

Evaluation of IL-1 α and IL-1 β , COX-2, and iNOS mRNA Expression in Orthodontic Patients Given Chitosan Mouthwash During Treatment with Miniscrew

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ABSTRACT **Objective:** Chitosan is a biomaterial with antibacterial properties that may benefit from maintaining peri-miniscrew hygiene and preventing inflammation. This study aimed to evaluate the expression of inflammatory-related molecules from the gingival crevicular fluid (GCF) after treatment of 1% chitosan when compared with 0.2% chlorhexidine mouthwash of patients with orthodontic miniscrew. **Materials and Methods:** A total of 30 subjects were divided into three groups: the first group received mouthwash containing 1% chitosan, the second group 0.2% chlorhexidine digluconate, and the control group received aquadest. The GCF was collected before and after 4 days of rinsing, and relative expressions of IL-1 α and IL-1 β , cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) were evaluated by real-time qPCR. **Results:** The expression of IL-1 α was the highest in chitosan-treated patients when compared with that of IL-1 β in between-groups. Patients receiving chlorhexidine have the highest expression of COX-2 and iNOS when compared with the chitosan and control groups, respectively. **Conclusion:** A mouthwash containing 1% of chitosan could suppress the expression of inflammatory mediators IL-1 β , COX-2, and iNOS.

KEYWORDS: Chitosan, chlorhexidine, implant, inflammatory mediators, mouthwash, orthodontic miniscrew

INTRODUCTION

Miniscrew as the anchorage is an essential part of orthodontic treatment and easy to be installed and removed; however, the main problem with orthodontic treatment is the failure of the anchoring system.^[1-3] Host immune response plays a vital role in the success of the orthodontic miniscrew.^[4] Immune responses in the early stage are mediated by inflammation as a host's response from mechanical, chemical, and thermal exposure.^[5] The balance between pro-inflammatory and anti-inflammatory molecules in response to stimuli due to orthodontic treatment plays an essential role in the success of orthodontic treatment.^[6] Interleukin-1 (IL-1) is very important in the process of T cell activation in inducing IL-2 expression. IL-2 will initiate the next immune response, which will manifest clinically.^[7]

A previous study demonstrated that IL-1 β induces naive and memory CD4⁺ T cells (Th1, Th2, and Th17) in response to antigen stimulation enhances their function.^[8]

The imbalance between pro-inflammatory and inflammatory mediators might occur in the development of peri-implantitis in orthodontic miniscrew. Evaluating these mediators involved in inflammation is important for further research for a better anchorage system in orthodontics. A few reports show the expression of some cytokines IL-1 β in crevicular fluid in response to

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orthodontic treatment using miniscrew as temporary anchorage.

The inducible nitric oxide synthase (iNOS) is another essential molecule that stimulates nitric oxide production for bone responses to mechanical stress.^[9] iNOS expressed by osteocyte, osteoclast, and chondrocytes during inflammation and mediated NO production stimulates bone resorption.^[9] Metal particles of miniscrew and surrounding bacteria also stimulate the osteoblasts to produce pro-inflammatory mediators that contribute to the inflammatory process in the tissue of the miniscrew implant.^[10] Cobalt ions can stimulate the secretion of prostaglandin E2 (PGE2) by osteoblasts induced by COX-1 and COX-2 cyclooxygenases.^[9,10] It has been shown that cobalt ions stimulate increased prostaglandin E2 (PGE2) secretion in primary human osteoblasts.^[11] This was preceded by upregulated cyclooxygenase COX-1 and COX-2 gene expression.^[12] Chitosan is a linear polysaccharide substance that is derived from the chitin of crustacean's shell. Previous studies have shown that chitosan has antibacterial effects.^[10] *In-vitro* study showed that the combination of chitosan and chlorhexidine has a great antibacterial effect, adequate biocompatibility, and decreased pro-inflammatory activation.^[13] However, it has been reported that the enzymatic mouthwash's cytotoxicity was lower than that of the chlorhexidine mouthwash.^[14] Our previous study shows that chitosan has reduced the total oral bacterial count around orthodontic miniscrews.^[15] Previous *in vitro* studies indicated that nano chitosan has an antibacterial and antifungal effect.^[16,17] Our previous study also revealed that chitosan in combination with anti-*Streptococcus mutans* IgY was able to inhibit *S. mutans* biofilm in the tooth surface of Sprague-Dawley rats.^[18] We hypothesized that chitosan would suppress the expression of inflammatory mediators. This study aimed to analyze the effect of chitosan rinse on mRNA expression of IL-1 α and IL-1 β , COX-2, and iNOS in patients with orthodontic miniscrew.

