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Research Report

The effect of nanochitosan hydrogel membrane on absorbtion of nickel, inhibition of *Streptococcus mutans* and *Candida albicans*

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ABSTRACT

Background: *The use of fixed orthodontic appliance for a long time can potentially cause nickel ion release, increase in the growth of Streptococcus mutans (S. mutans) and Candida albicans (C. albicans). Chitosan has the ability to bind metal, antibacterial and antifungal. Physical modification of chitosan into the nanoparticles size will expand the surface of the chitosan so that the absorption of nickel ions and the inhibition of growth bacteria and fungi can be increased.* ***Purpose:*** *The purpose of the study was to determine the effect of nanochitosan hydrogel membrane to the absorption of nickel ions, and the inhibition of S. mutans and C. albicans growth.* ***Methods:*** *Nanochitosan hydrogel membrane with chitosan weight variation of 0.6; 0.8 and 1 g immersed in artificial saliva containing nickel 0.075 mg / L for 15, 30 and 45 minutes. The nanochitosan hydrogel membrane was tested for nickel ion absorption by Atomic Absorption Spectrophotometry, whereas the antibacterial and antifungal tests were done by exposing the nanochitosan hydrogel membrane - nickel on S. mutans and C. albicans in the wells of plate.* ***Results:*** *Demonstrated that absorption of nickel ions was related with the increase in weight of chitosan and soaking time. Inhibition of growth of S. mutans and C. albicans showed a positive correlation with the increase in weight of chitosan.* ***Conclusion:*** *Variation on chitosan weight on hydrogel membrane and variation on immersion time have effect on nickel ion absorption, inhibition of S. mutans and C. albicans growth.*

Keywords: *Nanochitosan; nickel ion; Streptococcus mutans; Candida albicans*

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INTRODUCTION

Orthodontic appliance has three basic components i.e braces, archwires, buccal tubes, molar bands and accessories, which are made of stainless steel alloys. To get the best result of orthodontic treatment usually takes a long time, ranging from 1 to 3 years. During the period of orthodontic treatment, side effects can occur from the use of fixed orthodontic appliances.[1]

Some of the reported side effects include corrosion of metal alloy,[2] an increase in the accumulation of dental plaque,[3] an increase in the growth of some bacteria[4] and candida that causes disease dental hard tissues and soft tissues of the oral cavity.[5] The main results of corrosion on metal alloy orthodontic appliance is iron (Fe), chromium (Cr), and nickel (Ni).[6] Although all three components of the potentially harmful effects, Ni has been reported to cause allergic, toxic and carcinogenic reactions.[7] Several studies have reported that exposure to nickel ions result of 2.521 ± 1.764 mg/l can cause DNA damage and cell death by apoptosis, as shown by the growing number of comet cells and apoptotic cells.[2]

Increasing accumulation of plaque in orthodontic treatment will be a problem for the health of the teeth and mouth.[8] Colonies of bacteria were initially formed Gram positive colonies, namely *Streptococcus, Neisseria* and

Actinomices. Gram positive predominant bacteria that causes dental caries is *Streptococcus mutans* (*S. mutans*).[9] Beside the increased accumulation of *S. mutans*, *Candida* is also encountered during orthodontic treatment. Research on the effects of orthodontic treatment on the increased growth of *Candida albicans* (*C. albicans*) stated that *C. albicans* are found mainly on the surface of the orthodontic braces.[5] According to the study on 60 orthodontic patients, 15 orthodontic patients (25%) were found with increase *C. albicans* colonies in the oral mucosal cells. Increased growth of *C. albicans* in orthodontic treatment can trigger *candidiasis* and *angular cheilitis*.[10]

One of the natural ingredients that have the ability to absorb metal,[11] antibacterial and antifungal is chitosan.[12] The basic principle in the binding mechanism between chitosan and metal is the principle of ion exchange. Especially nitrogen in the amine group of chitosan will react and bind metals from solution.[11] Chitosan is able to inhibit the growth of various types of bacteria and fungi as chitosan has a polycationic natural shape, so chitosan can act as an antibacterial agent against bacteria and fungi through ionic interactions on the cell wall of bacteria, which can even damage the cell wall.[12]

