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The Effect of Stichopus hermanii to TLR-4 in Mediating Periodontal Ligament Remodeling During Orthodontic Relapse

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ABSTRACT

Stichopus hermanii contains an active compound that plays apart in the innate immune system. This research was aimed to investigate the effect of S. hermanii on the TLR-4 in mediating periodontal ligament (PDL) remodeling through fibroblast growth factor (FGF-2) and collagen type 1 parameter during orthodontic relapse. The true experimental using a post-test for the control group. Thirty-two male Guinea pig were distributed into four groups: K(–) group was the negative control group, K(+) group was the positive control group applied with orthodontic relapse forces, P1 group was applied with orthodontic relapse forces and 3% S. hermanii 3%, and the P2 group was applied with 3.5%. TLR-4, FGF-2, and collagen type-1 expression at the tension site was examined using immunohistochemistry. Analysis of Variance and Least Significance Difference tests were conducted for all specimens. TLR-4 in P1 and P2 were decreasing significantly ($p \leq 0.05$), while FGF-2 and collagen type 1 expression in P1 and P2 increased significantly ($p \leq 0.05$). Multiple regression test to correlate between parameters was also significant at 0.00 ($p \leq 0.05$). This study concluded that decrease of TLR-4 expression during orthodontic relapse showed a strong tendency to coincide with PDL remodeling along with increasing FGF-2 and collagen type 1 parameter. The optimum result in decreasing TLR-4 expression occurs when administered with S. hermanii 3.5%, which induces decreasing relapse to 29% and increasing periodontal remodeling, demonstrated by FGF-2 and collagen type-1 expression.

Keywords: Toll-Like Receptor-4, Orthodontic, Relapse, Periodontal ligament, Remodeling

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INTRODUCTION

Orthodontic treatment is used to correct the appearance and alignment of crooked, protruded, or crowded teeth, and these problems are solved based on evidence that when prolonged force is applied to a tooth, orthodontic tooth movement will occur due to remodeling of the periodontal ligaments (PDLs) and alveolar bone.[1,2] Orthodontic forces change, and thereby, transferred to the adjacent investing tissues, implying the initiation and propagation of signaling cascade cytoskeletal reorganization, gene expression and function, cell differentiation, cell proliferation, production, and secretion of specific proteins as a response. Apoptosis comprising the correlated blood vessels and neural tissue occurs during mineralized and nonmineralized tissue involved in tissue remodeling.[1,2,3] Pressure force induces bone matrix deformation and microcracks characterized by microcracks as primary damage induced by orthodontic mechanical force[4] and much determined on the degree of local strain.[5] The higher the strain degree, the more damage it produces. Microcracks are more predominant in the pressure-side than on the tension side of the tooth during orthodontic tooth movement.[6]

The change in the biological process that occurs during orthodontic tooth movement supporting newly aligned teeth is attributed to a physiologic regeneration of force balance, periodontal remodeling, and growth.[7] Teeth and supporting tissue will stabilize with time, but the risk of the teeth returning to their original position, a relapse, is always present. Orthodontic relapse can be triggered by periodontal bone support, tooth occlusal, soft tissue elements, and growth.[7,8] Experimental studies with *in vivo* orthodontic relapse models using male mice showed that cell apoptosis as a cell death marker in PDL was found in the area under pressure following orthodontic relapse.[9]

The TLRs have been indicated to have an affinity for molecules related to tissue damage.[10] Endogenous TLR ligands resulted from tissue injury are termed DAMPs, and established for functioning in immune regulation. DAMPs are produced in reaction to cell death and stress and create potent triggers of sterile inflammation. They may exert protective functions by notifying the immune system to the occurrence of apoptosis of cells resulting from mechanical stress.[11]

TLRs form a transmembrane receptor family by identifying DAMPs, which shows a vital role in the modulation of the immune response.[12,13] The up-regulation of TLR-2 and/or TLR-4 has been exposed in macrophages and fibroblast cells of inflamed periodontal tissue, which recommend that innate immune responses involve TLRs as signaling receptors.[12] The transmission of mechanical stimuli in orthodontic relapse involving host immune responses in particular cellular activity is not yet fully understood. Remodeling of PDL in response to mechanical stress and the strain-produced mechanical stimulation of cells and their associated extracellular matrix (ECM) can control integrin expression directly, focal adhesion proteins, cytoskeletal organization, cell morphology, cell adhesion to extracellular matrices, cell proliferation, and cell differentiation, thus impacting bone modeling.[5]

