# Bone-immune interaction in osteogenesis Relapse Orthodontic after Nanopowder Stichopus hermanii Application

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**Abstract**

Relapse occurred at the end of active orthodontic treatment and tendency occurs 33-90% after at least 10 years post treatment. Loss of periodontal bone support can cause relapse orthodontic Nanopowder Sticopus hermanii have an active compound that have role in osteogenesis . Bone and immune system often interact in their function.

The aim of this study is to investigate HSP-70 and ALP expression in tension area as Bone immune interaction in osteogenesis relapse orthodontic after giving Nanopowder Stichopus hermanii application.

The experiment was held by Post Test Only Group design. Twenty four male Cavia Cobaya were divided into three groups. K(-) group as negative control group (without treatment), K(+) group as positive control group which were applied with relapse orthodontic forces, and the other groups P, were applied with relapse orthodontic forces and Stichopus hermanii 3 %. After treatment the cavia cobaya were sacrificed. HSP-70 and ALP expression at tension site as bone immune interaction were examined with immunohistochemistry.

Anova test showed HSP-70 decreased significantly with p=0,00 (P≤0,05), while ALP expression increase significantly with p=0,00 (P≤0,05) especially in P group to show bone-immune interaction occured compare with K(-), and K(+). LSD Test showed significant differences between all groups. Pearson test to corelate test of HSP-70 and ALP expression showed the moderate negative linear relationship (r=-0,307).

There is corelation between decreasing HSP-70 and increasing ALP expression in tension area as bone immune interaction in osteogenesis relapse orthodontic after giving Nanopowder Stichopus hermanii application

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## Introduction

The long-term stability after orthodontic treatment seems to be more important than the final result itself because of possibility of relapse. Relapse is a dento-alveolar and skeletal change after orthodontic treatment towards the initial malocclusion. Mean relapse below 1 mm is reported 7 or 8.2 years after orthodontic treatment. Higher prevalence of relapse for patients in whom the dentitions were not completely leveled at the end of the treatment[1].

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The biological process changes during orthodontic tooth movement are attributed to a physiologic reestablishment of force equilibrium, periodontal remodeling and growth[2]. A return to the original teeth position, can be caused by periodontal bone support, occlusal, soft tissue factor and growth[2,3]. Stability of orthodontic treatment result is one of the biggest challenges in orthodontics[1].

Bone remodeling is a crucial process in orthodontic tooth movement. Orthodontic movement is a continual and balanced process characterized by bone resorption and bone apposition on pressure and tension area after mechanical forces[4]. Orthodontic forces altering the blood flow and localized environment. These force alterations lead to the generation and propagation of signaling cascades and associated tissue remodeling by delineating

biochemical and cellular reaction and molecular events, such as signal generation and transduction, cytoskeletal re-organization, gene expression, differentiation, proliferation, synthesis and secretion of specific products, and apoptosis occurring in mineralized and nonmineralized tissue including the associated blood vessels and neural elements[4,5].

Many evidence showed that there is the interaction between the immune and bone systems which is the immune and skeletal systems share cytokines, signaling molecules, transcription factors and membrane receptors[6]. Bone immune interaction during orthodontic tooth movement occurring in bone remodeling. Many of the soluble mediators produced by immune cells, including cytokines, chemokines, and growth factors, regulate the activities of osteoclasts and osteoblasts that responsible for crosstalk between the skeletal and immune systems[7]. A more complete of the interactions between immune and bone cells should lead to better therapeutic strategies for orthodontics treatment and stability.

The heat shock proteins 70 (HSP-70) have role in bone immunology. HSP-70 was identified as intracellular proteins, which facilitate protein refolding, chaperone proteins and increase during stress to provide cellular protection[8,9]. Continuous HSP-70 expression will cause expression of caspases an executor of Program Cell Death (PCD) apoptosis[10]. Under osteogenic induction, HSP70 upregulated the expression of osteo-specific genes, such as the runt family transcription factor (Runx2) and osterix (OSX). HSP70 promotes osteogenesis of human Mesenchymal Stem Cells (hMSCs) through activation of the ERK signaling pathway[11]. The role HSP-70 in orthodontic tooth movement was detected in periodontal ligament fibroblast pressure area with mechanical force 10 g, in first day showed that immunoreactivity of HSP-70 was weak and on days 2, 3, 4, 7, 14 there are greater immunoreactivity. HSP-70 function as a homeostatic factor in cell with orthodontic mechanical stress induced[12].

