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Effect of chitosan on a periodontal pathogen *Porphyromonas gingivalis*

G. İkinci [a], S. Şenel [a,*], H. Akıncıbay [b], S. Kaş [a], S. Erciş [c], C.G. Wilson [d], A.A. Hıncal [a]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey*
[b] *Department of Periodontics, Faculty of Dentistry, Hacettepe University, 06100 Ankara, Turkey*
[c] *Department of Microbiology, Faculty of Medicine, Hacettepe University, 06100 Ankara, Turkey*
[d] *Department of Pharmaceutical Sciences, SIBS, University of Strathclyde, Glasgow G4 0NR, Scotland, UK*

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Abstract

Local delivery systems of antimicrobial agents for treatment of the periodontal diseases received considerable attention during the past decade due to the disadvantages of the systemic administration. An ideal formulation should exhibit ease of delivery, a good retention at the application site, and a controlled release of the drug. The application of bioadhesive gels provides a long stay in the oral cavity, adequate drug penetration, high efficacy and acceptability. In dentistry and oral medicine, various applications of chitosan, which is a bioadhesive polymer have been proposed due to its favorable properties such as biocompatibility and biodegradability. The aim of this study was to determine the antimicrobial activity of chitosan formulations either in gel or film form against a periodontal pathogen, *Porphyromonas gingivalis*. The viscosity, bioadhesive properties and antimicrobial activity of chitosans at different molecular weight and deacetylation degree were evaluated in the absence or presence of chlorhexidine gluconate (Chx), incorporated into the formulations at 0.1 and 0.2% concentrations. The flow property of the gels were found to be suitable for topical application on the oral mucosa and to syringe into the periodontal pocket. Bioadhesion of the gels and films examined ex-vivo using fresh porcine buccal mucosa showed that both the film and gel formulations exert bioadhesive properties and was not affected by incorporation of Chx. Chitosan is shown to have an antimicrobial activity against *P. gingivalis* and this was higher with high molecular weight chitosan. The combination of chitosan with Chx showed a higher activity when compared to that of Chx alone, which would provide Chx application at lower concentrations thus avoiding its unwanted side effects. Chitosan films and gels seem to be promising delivery systems for local therapy of periodontal diseases with its bioadhesive property and antimicrobial activity.

Keywords: Chitosan film; Chitosan gel; Chlorhexidine; Periodontal drug delivery

* Corresponding author. Tel.: +90-312-310-1524; fax: +90-312-14777.
E-mail address: sevda@tr.net (S. Şenel).

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1. Introduction

The major approach in the prevention and treatment of periodontitis is the removal of supra and subgingival plaque. As this conventional therapy is not always successful, antimicrobial agents are widely used in cases of moderate to severe periodontal diseases to aid in eradication or at least suppression of plaque bacteria to an acceptable level. Among the methods of drug delivery used for periodontal disease therapy, the local administration of antibiotics has received considerable attention during the past decade (Steinberg and Friedman, 1999; Soskolne et al., 1997) as the systemic administration of these drugs have several drawbacks associated with their use, including a low patient compliance, a requirement for frequent dozing to maintain therapeutic drug concentrations in the serum and crevicular fluid, side effects including gastrointestinal and central nervous system disturbances, and super infections (Genco, 1981; Golomb et al., 1984).

Bioadhesive formulations are currently receiving much interest as drug delivery systems for periodontal pocket and oral mucosa (Nair and Chien, 1996; Bouckaert et al., 1992). Whilst inherent bioadhesive factors have an important role in retaining a formulation at epithelial surface, other physical properties such as the rate of hydration and the rheological properties of the polymeric formulation have a major impact on bioadhesion and consequently the eventual duration of retention (Smart, 1991). Gels and films are more suitable for this type of applications since they are able to cover a wider area of mucosa for the purposes of drug delivery and physical protection (Needleman et al., 1997; Şenel et al., 2000a). Chitosan, which is a hydrophilic biopolymer obtained by alkaline deacetylation of chitin, a major component of arthropod shells, has been claimed to act both as a bioadhesive and permeabilizer, making it a candidate system for oral mucosal drug delivery (Needleman et al., 1998; Şenel et al., 2000b). Chitosan has favorable properties such as nontoxicity, biocompatability and biodegradability. Moreover, chitosan itself possesses antimicrobial activity (Staroniewicz et al., 1994).