PDL is a considerably particular connective tissue that plays a function in orthodontic relapse found between the radicular and the alveolar bone. The main cell type found in this tissue is PDL fibroblasts. These cells are capable of performing cyclic response or static strains and are actively involved in numerous processes involving repair and regeneration, along with remodeling of adjacent hard tissue.[14] In a longitudinal section, the ligament cells appear to be located parallel to the oriented collagen bundles. Fibroblast activated by basic fibroblast growth factor (bFGF) or FGF-2 with similar function as a vascular

endothelial growth factor in this molecule. It is elaborated in the migration and proliferation of endothelial cells, angiogenesis under *in vivo* conditions, bone reconstruction, and is essential in collagen synthesis.[15,16] FGF-2 improves vascularization, enhances wound healing, regulates bone mass and its formation, increases the number of osteoclasts, decreases the production of type I collagen, and inhibits alkaline phosphatase activity. Mechanical stress changes the ECM composition of the PDL and induces the expression of several collagen types in PDL: fibril-forming, fibril-associated with interrupted triple helices, and non-fibril forming.[17]

The orthodontic treatment results consistency is one of the biggest problems to inhibit orthodontic relapse.[18] Inhibiting post-orthodontic relapses can be performed using removable retainers, fixed retainers, and orthodontic surgical appliances. All of these anchorages and retentive appliances have a role in keeping the best possible treatment outcomes, except for some limitations, such as difficulties in appliances fabrication, applications, and patient compliance.[19,20]

Stichopus hermanii exists in 1,250 varieties[21] and is a potentially valuable natural source, rarely explored, especially in the field of dentistry. *S. hermanii* belongs to the phylum of *Echinodermata* within the class of *Holothuroidea*.[22] It is a natural compound and contains numerous active components, such as hyaluronic acid, chondroitin sulfate, cell growth factor, Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA), and flavonoids that have the potential to reduce instances of orthodontic relapse. Previous research has shown that *S. hermanii* modulates inflammatory reactions, stimulates the modulation and proliferation of fibroblasts, and improves the rapid synthesis of the collagen fiber system with shorter healing time. The level of proinflammatory cytokines, which are interleukin (IL)-1α, IL-1β, and IL-6, were significantly decreased in *S. hermanii*-treated wounds and stimulated tissue regeneration.[23] Another study indicated that the *Stichopus* species extracts also negatively influences the capability or proliferation of human fibroblasts and osteoclast cells.[24]

This research provided an update on two critical points. Firstly, the effect of *S. hermanii* on TLR-4 expression, FGF-2, and collagen type 1 as PDL remodeling parameters during orthodontic relapse. Secondly, the mechanism of *S. hermanii* on TLR-4 expression that contributes to PDL remodeling and its correlation in orthodontic relapse. We also determined the most effective concentration.

MATERIALS AND METHODS

Samples preparation

This study used 32 male guinea pigs that are 2.5 months old, with bodyweights between 200 g and 300 g. Ethical approval for the research was obtained in January 2018 from the Ethical Commission of the Faculty of Dentistry Hang Tuah University no.055/ KEPK/ I/ 2018 and followed the National Institutes of Health Guide for the care and use of laboratory animals. The guinea pigs were distributed into four groups: K(-) group was the negative control group (without treatment); K(+) group was the positive control group to which orthodontic relapse forces were applied (orthodontic relapse was made by a helical spring for producing orthodontic tooth movement for 14 days. The helical spring was removed between day 15 and day 21 for producing orthodontic relapse); the P1 group was applied with relapse orthodontic forces and 3% *S. hermanii*; the P2 group was applied with 3.5% *S. hermanii*. The research was conducted at the Animal Laboratory of Department of Biochemistry of the Faculty of Medicine Airlangga University. After 21 days, the guinea pigs were slaughtered, and their jaws were subsequently sectioned.

Orthodontic relapse technique

Orthodontic relapse forces were produced by applying a helical spring with separating pliers to the mesial left maxillary incisors of the guinea pigs for 14 days. On the 15th day, the helical spring was removed for 7 days to induce orthodontic relapse. The helical spring force was 1 N, as determined by autograph, to achieve orthodontic tooth movement.

