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RESEARCH ARTICLE

In Vitro Study of Irrigation solution of Chitosan Nanoparticles to Inhibit the Adhesion and Biofilm Formation of *Enterococcus faecalis* in the Root Canal

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ABSTRACT:
Enterococcus faecalis (E. faecalis) plays a role in the pathogenesis of dental root canal infections. Chitosan has antibacterial properties and a chelating agent in the tooth root canal and is biodegradable and non-toxic. They analyzed the irrigation response of Chitosan nanoparticles to the virulence properties of *E. faecalis* in the dental root canal. Examination of *E. faecalis* virulence properties was carried out with violet crystals to obtain biofilm inhibition strength, Gram staining to determine lysis and coagulation of bacterial cells, and Atomic Force Microscopy (AFM) to analyze the surface roughness of the tooth root canal. Chitosan nanoparticles combined with 2.5% NaOCl has a strong inhibition of the formation of *E. faecalis* biofilm, both based on the control group ($p > 0.05$; 0.088) and incubation time of 48 h and 72 h, also inhibitory power, which was better at 24 h ($p > 0.05$; 0.185) than the other groups. Irrigation solution of Chitosan nanoparticles combined with 2.5% NaOCl has better lysis and cell agglutination of *E. faecalis* bacteria compared to other groups, especially at all incubation times, based on the control group ($p > 0.05$; 0.104) and incubation time ($p > 0.05$; 0.580) can reduce the surface roughness of the dental root canal, but the impact of giving irrigation materials to each treatment group with the incubation time showed significant differences ($p<0.05$). The Chitosan nanoparticles solution as an irrigation material has a strong ability to suppress the formation of biofilms, coagulation, and lysis of *E. faecalis* cells and better reduce the walls' surface roughness dental root canal at 24 hours incubation time.

KEYWORDS: Adhesion, Biofilm, Chitosan Nanoparticles, Dental Root Canal, *Enterococcus faecalis.*

INTRODUCTION:
Enterococcus faecalis (*E. faecalis*) is a persistent gram-positive microorganism that plays an essential role in periapex lesions' etiology after root canal treatment[1]. Wang (2012) found that 63% of failures in treating dental root canals caused *E. faecalis* infection[2].

Also, *E. faecalis* can adapt to an acidic and alkaline pH environment. The ability to form biofilms has been reported as a virulence factor that allows these bacteria to be more resistant to phagocytosis by antibodies[3].

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Endodontic treatment aims to eliminate microbes from the root canal and prevent reinfection from the microbiological aspect. Endodontic infections can only be treated with good chemical and mechanical procedures (cleaning and shaping[4]. In this process, irrigation plays a vital role because it can eliminate the smear layer and eliminate pathogens found on the dental root canal's wall surface. Based on previous research reports, it has been explained that each irrigation solution has a different role in eliminating pathogens and protecting the surface of the dental root canal[5].

Sodium hypochlorite (NaOCl) solution is recommended as an irrigation solution because it has intense antimicrobial and proteolytic activity but is known to be

very toxic and can irritate periapical tissue[6]. Moreover, Ethylenediaminetetraacetic acid (EDTA) is starting to be used as an effective irrigation agent in demineralizing dentin and removing smear layers. Unfortunately, its effectiveness is less in removing organic debris and has no antibacterial properties[7]. Ethylenediaminetetraacetic acid is also known to have a function to clean the root canal and has been proven to reduce human root dentin's mineral content[8]. Meanwhile, it is known that Chlorhexidine (CHX) is effective against *E. faecalis.* However, it is less effective against gram-negative bacteria and cannot dissolve necrotic tissue and debris, consequently covering the surface of dentinal tubules[9].

Side effects caused by using a combination of irrigation materials have encouraged many studies to obtain alternative materials with a better security level, more biocompatible, and more beneficial to the host[10]. As a natural polysaccharide obtained through chitin's deacetylation, chitosan has biocompatible properties, is bioadhesive, and is non-toxic to human cells. Chitosan is a chelating material that can lift the smear layer, be antibacterial, and improve the adhesive system[11]. Walczak (2018) dan Abidin (2007) reported for the first time using high molecular chitosan (DD 84.20% and molecular weight 893,000 Mv) in endodontic treatment[12,13]. Based on Silva's (2013), 0.2% chitosan solution can remove the smear layer from 1/3 middle and 1/3 apical of dental root canals[14]. This study aims to evaluate the biomaterial response of Chitosan nanoparticles irrigation materials to the virulence properties of *E. faecalis* in the dental root canals carried out in vitro.

