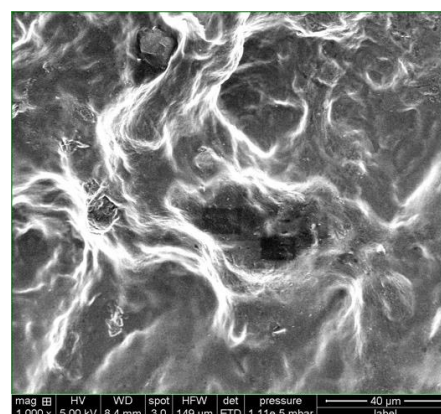


Figure 2: SEM image of 0.2% chitosan treated sample at X1000 magnification

Above figure shows chitosan coating which appeared as a non-porous membranous whitish coating on human tooth root surface.

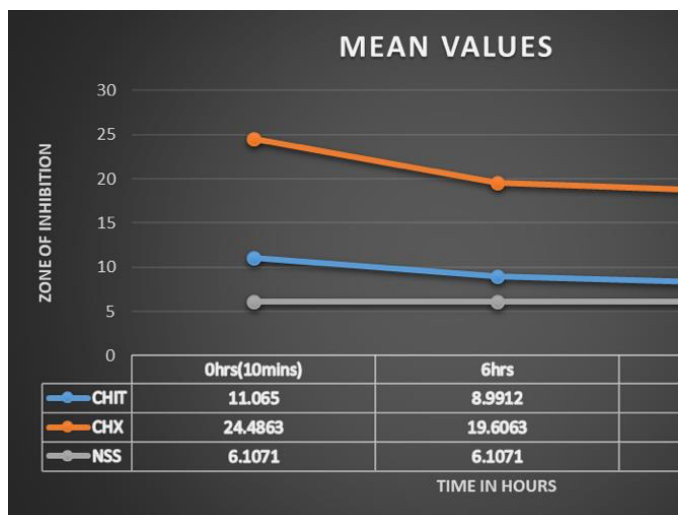


Figure 3: ZOI mean values of 0.2% Chitosan, Chlorhexidine and NSS

The above interaction plot shows mean values of the ZOIs for 0.2% chitosan, chlorhexidine and NSS at 10 mins, 6hrs and 24 hrs. For chitosan, the mean values are 11.65, 8.99 and 8.25 respectively, which when interpreted against the table of inference (Guevara et al, 2015), means that it initially had a partially active bacteriostatic effect, which gradually became inactive from six hours to 24 hours. Chlorhexidine, with mean values of 24.49, 19.61 and 18.55 respectively, showed a sustained very active effect until six hours, and only diminished to active at the 24hour mark. As expected, NSS

was inactive throughout the whole duration with a mean value of 6.11 for all the time intervals listed above.

Table 1: Shapiro Wilk’s Test

Group	Time	Statistic	Sig	Interpretation
0.2% CHT	10mins	.968	.885	Normal
	6hrs	.910	.351	Normal
	24hrs	.969	.888	Normal
0.2% CHX	10mins	.892	.244	Normal
	6hrs	.963	.839	Normal
	24hrs	.918	.413	Normal
NSS	10mins	.851	.098	Normal
	6hrs	.828	.056	Normal
	24hrs	.877	.175	Normal

Each test group consisted of three samples exposed at three different times namely, 10mins, 6 hrs and 24 hrs. All the resulting significance values for the three test groups at the three different exposure times are greater than the p-value of 0.05. This indicates that the results had a normal distribution and therefore parametric statistics were applicable

Table 2: Comparison of Chitosan, CHX at different immersion times in artificial saliva

Time	Groups	Mean±SD	95% CI	ZOI Range	Inference
10mins	CHIT	11.65±0.96	10.85-12.45	10-13mm	Partially Active
	CHX	24.49±1.98	22.83-26.15	19mm	Very Active
6hrs	CHIT	8.99±0.99	8.16-9.81	10mm	Inactive
	CHX	19.61±2.28	17.70-21.52	19mm	Very Active
24hrs	CHIT	8.25±0.83	7.56-8.94	10mm	Inactive
	CHX	18.55±2.76	16.24-20.86	14-19mm	Active

The 95% confidence interval as shown in the table indicated that, the estimated values for chitosan and chlorhexidine samples at different immersion times include the true population mean. The results indicate that the antimicrobial substantivity against F.nucleatum for 0.2% chitosan was partially active at 10 mins and inactive at 6hrs and 24hrs. On the other hand, CHX showed significant activity at 10minutes, 6hrs, and 24hrs.