Preparation, formulation and application of S. hermanii

In this study, the *S. hermanii* was taken from seaside areas nearby Sumenep, East Java, Indonesia. Each specimen was cleaned by making a longitudinal 3–5 cm incision with a scalpel on its ventral side without damaging the internal organs before being dehydrated in an oven at 28°C temperature for 7 days. The *S. hermanii* powder size was then reduced in a blender to get a micro powder. Ten milliliters of 3% *S. hermanii* gel was prepared from 0.3 g of *S. hermanii* watery powder, diluted with 2% NaCMC in 5% DMSO; 10 ml of 3.5% *S. hermanii* gel was prepared from 0.35 g of *S. hermanii* watery powder, diluted with 2% NaCMC in 5% DMSO; 0.025 ml of *S. hermanii* gel was applied with an insulin syringe twice a day into the gingival sulcus (figure 1).

Figure 1: *Stichopushermanii* gel was deposited on the gingival sulcus after the application of orthodontic force

Immunohistochemical staining of the TLR-4, FGF-2, and Collagen type 1 expression

TLR-4, FGF-2 and collagen type 1 expression were examined on the tension side with an immunohistochemistry method using monoclonal antibodies of TLR-4 (Invitrogen™), polyclonal antibodies of FGF-2 (antibodies-online GmbH™), and polyclonal antibodies of collagen type 1 (Santa Cruz Biotechnology®) before being observed through the microscope. Photographs were taken to measure the TLR-4, FGF-2, and collagen type 1 expressions observed through the microscope with 400x magnification. Meanwhile, the PDL size on one-third apical in the tension area was recorded. Each histological section was also observed, and its size was recorded up to three times in the view areas.

Statistical analysis

All descriptive data were analyzed using a Statistical Package for the Social Sciences (SPSS™) version 20, before being tabulated. A statistical hypothesis was conducted with a standard analytic significance of 95% (p=0.05) by ANOVA (Analysis of Variance) test to examine the difference of each parameter up to the control variables measured. If the ANOVA test indicated a significant difference, the data were subsequently subjected to the Least Significance Difference (LSD) Test (p<0.05). The correlation between TLR-4 (Stimulus) and the parameters of periodontal remodeling (FGF-2) and collagen type 1 (response) was tested by a linear regression test.

RESULTS

The purpose of this study was to examine the TLR-4 expression in mediating PDLremodeling, with FGF-2 and collagen type 1 parameters during orthodontic biometric relapse, after the application of 3% and 3.5% *S. hermanii* gels. The results showed biometric relapse, as shown in Figure 2A. Decreasing orthodontic relapse biometric up to 29% occurred when 3.5% of *S. hermanii* was applied. The expressions of TLR-4, FGF-2, and collagen type 1 (figure 2).