MATERIAL AND METHODS:

This research has passed ethical clearance No.248/TGL/KEPK FK USU-RSUP HAM/2019 from the Faculty of Medicine, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia. *Enterococcus faecalis* ATCC 29212 ($1.5x10^8$CFU/mL) obtained from the Laboratory of Microbiology, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia, and chitosan nanoparticle obtained from the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, University of North Sumatra University, Medan Indonesia.

Sample Preparation:

This study used 24 mandibular premolar, which were divided into 4 groups: positive control of 2.5% NaOCl, 2.5% NaOCl + 17% EDTA, 2.5% NaOCl + 17% EDTA + 2% CHX and 2.5% NaOCL + Chitosan nanoparticles. This study was a post-only control group analyzed in vitro by modeling infection of the dental root canal related to the biomaterial response of irrigation solution to *E. faecalis* in the tooth root canal. This study directs efforts to prevent the virulence of *E. faecalis* associated with biofilm formation, changes in the morphology of *E. faecalis* cells, and the surface roughness of the dental root canal.

Biofilm Assay:

On 96-well plates, the biofilm formation of *E. faecalis* was assessed using the 1 percent crystal violet method[15]. The 96-well plate was coated with 100µL of Tryptic Soy Broth (TSB) medium and incubated for 15 min, after which it was washed with Phosphate-buffered saline (pH 7.0), and 25µL of *E. faecalis* was added to each well, which was then adapted at room temperature for 15 min. In addition, a serial triple was applied to the test material (Chitosan nanoparticle). The test material and *E. faecalis* were homogenized for 10 min on a shaker with 500 xg before being incubated for 24 h, 48 h, and 72 h. The formation of E. faecalis biofilms is assessed by extracting all solutions from wells and then cleaning them with PBS and washing them in a dishwasher at 500 rpm for 10 min. This procedure was carried out twice more. Then, every 150µL, 1 percent violet crystal is inserted into the well plate. Then shake for 10 min at 200rpm with homogeneous crystalline violet dye and biofilm protein. After that, each well was washed for 5 min with 150µL of PBS, then discarded and refilled with 150µL of 70 percent ethanol for 1 min. After that, violet crystalline dyes were used to label 96-well plates containing biofilms, then incubated at room temperature for 15 min. The biofilm formation was measured by spectrophotometry at 520nm.

***In vitro* root canal treatment:**

The teeth were sterilized after being prepared for in vitro modeling.[16]. The prepared teeth were prepared for in vitro modeling and then sterilized. Then put 100µL TSB medium into each tooth treated and incubated for 15 min at room temperature. Furthermore, 25µL of *E. faecalis* (1: 3) suspension was injected into all treatment groups and incubated in an anaerobic atmosphere for six h. Then the procedure for modeling root canal treatment was repeated with the biomechanical preparation for each group. The teeth from all groups were also slowly irrigated in the root canal using chitosan nanoparticles 0.2% 75µL (1:3). All specimens were shaken for 5 minutes at 200 rpm and incubated three times: 24 h, 48 h, and 72 h. After that, the morphological profile of *E. faecalis* cells and changes in roughness on the dental root canal were examined.

Inhibition Assay:

Suspension solution from the dental root canal (test material and *E. faecalis*) as much as 25µl were cultured in chrome agar medium for 48 h at 37°C. Then, two morphologically different colonies were taken to examine the inhibition power of *E. faecalis* for the Gram

staining approach[15]. Subsequently, the bacterial isolate was dropped with a purple dye and left for 1 minute, washed, and dried. Then, an iodine solution is dropped for 1 min and washed with PBS until dry. After that, 96% alcohol is dropped for 30 sec, washed, and dried. It is fallen with safranin for 30 seconds in the final stage, then rinsed and dried. It is observed using a microscope with a magnification of 400x. Inhibition is shown as the cell area affected by the test material representing darker than normal cells and is characterized by the agglutinated size. Then, based on changes in bacterial cell morphology, the value of the inhibitory power is analyzed.