MATERIALS AND METHODS

The research protocol was approved by the Ethics Committee of Research Faculty of Dentistry, Universitas Indonesia (Ref. No. 4/Ethical Approval/FKG UI/I/2019). All procedures and materials and methods of this study have been published,^[15] and here we report regarding the host inflammatory responses that have not been published.

COLLECTING THE CREVICULAR GINGIVAL FLUID (CGF)

The method for collecting the CGF was followed, as previously reported by Guentsch *et al.*^[19] Briefly, samples

were collected in the morning, 2–3 h after breakfast. The sample was collected by paper points and was gently placed for 30 s into the pocket and then samples were eluted into 500 μ L phosphate-buffered saline (PBS) followed by centrifugation at 400g for 4 min. The paper points were removed, and the supernatants were stored at -20°C until RNA extraction.

QUANTITATION OF IL-1 α AND IL-1 β , COX-2, AND iNOS BY REAL-TIME PCR

The total cellular RNA from the crevicular fluid sample was extracted before reverse transcription to produce cDNA.^[20] Briefly, the samples were incubated for 5 min at $15\text{--}30^{\circ}\text{C}$ before adding 0.2 mL of chloroform per 1 mL of TRIZOL reagent. The RNA pellet was washed with 75% ethanol followed by dissolving in RNase-free water. The RNA samples were used for reverse transcription using the TaqMan Reverse Transcription kit (Applied Biosystems). The resulting cDNA was amplified by qPCR. IL-1 α and IL-1 β , COX-2, and iNOS cDNA were amplified using qPCR with their appropriate primers, as shown in Table 1. The PCR conditions were set as follow:

Stage	Condition
Pre-denaturation	95°C for 5 min
Annealing, extension	40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, and at 72°C for 5 min
Melting curve	95°C for 15 s, 60°C for 60 s, and 95°C for 15 s

The mRNA target gene expression was normalized to the level of β -actin. Expression of each gene is the difference in value of $2^{-\Delta\Delta C_t}$ before and after treatment.^[20]

RESULTS

Thirty patients (25 women and 5 men), according to the inclusion criteria, are involved and completed the procedure in this study with no complications. Subjects age ranges from 15 to 33 years (23.3 ± 4.7). From the 30 patients, 53 miniscrews inserted in various locations

Table 1: Oligonucleotide's primers used in this study

Primer name	Sequences	Reference
β -actin	F: 5'-gttgctatccaggetgtg-3' R: 5'-tgatcttgatcttcattgtg-3'	[20]
IL-1 α	F: 5'-cgcaatgactcagaggaaga-3' R: 5'-aggcgtcattcaggatga-3'	[21]
IL-1 β	F: 5'-acgatgcacctgtacgatca-3' R: 5'-tcttcaacacgcaggacag-3'	[21]
iNOS	F: 5'-tctccgaccaccactacagcaa-3' R: 5'-ggggaactgggcagactcaa-3'	[22]
Cox-2	F: 5'-gaatggggtgatgacagtt-3' R: 5'-cagaaggcaggatagcagc-3'	[21,23]

were evaluated for clinical signs of peri-miniscrew inflammation.

Our study has shown that IL-1 α in chitosan treatment patients was the highest when compared with the chlorhexidine and control groups. IL-1 α in chitosan treatment was 500 times higher than control, and the chlorhexidine group was 30-fold higher than the control [Figure 1]. In Figure 2, we can see that the expression of IL-1 β in the control group was the highest compared with that of chitosan treatment and the chlorhexidine patients' group. The control group was 11.6-fold higher than the chitosan group and 11.5-fold more elevated than the chlorhexidine treatment group. In addition, if we compare the expression of IL-1 α and IL-1 β , it can be seen that IL-1 α was the highest in chitosan-treated patients compared with IL-1 β , either in between-groups or within the group [Figure 2]. The Kruskal–Wallis test results among rinsing groups showed *P*-value less than 0.05, which means that the difference was statistically significant. The *post-hoc* analysis using the Mann–Whitney test showed that the expression of IL-1 α after rinsing differed significantly between the chitosan treatment and the other groups (*P* < 0.05). Moreover, IL-1 β , the mean, differed significantly between chlorhexidine and the other groups (*P* < 0.05).