Discussion

The purpose of this study was to evaluate adsorption and antimicrobial substantivity of 0.2% chitosan on human root surfaces. The SEM images of this study showed chitosan adsorption as a non-porous whitish smooth coating on the tooth root surface as examined on the SEM. These results are consistent with a study done by Demetgul et al., 2018 which showed that the pure chitosan had a nonporous and smooth membranous phase surface,

while the SEM image of CSCH (chitosan chromone) showed a polymorphic porous structure.

The disk diffusion test was done to assess retention (antimicrobial substantivity) of the different solutions as seen by their ability to inhibit F. nucleatum after immersion in artificial saliva over three time periods 10mins, 6hrs and 24hrs (exhibited as zones of inhibition ZOI). This in-vitro study simulated the clinical situation in the oral cavity by subjecting the root discs in artificial saliva and incubating them at 37°C, in a similar study by Demeriel et al., (1991), daily changes of serum were used to simulate the clinical situation.

Evaluation of 0.2% chitosan, demonstrated that at (10 minutes immersion in artificial saliva), chitosan was capable of inhibiting F. nucleatum growth at 48 hours and therefore was retained onto tooth root surface for that

given time period, whereas no zone of inhibition was observed at 6hrs and 24hrs unlike the results demonstrated in the CHX groups at 6 hrs and 24 hrs.

The results of this study demonstrated that the chitosan group (experimental group) showed antimicrobial retention effect at 10 minutes which translated to partially active while chlorhexidine showed activity at 10minutes, 6hrs and 24 hours indicating activity at all the exposure times as interpreted Guevarra et al 2005. These results may indicate a short-acting effect of chitosan. Owing to the limitations of the experimental procedures, the effect of chitosan was not determined in the intervening time from 10 minutes to before six hours, at any which time, chitosan would probably still have been exhibiting some bacteriostatic effect against *F. nucleatum*.

In this study it was demonstrated that 0.2% chitosan solution showed positive antimicrobial results against *F. nucleatum*, although it was only for a short period of time. Notably, the results correlate to a study done by Lian – Yiang Zheng et al. (2003) which evaluated the antimicrobial activity of 0.25% chitosan solution against *E. coli*, a gram-negative bacteria by its ability to inhibit the growth of *E. coli* similar to *F. nucleatum* which is a gram negative bacteria. However, contrary to this study the results demonstrated that they observed more colonies of *E. coli* at 1 hr, scarce colonies at 16hrs and no colonies at 24 hrs. Therefore, this illustrated that the antimicrobial process of chitosan against *E. coli* was gradually increasing over time during which the bacterial cell wall is believed to be damaged and thereafter killed by the coating and bonding of chitosan

The results of the current study revealed that both chitosan and CHX had an antimicrobial activity against *F. nucleatum*. However, the antimicrobial activity of chitosan was observed for a short time compared to that of chlorhexidine. This is because the partially adsorbed chitosan was easily washed away in the artificial saliva resulting in reduced antimicrobial effect. Nonetheless, the results attained in this study indicated that the mechanism of antimicrobial activity is undoubtedly more complex as the results showed decreased activity of chitosan over time. More studies are needed to explain the mechanism

Conclusion

Based on the results of this study, 0.2% chitosan solution (experimental) and chlorhexidine (control) groups demonstrated retention thus resulting in antimicrobial activity against *F. nucleatum* with both groups showing zones of inhibition. It was further established that, the antimicrobial substantivity of chitosan at 10 minutes could be categorized as "partially active" (Guevarra et al., 2005).

Recommendations

Future researchers can also investigate the adsorption and antimicrobial substantivity of chitosan using other concentrations of chitosan at shorter time intervals. Perhaps greater zones of inhibition indicating greater antimicrobial activity can be observed. Future recommendations can be made to manufacturers to

supplement chitosan with other materials that will enhance its retention on the tooth surface and enhance its antimicrobial substantivity.

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