Surface Roughness of Dental Root Canal:

Twenty-four mandibular premolars were prepared and then stored in glycerol solution, then rinsed with PBS solution for 10 sec. The tooth's root is cut transversely (distal-mesial or vice versa) in the Cementoenamel Junction (CEJ) section using a carborundum disc in moist conditions. After that, the specimen is put in a sterile container (vial) which contains a glycerol solution to maintain tooth enamel moisture. Roughness assessment for the dental root canal's wall surface using an Atomic force microscope (AFM) with coverage of scanning surface area is 10x10µm.[17] The study specimens were labeled according to 4 types of treatment groups. Then the sample is placed in the holder, and the NanoSurf2 icon is clicked on the computer screen and waits until the computer and controller are connected. After that, the image size is selected by clicking IMAGING by choosing the range 0-0.5µm or equal to 0-500 nm. Then clicked on POSITIONING followed by ADVANCE, and our eyes were checking on the peep lens found on the scan head. It is intended to control the position of the scanning needle not touching the sample surface. Subsequently, click on APPROACH and wait until the controller sounds "tet" to signal that the scanning needle is touching the sample's surface and ready to start the scanning process. This process is finished after ± 8 minutes of scanning to get the image in JPEG format. When the results are obtained, click on POSITIONING followed by WITHDRAW, then to end all processes click on RETRACT until it stops. Then the sample is removed from its position, which shows the test procedure is complete, and the device states that the whole process has finished operating.

Statistical Analysis:

Data on *E. faecalis* biofilm formation, adhesion, and root canal surface roughness was analyzed by Kruskal Wallis with a significance level of $p<0.05$. The *E. faecalis* inhibition data were interpreted qualitatively based on cell morphology.

RESULTS AND DISCUSSION:

Fig 1 shows that incubation time determines the frequency of biofilm inhibition power, although there is no statistically significant difference based on incubation time ($p>0.05$; 0.185), similar to the absence of significant differences in the treatment group ($p>0.05$; 0.088), meaning that the strength of Chitosan nanoparticles antibiofilm with a mixture of other irrigation materials does not have a significant effect, in other words, each irrigation material has an anti-biofilm effect that varies according to the time of exposure.

Fig 2 explained that the incubation time affects the morphological changes of bacterial cells. At 24 h, although the size of bacterial cells that were seen were still small, lysis cells were seen in group B (NaOCl 2.5% + EDTA 17%); Group C (NaOCl 2.5% + EDTA 17% + CHX 2%) and Group D (NaOCl + Chitosan nanoparticles. In group D, many bacterial cells were lysed. Furthermore, the 48 h group showed that the number of bacterial cells lyzed increased. Even the remnants of dead bacterial cells could be seen in the treatment of group C, whereas in group D, it was shown that the number of bacterial cells had decreased and tended to be the same as in group B (NaOCl) 2.5%+ EDTA 17%). In the 72 h group, it can be explained that all groups underwent lysis even though some were still alive. This tendency is influenced by the test material's working power, which can agglutinate and lyse bacterial cells' surfaces.

Fig 3 shows Chitosan nanoparticles possess the ability to reduce the surface roughness of the dental root canal, based on the control group 2.5% NaOCl and 2.5% NaOCl + 17% EDTA + CHX.2%. It is assumed that the use of chitosan for in vitro root canal treatment impacts *E. faecalis*. This result is in line with the adhesion power of *E. faecalis* biofilm formation. The Kruskal Wallis analysis showed that the surface roughness value in all treatment groups was insignificant ($p> 0.05$; 0.104). While based on the incubation time, there were also negligible differences between 24 h, 48 h, and 72 h groups ($p> 0.05$; 0.580), on the contrary, the impact of irrigation material application on each treatment group with the incubation time showed a significant difference ($p<0.05$). These results indicate that the addition of chitosan has no effect on the surface roughness of the dental root canal, but on the other hand, it shows a better product at 24 h compared to 48 h and 72 h.

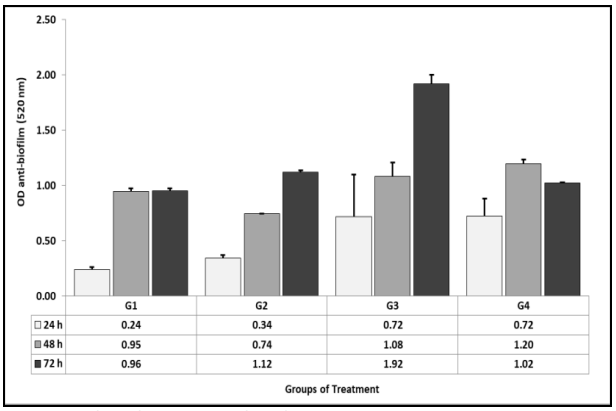

Fig.1: Biofilm formation of *E. faecalis*.
G1 = 2.5% NaOCl + *E. faecalis*; G2 = NaOCl 2.5% + EDTA 17% + *E. faecalis*; G3 = NaOCl 2.5% + EDTA 17% + CHX 17% + *E. faecalis*; G4 = 2.5% NaOCl + Chitosan nanoparticles + *E. faecalis*. The lowest inhibitory power of *E. faeclis* biofilms was shown at 24 hours in all groups. Chitosan nanoparticles after mixing with 2.5% NaOCl has the best biofilm inhibition effect at 48 and 72 hours. Bar (Biofilm *E. faecalis*) and Bar erorr (Standard deviation).