The mechanism HSP-70 involving in relapse orthodontic is not clear. Relapse orthodontic can be anticipated with osteogenesis in tension area[13]. Alkaline Phosphatase is an early osteoblast marker in for cell differentiation from primary osteoblast, osteoblast precursor such as bone marrow-derived mesenchymal stromal cells (BMSCs) in osteogenesis. Levels of alkaline phosphatase activity can be used for examining cell differentiation in osteogenesis and are not proportional to observed mineralization levels[14]. The Bone immune interaction between HSP-70 and ALP in relaps orthodontic have not been investigated yet.

Osteogenesis with natural ingredients Stichopus hermanii for relapse orthodontic have been investigated[13]. Stichopus hermanii contain various active ingredient such as hyaluronic acid, chondroitin sulphate, cell growth factor, EPA DHA, flavonoid[15]. Stichopus hermanii is cholesterol-free and high in protein (55 % of dry body weight) and contains 10-16 % mucopolysaccharides and saponins[16]. Stichopus hermanii contain nutrients such as Vitamin A, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin), and minerals, especially calcium, magnesium, iron and zinc. A number of unique biological and pharmacological activities including anti-angiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant Stichopus hermanni can act as painkiller, anti-inflammatory and anti-itching[17]. That mucopolysaccharides active content reducing arthritic pain and arthralgia. Research studies indicate that the saponins of Stichopus hermanii have anti-inflammatory and anticancer properties[16]. Previous studi showed that *Stichopus hermanii* modulated the inflammatory responses, The level of proinflammatory cytokines; IL-1α, IL-1β, and IL-6, were significantly reduced in Stichopus hermanii application for treating wounds and also stimulate tissue regeneration and stimulated the activation and proliferation of fibroblasts, and also enhanced rapid production of collagen fiber network with shorter healing time[18]. The other study show that studies have shown that the extract of Stichopus species also affects viability or proliferation of human fibroblasts and osteoclast cells in a negative manner[19].

In the current study, we investigated the interaction HSP70 and ALP in osteogenesis differentiation relapse orthodontic after Nanopowder Stichopus hermanii application.

## Materials and methods

This study was performed on 24 male *Cavia cobaya* 2,5 months old with 200-300 g

weight. Ethical Approval for this research was obtained from Ethical committee of Dentistry Faculty Airlangga University in April 2015. The Cavia cobaya was divided into 3 groups. K(-) group as negative control group (without any treatment), K(+) group as positive control group which were applied with separator rubber for resulting orthodontic tooth movement for 14 days and removed the rubber after 7 days later for resulting relapse orthodontic, and the treatment groups P were applied with relapse orthodontic forces and *Stichopus hermanii* 3 %.

**Preparation of Relapse orthodontic**

Relapse orthodontic forces was produced with giving applied separator rubber by separating plier in mesial left insisivus maxilla cavia cobaya 14 days and after day 15 separator rubber was removed for 7 days for becoming relapse orthodontic. Separator forces was 0,0474 kN, measured by autograph to gain orthodontic tooth movement.

**Preparation of Powder Nanopowder *Stichopus hermanii***

Nanopowder *Stichopus hermanii* were used in this study from coastal regions around Sumenep, East Java Indonesia. *Stichopus hermanii* was cleaned by making a longitudinal incision 3-5 cm on the ventral side of stichopus hermanii without damaging the internal organs using scalpel. *Stichopus hermanii* was dried in oven 28°C for 7 days. After this, *Stichopus hermanii* was blender until get the powder. Stichopus hermanii was made to nanopowder by using High Energy Milling method.

**Figure 1.** Nanopowder Stichopus hermanii gel was applied in gingival sulcus with insulin syringe.

**Preparation and Applied *Stichopus Hermanii* gel**

Nanopowder *Stichopus hermanii* gel 3% was made from 0,3 gr *Stichopus hermanii* powder was diluted with NaCMC 2% in DMSO 5 % until 10 ml. *Stichopus hermanii* gel was applied in gingival sulcus with insulin syringe 0,025 ml twice per day, shown as fig 1.

The research was conducted in Biochemistry Laboratory Medical Faculty of Airlangga University. After 21 days, the *Cavia cobaya* were sacrificed. The jaw was sectioned. HSP-70 and ALP bone-immune interaction were examined with immunohistochemistry method in tension side and then observed by using a microscope. The photos were taken to measure the HSP 70 and ALP expression seen on the microscope with an enlargement 400x. Meanwhile, the size of the periodontal ligament on 1/3 apical in the tension area was observed. Each histological section was observed and calculated as many as three times in the field of view.