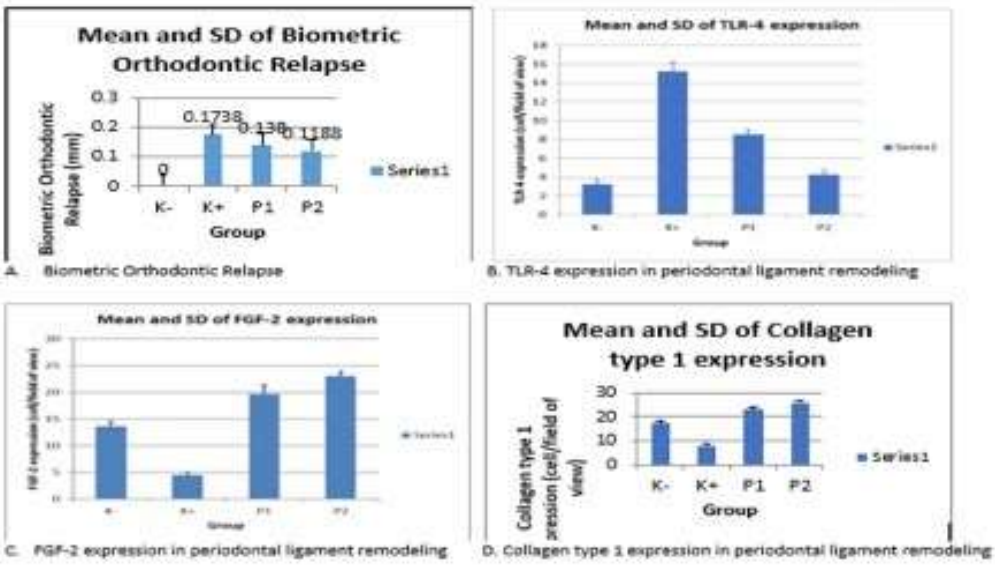

Figure 2: Bar chart of the *Stichopushermanii* effect during periodontal ligament remodeling in orthodontic relapse to biometric relapse in mm (A), TLR-4 expression (B), FGF-2 expression (C), and collagen type 1 expression (D).

The figure 2 and figure 3 shows the mean and SD of the expression of TLR-4 in the K(–), K(+), P1, and P2 groups (3.25±0.45, 15.25±0.96, 8.5±0.5, and 4.25±0.45, respectively). The data were then subjected to a normality test and a homogeneity test, which confirmed that all data were homogeneous and normally distributed. The ANOVA test value was p=0.00 (p≤0.05) for the TLR-4 expression in orthodontic relapse. Guinea pig administered with *S. hermanii* demonstrated significant differences. Thus, the LSD test was performed in advance, which confirmed that the P1 group was showing a significant decrease in the TLR-4 expression compared with the K(+) and K(–) groups. Moreover, the P2 group TLR-4 was significantly decreased compared with the K(+) group. However, the difference was not significant compared with the K(-) group. This result indicated that the P2 group applied with 3.5% *S. hermanii* have lower TLR-4 expression. This result also proved that 3.5% of *S. hermanii* could decrease the TLR-4 expression as DAMPs recognition.

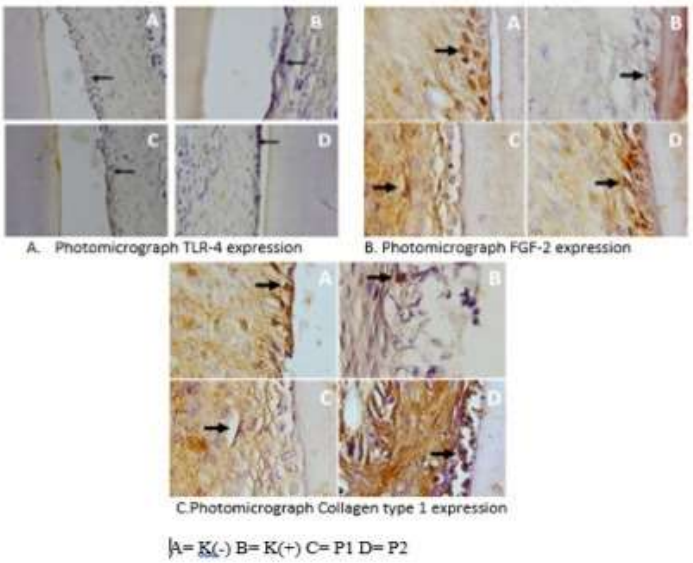

Figure 3: Photomicrograph of TLR-4 (A), FGF-2 (B), and (C) collagen type 1 expression in the K(−), K(+), P1, and P2 groups after *S. hermanii* application in the gingival sulcus during periodontal ligament remodeling orthodontic relapse.

FGF-2 and collagen type-1 expression were examined as PDL fiber remodeling parameters. Figure 2C and 3B show that the FGF-2 expression means and SD in the K(−), K(+), P1, and P2 groups are 13.63±0.28, 4.5±0.5, 19.63±1.72, and 23±0.87, respectively. All data are homogeneous and normally distributed. The ANOVA test p-value=0.00 ($p \leq 0.05$) for FGF-2 expression with the application of 3.5% *S. hermanii* showed a significant increase compared with the orthodontic relapse group. This result indicated that there was increased periodontal remodeling when the *S. hermanii*gel was applied, especially in the concentration of 3.5%.

An LSD test was performed in advance, which confirmed that the P1 and P2 groups showed a significant increase in FGF-2 expression compared with the K(+) and K(−) groups but not significant when compared with the P2 group. This result means that the application of *S. hermanii* gel in P2 and P1 groups have the same implication of increasing the FGF-2 expression.