Chitosan is expected to be the active ingredients that can reduce the negative effects of the use of orthodontic appliances. Chitosan was modified into the nanoparticle size, in order to increase the surface area so that the amine group increases and form a nanochitosan preparations to be a nanochitosan hydrogel membrane with the aim to improve the absorption of nickel ions, capable of inhibiting the growth of *S. mutans* and *C. albicans.*

The purpose of this study was to determine the effect of nanochitosan hydrogel membrane to the absorption of nickel ions, and the inhibition of growth of *S. mutans* and *C. albicans*. In previous studies it is known that chitosan 0.8 g can absorb Zn ions well. [13] In this experiment, chitosan weight ratio of 0.6 ; 0.8 and 1 g were used to determine the pattern of optimal absorption of nickel ions. The results of this study are expected to be useful nanokitosan hydrogel membrane to reduce the risk of release of metal ions and inhibit the growth of bacteria *S. mutans* and *C. albicans* during orthodontic treatment.

MATERIALS AND METHODS

This research use chitosan from crab shells (Sigma-Aldrich, st. louis, mo, usa), sodium acetate 1% 5 mM (Brand), sodium phosphate (Fluka) 50 mM, 1 M NaOH solution, concentrated NH_3 pa (Merck), gelatin powder Sigma from bovine skin type B (Nitta), Preparations of *C. albicans* 108 CFU/ mL, Mueller Hilton agar (MHA), brain heart infusion (BHI), preparation of *S. mutans*, Brown agar, nickel solution (Laboratorium Penelitian dan Pengujian Terpadu UGM), artificial saliva.

A total of 0.6; 0.8 and 1 g of chitosan, dissolved in 500 ml (0.75 ml of acetic acid and distilled water *add.* 500 ml) was stirred until evenly dispersed chitosan. Each solution of chitosan 0.6; 0.8 and 1 g was added 0.8 ml of NH_3 (21%) dropped slowly over the stirrer while checked with a pH meter, until the pH reached 6.2 to 6.3. The solution became cloudy whitish color, then put in ultrasonic bacth for ± 1 hour, then allowed to stand for 30 minutes at room temperature. Gelatin 1.8 g and 30 ml nanochitosan (each of the weight chitosan of 0.6; 0.8 and 1 g) was dissolved into 60 ml of distilled water and then subsequently homogenized using a magnetic stirrer for 3 hours, then allowed to stand for 30 minutes. After swelling it was diluted with water bath 37^0 C. Nanochitosan gelatin liquid was inserted into the mold (petri dish with a diameter of 6 cm). Nanochitosan hidrogel was then put in the refrigerator at a temperature of 4^0 C for 7 days to dry. To obtain crosslinked gelatin hydrogel dihydrothermal (DHT) was done with a vacuum oven at Integrated Laboratory in Faculty of Dentistry Universitas Gadjah Mada at a temperature of 140^0 C to 160^0 C for 48 hours. After the hydrogel membranes dry out and form a thin sheet, the hydrogel membrane was cut to the size of 20 x 20 mm.

Nanochitosan hydrogel membrane (chitosan weight of 0.6 g) was soaked into 50 ml artificial saliva containing nickel 0.075 mg/l for 15 minutes.[13] Then nanochitosan hydrogel membranes were analyzed using absorption atomic spectrometry (AAS) to measure the concentration of nickel metal are absorbed in the membrane. Nanochitosan hydrogel membranes that contained nickel was diluted with distilled water. Nanochitosan hydrogel membrane solution was diluted 1000 ppm to 100 ppm, then diluted further to a concentration of 3-12 ppm. After all the solution is ready, the computer is turned on by using AAS analysis program, namely GBC version 1.33. Conduct program settings was done by adjusting the nickel element to be analyzed. Then the tool was turned on, the program will ask for blank and all standard solutions sequentially. Capillary tube was inserted into the solution, the program will read the content of nickel ions contained in the solution. After that will come a calibration graph of the results of analysis of nickel element contained within the solution. The same thing is done in the nanochitosan hydrogel membrane that containing chitosan weight 0.8 and 1 g.

Nanochitosan hydrogel membrane is soaked into 50 ml of artificial saliva containing nickel 0.075 mg/l for 15 minutes.[14] Then nanochitosan hydrogel membranes were analyzed using AAS to measure the concentration of nickel metal are absorbed in the membrane. The same thing was done with a variation of contacts 30 and 45 minutes.