Chlorhexidine gluconate (Chx) at concentrations of 0.1 and 0.2% is used widely in clinical dental practice due to its activity against a wide range of oral microbial species (Salem et al., 1987). *Porphyromonas gingivalis* has been considered as an aggressive periodontal pathogen because of its high association with periodontal destruction in human beings, and it is reported to prolong the inflammation and progression of attachment loss (Slots and Taubman, 1992; Kojima et al., 1993).

In this study, the antimicrobial activity of chitosan with different molecular weight and deacetylation degree as well as their combination with chlorhexidine was investigated against the periodontal pathogen, *P. gingivalis*. For this purpose the gel and film formulations were prepared, and bioadhesion, viscosity and antimicrobial activity studies were performed on these formulations.

2. Material and methods

2.1. Materials

Chitosans used in this study are given in Table 1. Chlorhexidine digluconate (Lot 65H0427) (Sigma Chem. Co., USA), tripolyphosphate pentasodium salt (TPP) (Sigma Chem. Co., USA), glycerin (Birpa Chem., Turkey) and lactic acid (E. Merck, Germany) were used as received.

Table 1
Chitosans used in the study

High molecular weight (*HM*)[a]	Degree of deacetylation (%)
Chitosan-H (HM-80)	80
Low molecular weight (*LM*)	
Protasan 212 (LM-73)	73
Protasan 213 (LM-84)[b]	84
Protasan 214 (LM-95)	95

[a] MW: 1 400 000 (Dainishiseika Colour and Chem. MGF Co.Ltd., Japan).
[b] MW: 272 000 (Pronova-Biomedical, Norway).

2.2. Preparation of gels and films

HM-80 gels were prepared at 1 and 2% concentrations in dilute lactic acid solution (1%). LM gels were prepared at 3% concentration. Since the viscosity of the gels increased with the molecular weight, these concentrations were chosen for ease of application. Chx was incorporated into the formulations at 0.1 and 0.2% concentrations.

Chitosan films with a thickness of 400 μm were prepared with HM-80 by solution casting method. Ten percent glycerine was used as plasticizer. The films were cross-linked with TPP solution at two different concentrations (0.1 and 0.5%) (Şenel et al., 2000a).

2.3. Viscosity

Viscosity measurements of gels were performed on a Brookfield digital viscometer (Model DV-II, cone-SD1) at room temperature.

2.4. Bioadhesion measurements

Bioadhesion was examined ex vivo using fresh porcine buccal mucosa without any further treatment. The maximum force of detachment was measured on a texture analyzer (TA-XT2, Stable Micro System). Chitosan gels were freeze–dried before bioadhesion measurements. Chitosan films and freeze–dried gel disks (18 mm diameter) were fixed to the support of the texture analyzer using a double side adhesive. The tissue ($\sim 5 \times 5$ cm) was needled on a polystyrene and placed in the texture analyzer. The film or gel was brought into contact with the tissue under a very slight pressure (2 g) and was kept in this position for 1 min. Then the tensile test was performed at a constant extension rate of 0.5 mm/s.