Collagen type 1 expression in Figure 2D shows that the mean and SD in the K(−), K(+), P1, and P2 groups were 17.13±0.88, 8±0.73, 22.75±1.01, and 25.88±0.88, respectively. All data are homogeneous and normally distributed. The ANOVA analysis p-value=0.00 ($p \leq 0.05$) for the collagen type 1 expression with *S. hermanii* gel application, indicated a significant difference. The LSD test confirmed that both P1 and P2 groups showed a significant increase in the collagen type 1 expression compared with the K(+) and K(−) groups. Increasing expression of collagen type 1 can be caused by 3.5% *S. hermanii* gel application. The correlation test was conducted with multiple regression test results, after the linear relationship between independent variables and dependent variable in the linear regression model with scatter plots and multivariate normality had been checked. Probability test (F=0.000, p<0.05) indicated the acceptance of H1 thus, the FGF-2 and Collagen type 1 expression were related simultaneously to TLR-4 expression with a correlation coefficient of 0.686 (strongly correlated). The TLR-4 expression contributed to the FGF-2 and collagen type 1 expression (0.471). Some other factors might also contribute to periodontal remodeling.

DISCUSSION

Danger signals can be represented by the release of endogenous molecules that follows cell damage and/or death (DAMPs).[25] In this research, the increased expression of TLR-4 has been exposed in the fibroblasts of inflamed PDL in the orthodontic relapse (K+) group that indicated innate immune reactions, including the TLRs as signaling receptors.[12] In the short period, modulation of the innate immune system is advantageous to the vasculature by affording cytoprotective mechanisms and accelerating tissue repair after damage or infection.[25] TLR-4 in recognition of DAMPs passes into the bloodstream to stimulate the innate immune system as a result of modest elevations in peripheral vascular resistance. After cell damage and/or death, TLR-4 stimuli could subsequently lead to severe ischemic- or pressure-induced reactions; thereby, providing more DAMPs is necessary for innate immune system stimulation with consequences of inflammation, vasoreactivity, and vascular remodeling during orthodontic relapse.[12,25] Increased TLR-4 expression showed that there was damage to the orthodontic relapse tension area, which seemed contrary to the findings of previous research that suggested apoptosis is restricted in the pressure area.[9]

TLR activation stimulates catabolic reactions by enhancing matrix metalloproteinases (MMPs), nitric oxide, and prostaglandin 2 production and downregulating biosynthesis of matrix macromolecule.[26-29] A modulated expression of TLR4 compared with the normal condition may reflect further prevention of ECM destruction.[29] In this research, the K(+) group showed that orthodontic relapse decreased the FGF-2 and collagen type 1 expression compared with the normal K(-) group. Increased TLR

expression in orthodontic relapse was followed by decreased FGF-2 expression due to the modulation of MMPs. FGF-2 is known as a growth factor with multiple results on biomolecules, cells, and tissues and functions as a fibroblast mitogenic substance. However, further studies have emphasized its other activities, including angiogenesis stimulation, induction of endothelial cell proliferation, involvement in tissue remodeling, enhancement of wound healing, stimulation of proliferation and differentiation of osteoblasts and osteoclasts, chemotaxis of macrophages, involvement in fetal development, and protection of nerve cells against apoptosis.[30,31] Decreased FGF-2 and collagen type 1 expression in orthodontic relapse are followed by limited ECM remodeling in the PDL.[22]

S. hermanii contains numerous active ingredients, for example, hyaluronic acid, glycine chondroitin sulfate, heparan sulfate, cell growth factor, EPA, DHA, and flavonoid. *S. hermanii* is cholesterol-free, with high protein (55% of dry body weight) content, and comprises of 10%–16% mucopolysaccharides and saponins.[32] It also contains nutrients such as vitamin A, thiamine (B1), riboflavin (B2), niacin (B3), and minerals, particularly calcium, magnesium, iron, and zinc. Biological and pharmacological activities of *S. hermanii,* including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, and antioxidant makes it able to serve as a pain killer, anti-inflammatory, and anti-itching agent.[32,33]