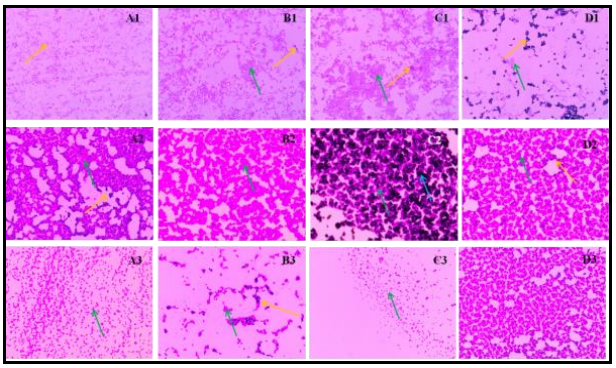

Fig. 2: Profile of *E. faecalis* cells after interacting with test material in a dental root canal.
Line I (A1, B1, C1, D1) group 24 h. Line II (A2, B2, C2, D2) groups 48 h, Line III (A3, B3, C3, D3) groups 72 h. A (NaOCl 2.5% + E. Faecalis); B (NaOCl 2.5% + EDTA 17% + *E. faecalis*); C (NaOCl 2.5% + EDTA 17% + CHX 17% + *E. faecalis*); and D (NaOCl 2.5% + Chitosan nanoparticles + *E. faecalis*). Yellow arrows (*E. faecalis* cells); green arrow (lysis cells); blue arrow (death cells). 400 x magnification

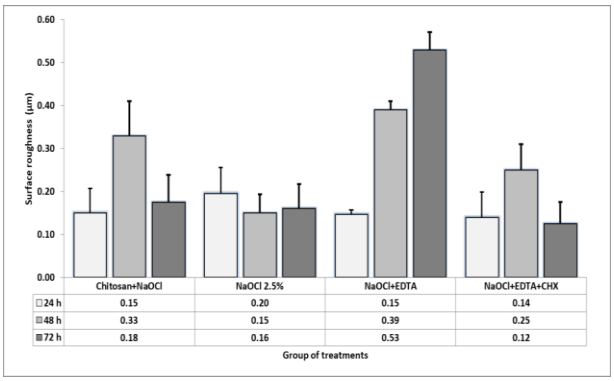

Fig. 3: Surface Roughness Analysis.
At 24 hours incubation, the Chitosan nanoparticles + NaOCl group had the same roughness value as the NaOCl + EDA group. The two groups' roughness values were almost the same as the control group (NaOCl + EDTA + CHX). At the incubation time of 48 h and 72 h, the roughness value was unstable in all treatment groups. Bar (roughness value) and error bar (Standard deviation)

This study evaluates the inhibitory effect of Chitosan nanoparticles irrigation solution on the formation of biofilms and changes in the morphology of *E. faecalis* cells. Besides, it is assessing its ability to reduce the surface roughness of the dental root canal. Based on the study results, the power of inhibition of the formation of *E. faecalis* biofilms is related to the lysis and coagulation of *E. faecalis* cells. It contributes to the decrease in surface roughness of the dental root canal. All three forms of tests were carried out to measure the virulence of *E. faecalis* related to the ability of *E. faecalis* in biofilm formation, cell growth and spread, and its ability to colonize to form quorum sensing. Based on the findings of this study, it can be stated that the molecular irrigation solution of Chitosan nanoparticles can prevent *E. faecalis* from forming porosity on the surface of the dental root canal as a result of its failure to form biofilms, colonization, and extraction of calcium dentin minerals in the pathogenesis of dental root canal.