2.5. Antimicrobial susceptibility tests

P. gingivalis was isolated from subgingival plaque of an adult periodontitis patient. Plaque sample was cultured on Schaedler agar supplemented with hemin (5 μg/ml) and vitamin K_1 (0.1 μg/ml). Identification of the microorganism was based on colony pigmentation, gram stain and long wave UV light fluorescence and biochemical tests (Slots and Homer, 1982; Sutter et al., 1986). Susceptibility testing of the above mentioned organism was carried according to Wadsworth Anaerobic Bacteriology Manual, susceptibility testing of anaerobes. Briefly, serial twofold dilutions of the antimicrobial agents were prepared in *Brucella* broth containing hemin (5 μg/ml) and vitamin K_1 (0.1 μg/ml). Three or four colonies of the organism were picked from an overnight culture on blood agar plate and inoculated into a tube of 5–6 ml supplemented thioglycolate medium. This medium was enriched with hemin (5 μg/ml) and vitamin K_1 (0.1 μg/ml) prior to sterilization and sodium bicarbonate (1 mg/ml) added just prior to use. After a night incubation at 37 °C, the culture was diluted in *Brucella* broth to the turbidity of 0.5 McFarland standart (10^8 CFU/ml), and was then further diluted to 1:200. A volume of inoculum equal to the amount of broth containing the drug was added, and tests were incubated at 37 °C in GasPak jars for approximately 48 h. An inoculated broth containing no antimicrobial agent was included as a growth control.

Chitosan films were cut into 8 mm diameter discs and weighed before placing onto dishes. Application volume of the gels and drug solution was 150 μl which corresponded to the same drug content as the films. Samples were pipetted into uniformly spaced 8 mm diameter wells. Inhibition zone diameters were measured after 48 h incubation. The minimum inhibitory concentration (MIC) was read as the lowest concentration of drug or chitosan showing no visible growth.

3. Results

3.1. Viscosity

It was seen from the flow curves (Fig. 1) that viscosity increases significantly with increasing molecular weight of chitosan as well as increasing chitosan concentration. Chitosans exhibited pseudoplastic behaviour, which indicates a gel structure. Incorporation of Chx into the gels did not change the viscosity.

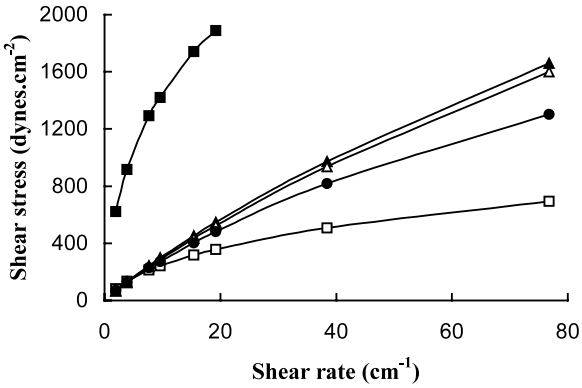

Fig. 1. Flow curves of chitosan gels (■ 2% and □ 1% HM-80; ▲ 3% LM-95; △ LM-84; ● LM-73) ($n = 3$).

3.2. Bioadhesion studies

The presence of Chx did not affect the bioadhesion of chitosan films but a slight decrease in the detachment force was observed after cross-linking (Fig. 2). No difference was found in adhesion force of gels prepared with different types of chitosan (Fig. 3).

3.3. Antimicrobial activity

Inhibition zone diameters obtained for chitosan gels and films are given in Figs. 4 and 5, respectively. The results indicate that *P. gingivalis* is susceptible both to chitosan and Chx. The activity

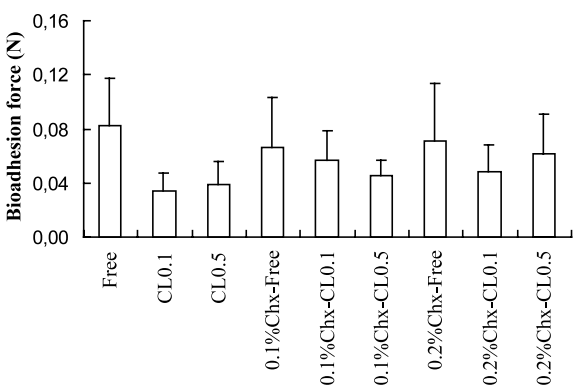

Fig. 2. Adhesion forces for chitosan films ($n = 6$, mean ± SD) (CL0.1: film cross-linked with 0.1% TPP; and CL0.5: 0.5% TPP).