S. mutans was obtained from cultures that had been available in the Laboratory of Microbiology, Faculty of Veterinary Medicine Universitas Gadjah Mada. This test used a medium brown agar. Pure cultures of *S. mutans* were inoculated into medium brown agar using a sterile cotton swab, allowed to stand for 10 minutes. Blank disc was inserted into the nanochitosan hydrogel membrane solution-nickel and allowed to stand for 1 hour. The discs were then placed on the surface of the brown agar using

sterile tweezers. The media put in the incubator for 24 hours at 37^0 C. After incubation for 24 hours would appear the presence of bacterial inhibition zone. Measurements were performed by measuring the inhibition zone using a digital caliper accuracy of 0.01 mm.

Suspension of *C. albicans* was obtained from cultures that had been available in the Laboratory of Microbiology, Faculty of Veterinary Medicine UGM. This test uses Mueller Hinton agar (MHA) medium. Pure cultures of *C. albicans* were inoculated into medium MHA agar using a sterile cotton swab, allowed to stand for 10 minutes. Blank disc was inserted into the nanochitosan hydrogel membrane solution - nickel and allowed to stand for 1 hour. The discs were then placed on the surface of the brown agar using sterile tweezers. The media put in the incubator for 24 hours at 37^0 C. After incubation for 24 hours would appear the presence of fungal inhibition zone. Measurements were performed by measuring the inhibition zone using a digital caliper accuracy of 0.01 mm.

RESULTS

Results of the nickel ions absorption study showed that nanochitosan hydrogel membrane containing 1 g of chitosan with a 45 minute soak time is able to absorb the highest nickel ions are 0,0674 ± 0,002 ppm (Table 1). Nickel ions absorption pattern shows the pattern increases with increasing chitosan content in the nanochitosan hydrogel membrane (Figure 1).

Variations on immersion time of 15, 30 and 45 minutes in all variations of weight chitosan showed an increasing pattern. fifteen minutes soaking time the nanochitosan hydrogel membranes 0.6 shows the average nickel ion absorption by 0.0280 ± 0.003 ppm, while the highest absorption of nickel ions on a 45-minute immersion in the nanochitosan hydrogel membrane 1 g of 0.0674 ± 0.002 ppm. All treatment groups of nanochitosan hydrogel membrane, the highest nickel ion absorption is still below the absorption of nickel ions in the control group of nanochitosan membrane. Based on a two-ways Anova test showed that the weight variation of chitosan on nanochitosan hydrogel membranes and variation of soaking time significantly affect the absorption of nickel ions.

The results of the *S. mutans* growth inhibition studies showed that the higher more weight of chitosan on nanochitosan hydrogel membranes, the greater inhibition zone *S. mutans*. Nanochitosan hydrogel membrane 1 g has the highest inhibition zone diameter of 1.018 ± 0.034 mm (Table 2).

Based on the variation of soaking time, the highest inhibition zone diameter at 15 minutes soaking time of 1.018 ± 0.034 mm (Figure 2). Based on a two-ways Anova test, significance value of $p<0.05$ in all groups so that it can be concluded that the variation of the weight of chitosan on nanochitosan hydrogel membranes and variation of soaking time significantly effect on *S. mutans* inhibition zone.

The results of inhibition of *C. albicans* growth studies showed heavier chitosan on nanochitosan hydrogel

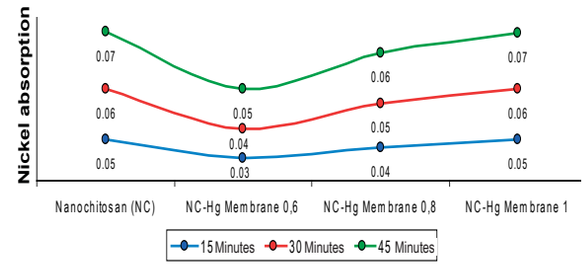

Figure 1. The average of nickel ions absorption by nanochitosan hydrogel membrane.