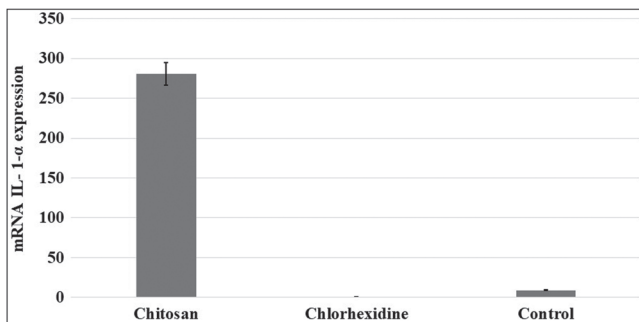


Figure 1: The relative expression of IL-1 α of mRNA isolated from the CGF

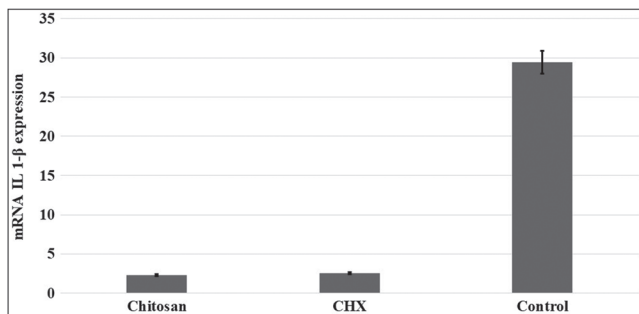


Figure 2: The relative expression of IL-1 β of mRNA isolated from the CGF

This study has shown that patients who received chitosan have the lowest level of iNOS and COX-2 expression. The highest expression of iNOS and COX-2 is in the chlorhexidine treatment group (*P* < 0.05) [Figures 3 and 4]. Further analysis of COX-2 and iNOS indicates a similar mRNA expression pattern between the groups. These results showed us that chitosan was suppressing inflammatory mediators expression.

The data reveal that the expression of IL-1 α and IL-1 β mRNA in patients given chitosan and chlorhexidine shows that chitosan suppresses IL-1 β expression along with increased IL-1 α expression [Figure 5A]. Chlorhexidine mouthwash showed a different pattern, whereas both IL-1 α and IL-1 β showed very low expression, and this pattern was similar in the control group [Figure 5A]. Further analysis of iNOS and COX-2 expression showed that COX-2 expression was higher than iNOS in all groups and was the highest in the chlorhexidine group. The expression pattern also showed similarity in all groups, including the control group [Figure 5B].

DISCUSSION

Chitosan, as an antibacterial in the form of mouth rinse, was used to eliminate oral microorganisms' adherence