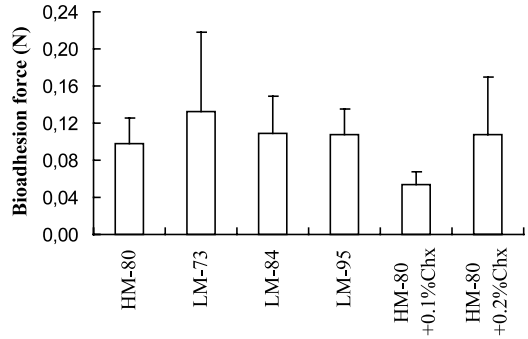

Fig. 3. Adhesion forces for chitosan gels ($n = 6$, mean ± SD).

obtained with 1% gel prepared with high molecular weight chitosan (HM-80) was similar to that of 3% gels prepared at low molecular weight chitosan (LM-73, LM-84, LM-95). Within the range investigated (73–95), change in degree of deacetylation did not have any effect on microbiological activity (Table 2). Similarly, no increase

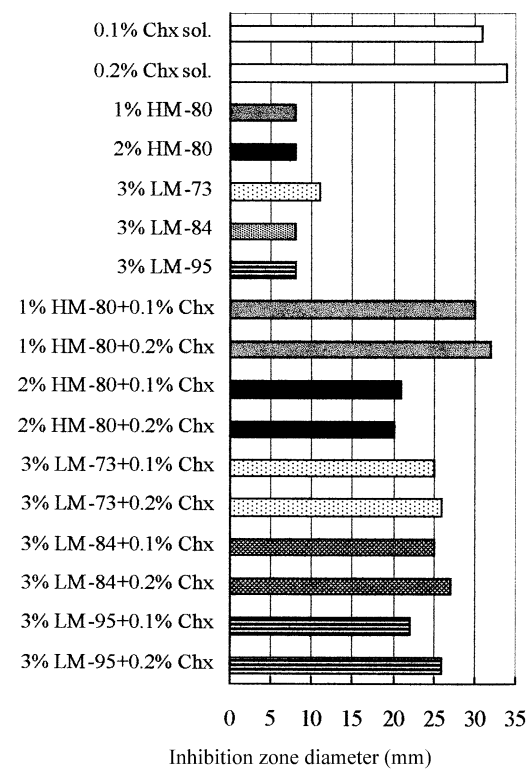

Fig. 4. Inhibition zone diameters for chitosan gels with or without Chx against *P. gingivalis* ($n = 3$).

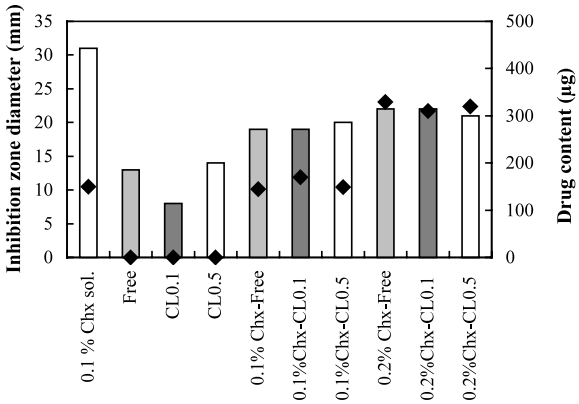

Fig. 5. Inhibition zone diameters for chitosan films with or without Chx against *P. gingivalis* (CL0.1: film cross-linked with 0.1% TPP; and CL0.5: 0.5% TPP, bars indicate inhibition zone diameter, diamonds indicate drug content) ($n = 3$).

was observed in microbiological activity when the Chx concentration was increased from 0.1 to 0.2%.

4. Discussion

In our previous study, bioadhesive gel and film formulations were prepared using chitosan to deliver Chx into the oral cavity. The results of in vitro release studies indicated a promising application of Chx at a low concentration (0.1%) incorporated into chitosan gel for candidiasis in the oral cavity (Şenel et al., 2000b). In this study, we studied the antimicrobial activity of these formulations as well as other formulations prepared using different types of chitosan on a periodontal pathogen, *P. gingivalis* which invades human periodontal pocket epithelium. The viscosity measurements showed that the flow behaviour of the chitosan gels examined in this study is ideal for introducing material into the periodontal pocket.