Table 1. The mean nickel ion absorption by the nanochitosan hydrogel membrane

No	Materials	Sample	Nickel ion absorption (ppm)		
			15 Minutes	30 Minutes	45 Minutes
1	NCh membrane	9	0.048±0.003	0.061±0.002	0.068±0.002
2	NCh Hgel membrane 0.6	9	0.027±0.002	0.035±0.002	0.040±0.037
3	NCh Hgel membrane 0.8	9	0.039±0.002	0.051±0.002	0.061±0.002
4	NCh Hgel membrane 1.0	9	0.048±0.002	0.060±0.002	0.067±0.002

Table 2. Average inhibition zone growth of *Streptococcus mutans*

No	Materials	Sample	Inhibition zone of *Streptococcus mutans* (mm)		
			15 Minutes	30 Minutes	45 Minutes
1	NCh membrane	9	1.056± 0.017	0.992± 0.011	0.930± 0.023
2	NCh Hgel membrane 0.6	9	0.873 ± 0.042	0.838 ± 0.031	0.817 ± 0.042
3	NCh Hgel membrane 0.8	9	0.922 ± 0.020	0.899 ± 0.018	0.855 ± 0.025
4	NCh Hgel membrane 1.0	9	1.018 ± 0.034	0.960 ± 0.012	0.904 ± 0.028

Table 3. Average inhibition zone growth of *Candida albicans*

No	Materials	Sample	Inhibition zone of *Candida albicans* (mm)		
			15 Minutes	30 Minutes	45 Minutes
1	NCh membrane	9	1.594± 0.014	1.554± 0.013	1.511± 0.013
2	NCh Hgel membrane 0.6	9	1.456± 0.014	1.423± 0.013	1.378± 0.013
3	NCh Hgel membrane 0.8	9	1.504± 0.016	1.474 ± 0.016	1.448 ± 0.019
4	NCh Hgel membrane 1.0	9	1.541± 0.028	1.504± 0.020	1.489± 0.018

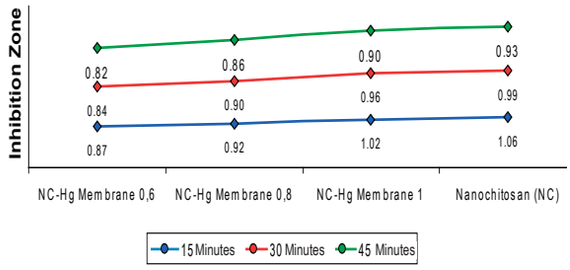

Figure 2. The average of *Streptococcus mutans* inhibition zone by nanochitosan hydrogel membrane.

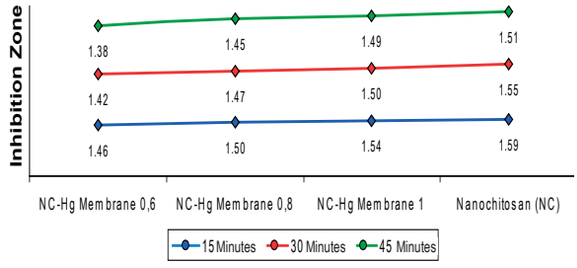

Figure 3. The average of *Candida albicans* inhibition zone by nanochitosan hydrogel membrane.

membranes make up the greater inhibition zone *C. albicans*. Nanochitosan hydrogel membrane 1 g has the highest inhibition zone diameter 1.541 ± 0.028 mm (Table 3).

For a variation of soaking time 15, 30 and 45 minutes in all variations of chitosan weight showed a declining pattern. The highest inhibition zone diameter at 15 minutes soaking time 1.541 ± 0.028 mm (Figure 3). Based on a two-ways Anova test obtained significance value of $p<0.05$ so that it can be concluded that the variation of the weight of chitosan on nanochitosan hyrogel membranes and variation of soaking time significantly have effect on *C. albicans* inhibition zone.

DISCUSSION

The basic principle in the binding mechanism between chitosan and metal contained in the solution is the principle of ion exchange.[11] Especially nitrogen in the amine group of chitosan will react and bind metals from solution. Increased absorption of nickel ions occurs because of the greater weight of chitosan that make nanochitosan, nanochitosan surface area is increased and the amine group formed the more so the higher the ability to absorb metals.[14] Chitosan has a very good the properties of metal ion absorption due to a) the nature of the hydrophilic chitosan; b) primary amine group with high activity; c) chitosan polymer chain structure which can form a flexible configuration of chitosan to bond with metal ions.[16]