Fig 1 shows that all treatment groups had relatively strong inhibition of *E. faecalis* biofilm. Chitosan nanoparticles with 25% NaOCl significantly affect the inhibition of biofilms, which means that each irrigation material has various anti-biofilm effects based on tolerance and adaptation time. All irrigation materials tested in this study had a strong influence on the biofilms formation of *E faecalis*. The irrigation material tested has an optical density value above ≥0.4, except for the 24-hour incubation time. The NaOCl group is 2.5%; NaOCl 2.5% + EDTA 17%. The Odeyemi (2017) and Kim (2017) reports confirm that optical density values above ≥0.4 indicate a strong ability to prevent bacterial biofilm formation[18-20]. This phenomenon shows the relationship between the biological and chemical properties of the two compounds. Batubara (2014) has reported that chitosan (poly-β-1,4-glucosamine) is a polysaccharide with biocompatibility, biodegradability, bioadhesive, and non-toxic properties in human cells[21]. These properties could be why chitosan can prevent the formation of *E. faecalis* biofilm because it has good bioadhesive properties[22]. Based on this concept, it can be stated that chitosan can inhibit the development of bacteria by suppressing the formation of biofilms.

Based on these results, it can also be assumed that chitosan has biosensors properties and can detect several communication signals between bacteria. This property causes the failure of bacteria to communicate between bacteria to form quorum sensing in biofilm formation.[23] Besides, chitosan has high dissolved oxygen, which will disrupt communication between bacteria, mainly through the Respiration oxygen species (ROS[24]. All treatment groups have the properties to lyse and agglutinate *E. faecalis* bacterial cells. These properties indicate that all irrigation materials used in this study, such as 2.5%

NaOCl, 17% EDTA, and CHX 0.2% have antibacterial properties. Still, these properties only contribute to bacterial growth and development, while in terms of mechanical and chemical properties, it is sure to provide adverse effects on the dental root canal[25]. The Chitosan nanoparticles used in this study tend to have a better impact than other materials. Although lysis of chitosan nano high molecules 0.2% shows the same strength as other groups, the cell damage shows a different profile (Fig 2).

Based on this concept, bacterial cells that are lysed and coagulated are known to be associated with interactions between chitin/chitosan which are positively charged, and microbial cell membranes that are known to be negatively charged mediated by electrostatic forces between protonated NH3 + groups and harmful residues, which are related to Ca2 + bonds to maintain the electronegative properties of the surface of bacterial cell membranes [26,27]. Electrostatic interactions cause changes in membrane walls' permeability properties, thereby triggering internal osmotic imbalance and inhibiting microorganisms' growth. It also causes peptidoglycan hydrolysis in the walls of microorganisms that cause intracellular electrolyte leakages such as potassium ions and proteinase bonds[28]. Electronegative or polyanion properties in Gram-positive bacteria are lipoteichoic acid (LTA) which will react with polycation in chitosan[29]. Therefore, as reported in this study, this interaction can explain chitosan's ability to lyse and neutralize several bacterial cells exposed to chitosan solution. The antibacterial activity of chitosan is affected by chitosan polymerization and physicochemical properties. Chitosan molecules can pass through bacterial cell walls with multilayer murein interconnected and reach the plasma membrane. This effect is due to electrostatic interactions between chitosan molecules and microbial cell membranes that cause damage to cell wall surface proteins, increase penetration of chitosan to the nucleus, and affect protein sentiment [29].

Results in the control group of 25% NaOCl and NaOCl + EDTA + CHX imply that chitosan nano high molecules (0.2%) can reduce the dental root canal's surface roughness (Fig 3). This result correlates with its ability to inhibit biofilms and damage *E. faecalis* cells. This finding is related to the power of chitosan nano high molecules 0.2% to inhibit biofilms and damage *E. faecalis* cells. This aspect shows chitosan's role as a bio protector in developing bacteria and chelating agents in the dental root canal to model dental root canal treatment. Although chitosan addition did not significantly affect the decrease in surface roughness than the control, it became better at 24-hour incubation than 48 h and 72 h. Its is shows chitosan's ability as a chelation material that can remove the smear layer and has antibacterial properties[30]. Thus, there is a correlation with its ability to reduce root surface canal roughness.

This phenomenon is related to the molecular size of Chitosan nanoparticles. This site can be absorbed by the dental root canal's wall surface[31]. Although the mechanism of action has not been reported clearly, it is assumed that the absorption process can cause ion exchange and chelation. Two chitosan theories explain the chelating process, including: (i) a bridge model, two or more amino groups from one chitosan chain will bind to the same metal ion (ii) the amino group of the structure involved in securing only one, and namely metal ions bind to amino groups.[32]

CONCLUSION:

The Chitosan nanoparticles solution as an irrigation material has a solid ability to suppress the formation of biofilms, coagulation, and lysis (adhesion) of *E. faecalis* cells and a better ability to reduce the surface roughness of the walls of the dental root canal at 24 h incubation time.

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CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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