Table 2
MIC values of chitosan, Chx and their combinations

	MIC (mg/ml)
Chx	0.062
HM-80	0.082
LM-73	3.638
LM-84	3.855
LM-95	3.795
1% HM-80 + 0.1% Chx	0.004
1% HM-80 + 0.2% Chx	0.003
2% HM-80 + 0.1% Chx	0.003
2% HM-80 + 0.2% Chx	0.008
3% LM-73 + 0.1% Chx	0.0007
3% LM-73 + 0.2% Chx	0.0005
3% LM-84 + 0.1% Chx	0.0001
3% LM-84 + 0.2% Chx	0.0002
3% LM-95 + 0.1% Chx	0.004
3% LM-95 + 0.2% Chx	0.007

The results showed that both chlorhexidine and chitosan exert an antimicrobial activity against *P. gingivalis*. A markedly higher activity was obtained with the high molecular weight chitosan. An ionic interaction between the cations due to the amino groups of chitosan and anionic parts of bacterial cell wall such as phospholipids and carboxylic acids has been proposed as the mechanism for the antimicrobial activity of chitosan (Seo et al., 1994). However, the results obtained in our study showed that the mechanism of the antimicrobial activity is probably more complex as no difference was observed in activity with chitosans within the range of deacetylation degree of 73–95%. More studies are needed to elucidate the mechanism. The activity increased with the combination of Chx and chitosan. Increasing the concentration of Chx did not have significant effect on activity indicating that in the presence of chitosan, it is possible to deliver this compound at a lower concentration which will avoid unwanted side effects including staining and altered taste sensation.

The bioadhesion studies performed ex vivo demonstrated that both the film and gel formulations of chitosan show significant bioadhesive properties, which will provide a significant contribution to the retention of the drug in the periodontal pocket as well as release of drug in a prolonged fashion. It is suggested that this is mediated through the positive charge on chitosan, which also contributes to its continued effect on the epithelial permeability after physical removal from the surface. It was shown that after the removal of chitosan from the surface of Caco-2 cell monolayers, it did not readily dissociate from the cell membrane (Schipper et al., 1997). Simi-

larly, in a study where the bioadhesive properties of chitosan was compared to poly (ethylene oxide) and xanthan gum as a delivery system for periodontal pocket, chitosan demonstrated the most prolonged drug levels and the rate of removal of the chitosan formulation was found to be slower than that of the others (Needleman et al., 1997).

No significant difference in bioadhesion force was observed between the different types of chitosans examined in this study yet these results must be undertaken with caution, as the samples prepared for bioadhesion studies may not reflect the actual situation. A slightly higher bioadhesion was obtained with chitosan gels when compared to films. The presence of Chx did not have any influence on bioadhesion but, certainly, the nature of the drug compound incorporated into the formulation plays an important role in changing the bioadhesive properties of chitosan.

With the films, bioadhesion decreased with cross-linking which can be attributed to a decrease in the positive charge of chitosan after cross-linking emphasizing the role of electrostatic interaction in adhesion of chitosan on the tissue. This also correlates well with the results of the study where the mucoadhesives properties of chitosan microspheres were investigated in vitro (He et al., 1998). It was shown that the amount of chitosan adsorbed on the tissue increased with the decreasing cross-linking levels that led to increase in the positive potential of chitosan microspheres.

Gels as syringeable systems can be easier to use than the films but may require repeated visits. With film formulations, a markedly prolonged release was observed for Chx when compared to that from the gel formulations (Şenel et al., 2000a). Therefore, it would be ideal to choose either the gel or the film form of chitosan depending on the case.

It can be concluded that with its bioadhesive and antimicrobial properties, chitosan in gel or film form can be a promising delivery system not only for Chx but also for other antibacterial drugs in periodontal therapy. These formulations are to be professionally delivered so unlike systemic regimens should require little or no patient compliance for success. In vivo studies on substantivity and durability of the formulations are in progress.

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