In a variation of chitosan weight of 0.6; 0.8 and 1 g contained in nanochitosan hydrogel membrane known that nickel absorption increases with the increase chitosan weight that contained in nanochitosan hydrogel membrane. Chitosan adsorption ability can be improved by modifying both physically and chemically. Modification of physics related to the crystallinity structure that reduces the size of chitosan to be nanoparticles. Nanoparticles are particulate material with at least one dimension smaller than 1000 nanometers. One nanometer is 10^{-9} m, so that the nanoparticles have a greater surface area to volume ratio. The more surface area of chitosan by changing its size to nanoscale, then getting bigger too absorptive capacity.[17] Absorption of nickel by nanochitosan hydrogel membrane as the highest in the weight of 1 g chitosan. Increased absorption of nickel ions occurs due to the greater weight of chitosan that compile nanochitosan the increasingly widespread nanochitosan surface amine groups formed led to more and more, so the ability to absorb the metal increase.[16,17] Based on the variation of immersion time 15, 30 and 45 minutes, data show the nanochitosan hydrogel membrane 1 g with contact time 45 had the greatest absorption. At 15 minutes soaking time is not optimal absorption, because the process of adsorption and the formation of bridges between the particles is not perfect. Whereas at 30 and 45 minutes absorption of nickel ions is increased, due to the amine group on nanochitosan hydrogel membrane still able to bind nickel ions. In the 45-minute contact time highest absorption occurs in the weight variation nanochitosan 1 g. Compared with the control group nanochitosan membrane then the value of nickel ion absorption by the nanochitosan hydrogel membrane 1 g is almost the same. This is probably due to the composition of gelatin contained in nanochitosan hydrogel membrane, which can also bind to gelatin amine and hydroxyl groups of chitosan.

Nanochitosan hydrogel membrane can bind nickel ions via the amine group (NH_3), this amine group is also involved in the antimicrobial mechanism of chitosan against bacteria and fungi.[18] The results showed nanochitosan hydrogel

membrane with variation of chitosan weight and variation of immersion time can inhibit the growth of *S. mutans* and *C. albicans.* The higher weight of chitosan that contained in nanochitosan hydrogel membrane, the greater inhibition zone of *S. mutans* and *C. albicans*, but by the variation of immersion time, the longer soaking time, inhibition zone of *S. mutans* and *C. albicans* decreases. *S. mutans* and *C. albicans* inhibition zone is still high due to the nickel ion is still not completely absorbed by nanochitosan hydrogel membrane, so the ability of the amine group to destroy the bacterial membrane can be optimized. Amine functional group (-NH2) on chitosan, which is a very strong positive charge, may be able to bind to the bacterial cell wall which negatively charged. This bond may occur in electronegative sites on the surface of the bacterial cell wall. In addition, because -NH2 also has a lone pair, then amina group can bind Ca^{2+} minerals contained in the cell wall of bacteria to form a coordinate covalent bond.[18] At the variation of immersion time, the longer soaking of the amine group on the nanochitosan hydrogel membrane will increasingly bind to nickel ions, so the ability to damage the cell walls of bacteria decreased.

Mechanism between antimicroba and antifungal is same, that through the interaction between the charge positive group NH_3^+ on the unit glucosamine chitosan and the negative charge on the cell membrane of microbes resulting in electrostatic interactions that cause changes in the permeability of the membrane walls of microbes that alter the osmotic balance internally that can inhibit microbial growth, and hydrolysis of peptidoglycan in the wall of microbes resulting in loss of intracellular electrolytes, proteins, nucleic acids and glucose in microbes.[18,19] NH_3 group plays an important role in the process of absorption of nickel ions and the antibacterial/ antifungal. If the absorption of nickel ions is not optimal, there is still an active NH_3 group so that the process antibacterial and antifungal mechanism can be improved.[15] If the NH_3 group has bonded optimum with nickel ions, nanochitosan hydrogel membrans still able to be an antibacterial and antifungal because of the presence of acetic acid content in the solution during the process of making nanochitosan membrane. Acetic acid has the ability to inhibit bacterial or fungal.[17]

In conclusion, variation of chitosan weight in the nanochitosan hydrogel membrane and variations of soaking time in artificial saliva have effect on the absorption of nickel ions, the inhibition of growth of *S. mutans* and *C. albicans*. The nanochitosan hydrogel membrane is able to absorb the nickel ions in artificial saliva, with the highest absorption of nickel ions on the nanochitosan hydrogel membrane containing 1 g chitosan. The ability of nanochitosan hydrogel membrane-nickel in inhibiting the growth of *C. albicans* and *S. mutants* is the highest on nanochitosan hydrogel membranes 1g-nickel but the highest effect in the first 15 minutes of soaking time.

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