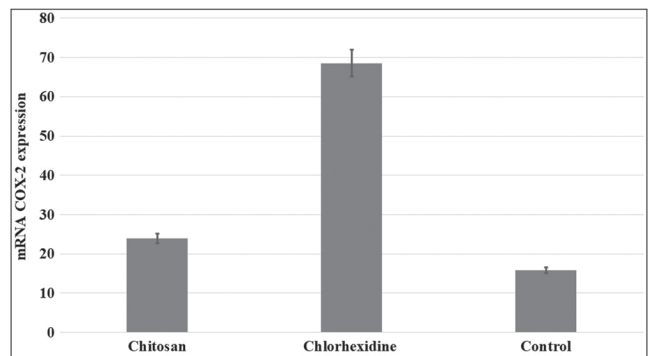


Figure 3: The relative expression of COX-2 of mRNA isolated from the CGF

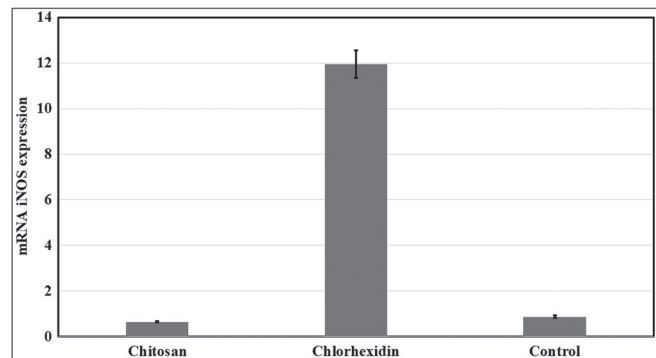


Figure 4: The relative expression of iNOS of mRNA isolated from the CGF

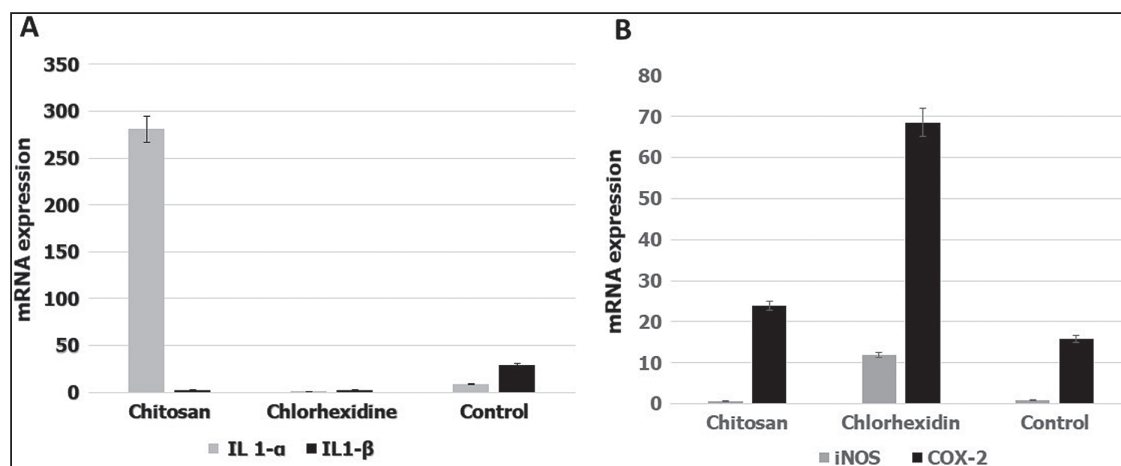


Figure 5: The comparison of CGF IL-1 α and IL-1 β (A) and of COX-2 and iNOS mRNA (B) in all groups of experiments

to the surface of the miniscrew during orthodontic treatment. Our report regarding the antibacterial effect from this study has been published and indicated that chitosan has a comparable antibacterial activity to chlorhexidine in reducing the number of oral bacteria.^[15] In this paper, we analyze the profile of inflammatory mediators which reveals that IL-1 α suppressed the expression of IL-1 β in the chitosan treatment group. In contrast, this phenomenon did not happen in the chlorhexidine group as there was a similar amount of IL-1 α and IL-1 β in the chlorhexidine group. In contrast, in the control group, the expression of IL-1 β was the highest among the treatment groups. We can assume that IL-1 β as an inflammatory cytokine was induced by the control group's microorganisms as no chitosan or chlorhexidine was applied.

We know that IL-1 α and IL-1 β are the major agonists of IL-1, whereas IL-1Ra is a physiological inhibitor of pre-formed IL-1. IL-1 β is a potent pro-inflammatory cytokine crucial for host-defense responses to infection and injury.^[21] In the periphery, IL-1 β is required for the efficient clearance of bacterial infections.^[7]

This study has shown that patients who received chitosan have the lowest level of iNOS and COX-2 expression. This condition might be due to the antibacterial effect of chitosan, resulting in the suppression of iNOS and COX-2 expression.

Further analysis indicates that the iNOS mRNA expression reveals a similar pattern to COX-2, with chlorhexidine treatment group being the highest compared with the other groups. It was well known that the NO and COX have a synergetic function during early response inflammation. It was reported that NO activates the COX enzymes to produce prostaglandins.^[11] Our study reveals that patients with

chlorhexidine treatment express higher iNOS and COX-2 than the other groups. In this case, it may be due to the fact that chitosan has more antimicrobial properties. Consequently, the inflammatory mediators were suppressed. The iNOS stimulates NO to activate the COX enzymes, suggesting that COX enzymes represent importance for modulating NO's multifaceted roles.^[22] This study supports the previous report, and the level of gingival iNOS has been detected in orthodontic treatment and the osteocytes.^[23] The literature reported that the success rate of miniscrew application during orthodontic treatment is more than 80%; the factors determining success rates were inconsistent between the studies analyzed.^[24] Minimizing infection surrounding the miniscrew tissue might be a strategy to prevent the failure of this orthodontic miniscrews application. From this study, we suggest that IL-1 α and IL-1 β , COX-2, and iNOS from crevicular gingival isolated mRNA were potent markers of inflammation in the early stage of applying orthodontic miniscrew. Although this study indicated a promise using chitosan mouthwash to decrease the possibility of inflammation, there are some limitations that need to be overcome in the future work, such as the responses when using a mixture of chitosan and chlorhexidine or mixed chitosan with enzymatic-based mouthwash.

CONCLUSION

This study reveals that chitosan has suppressed IL-1 β , a kind of pro-inflammatory cytokine, and chemokines, namely, COX-2 and iNOS. The elevation of IL-1 α expression may result in the downregulation of IL-1 β .

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CONFLICTS OF INTEREST

The authors report no conflict of interest.

AUTHORS CONTRIBUTIONS

HSA: conceptualization, supervision, drafting the manuscript. EH: data collection, laboratory work. EWB: laboratory supervision, data interpretation and proofreading the manuscript.

ETHICAL POLICY AND INSTITUTIONAL REVIEW BOARD STATEMENT

The research protocol was approved by the Ethics Committee of Research Faculty of Dentistry, Universitas Indonesia (ref. no. 4/Ethical Approval/FKG UII/2019).

PATIENT DECLARATION OF CONSENT

All research subjects have signed 'Patient declaration consent'.

DATA AVAILABILITY STATEMENT

Data can be obtained via email of corresponding author.

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