Previous studies have shown that *S. hermanii* modulated inflammatory responses. Proinflammatory cytokines, such as IL-1α, IL-1β, and IL-6, were expressively decreased after *S. hermanii* application in wound treatment.[23] In this research, the TLR-4 expression was decreased in orthodontic relapse after administration of *S. hermanii* gel, as an effect of glycine, one of the *S. hermanii* content, which had a cytoprotective effect on stress.[34] Dietary glycine produced a downregulatory effect on TLRs.[35] Flavonoid also has a negative impact on TLR-4 that plays an important role in associated inflammation, consequently directing to the amelioration of PDL cell damage, thus enhanced the cell's survival. Downregulated downstream of TLR4 influence cellular signaling pathways that will impair transcriptional actions to downregulate the proinflammatory effectors commonly through two signaling pathways, one is the MyD88-dependent pathway and involves the NF-κB and MAPK activation.[36]

In this research, the P1 and P2 groups showed an increase in the FGF-2 and collagen type 1 expression in orthodontic relapse when administered with *S. hermanii* gel. According to the previous study, *S. hermanii* also stimulated the tissue regeneration along with the stimulation and proliferation of fibroblasts, while also enhancing the rapid production of a collagen fiber tissues, thus encouraging shorter healing time.[23] Another study supported previous research findings, which confirmed that *Stichopus* species extract negatively affected the viability and proliferation of human fibroblasts and osteoclast cells.[24] *S. hermanii* also contained the heparan sulfate that has a function of modulating the FGF-2 production and mediating physiological mechanisms in cell development, growth, migration, and wound healing. The effects of bFGF-2 are recognized to regulate ECM synthesis produced by fibroblast cells.[22] Furthermore, FGF-2 also stimulates alveolar bone formation,[37] which supports relapse prevention.

S. hermanii also contains collagen, but this compound does not have a significant effect on increasing the amount of collagen expression in the PDL, instead, supporting higher collagen stability.[38] Also, the activity of prolidase, which catalyzes the final step of collagen and plays an essential role in collagen biosynthesis on the PDL structures, which are accelerated by flavonoids.[39] Collagen stability will improve recovery from inflammation and increase the number of fibroblasts and osteoblasts.[38] It exerts a beneficial effect on osteogenesis to prevent orthodontic relapse.[22]

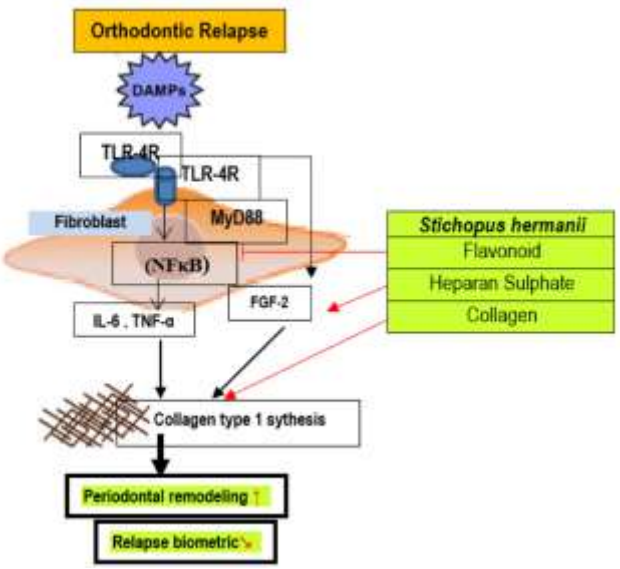

Figure 4: The role of TLR-4, FGF-2, and collagen type 1 expression in periodontal ligament remodeling during orthodontic relapse induced by *S. hermanii* application

This study showed that FGF-2 and collagen type 1 expressions were positively and simultaneously related to TLR-4 expression with a strong correlation. This result means that increased expression of FGF-2 and collagen type 1 was correlated with TLR-4 expression. When TLR-4 decreased, periodontal tissue repair will occur through the decrease of MMPs, resulting in the increased production and synthesis of FGF-2 and collagen type 1 needed for periodontal remodeling (figure 4).[25-31]

CONCLUSION

This study concluded that decreased TLR-4 expression during orthodontic relapse showed a strong tendency to coincide with PDLremodeling, along with increased FGF-2 and collagen type 1 expressions. The highest decrease occurs after administration of 3.5% *St. hermanii* gel, which induces a decreased biometric relapse down to 29%, and increased periodontal remodeling, reflected in the FGF-2 and collagen type 1 expression.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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