Finally, the data were statistically measured by using Statistical Package for the Social Science (SPSS) version 20. The research data result tabulated, the statistical hypothesis was conducted with a standard analytic significance of 95 percent (P=0.05) by ANOVA test (analysis of varians) to analyze the difference of each variable compared with control. Then the data were tested with LSD Test ($p<0.05$). Corelation test with Pearson.

**Results**

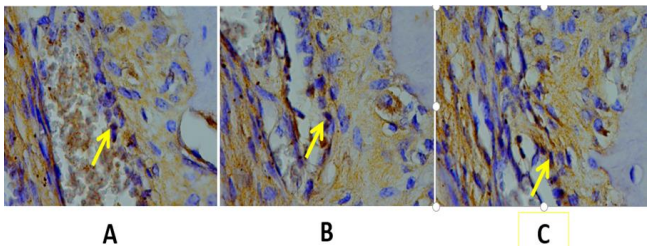


**Figure 2.** Photomicrograph of HSP-70 expression in K(-), K(+), and P.

The aim of this study is to investigate bone-immune interaction through HSP-70 and ALP expression at tension site relapse orthodontic by giving Nanopowder *Stichopus hermanii*. The result in this experiment showed HSP-70 expression as shown as fig 2 and ALP expression as shown as fig 3.

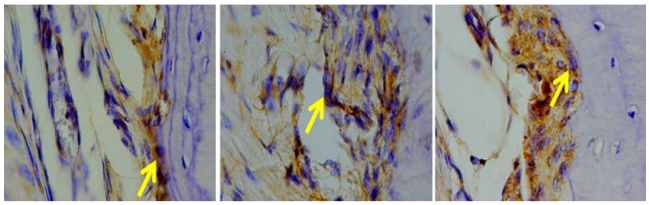


**Figure 3**. Photomicrograph of ALP expression in K(-), K(+), and P.

| Group | Mean± Standart Deviation |
|---|---|
| K(-) | 2,75±1,28 |
| K(+) | 7,13±1,46 |
| P | 4,75±1,58 |

**Table 1.** The Expression of HSP-70 in relapse orthodontics *Cavia cobaya* applied with *Stichopus hermanii.*

| Group | K(-) | K(+) | P |
|---|---|---|---|
| **K(-)** | | 0,000 | 0,000 |
| **K(+)** | 0,000 | | 0,000* |
| **P** | 0,000 | 0,000* | |

**Table 2**. LSD Test expression of HSP-70 in relapse orthodontics *Cavia cobaya* applied with Stichopus hermanii.

Table 1 showed means and SD in K(-), K(+), P are 2,75±1,28; 7,13±1,46; and 4,75±1,58 .Then the data were tested with normality test, homogenity test and show the data was homogen and have a normal distribution. ANOVA test value p=0.00 (P≤0.05) for the HSP-70 expression in relapse orthodontics *Cavia Cobaya* applied with *Stichopus hermanii* showed significantly differences. With the LSD test as seen as table 2, showed that P showed significantly decreased HSP-70 expression compare to K(+) and K(-).

| Group | Mean± Standart Deviation |
|---|---|
| K(-) | 7,25±1,91 |
| K(+) | 11,00±2,00 |
| P | 19,00±1,6 |

**Table 3**. The Expression ALP as osteoblast activity in relapse orthodontics Cavia Cobaya applied with Stichopus hermanii.

Table 3 showed means and SD in K(-), K(+), P are 7,25±1,91; 11±2,00; and 19±1,6. Then the data were tested with normality test, homogenity test and show the data was homogen and have a normal distribution. ANOVA test value p=0.00 (P≤0.05) for the expression of ALP as osteoblast activity in relapse orthodontics Cavia cobaya applied with Stichopus hermanii showed significantly differences. With the LSD test as seen as table 4, showed that P showed significantly increased ALP expression compare to K(+), and K(-). Pearson test to corelate test of HSP-70 and ALP expression showed the moderate negative linear relationship (r=-0,307).

| Group | K(-) | K(+) | P1 |
|---|---|---|---|
| **K(-)** | | 0,000 | 0,003 |
| **K(+)** | 0,000 | | 0,000* |
| **P** | 0,003 | 0,000* | |

**Table 4**. LSD Test expression ALP as osteoblast activity in relapse orthodontics Cavia Cobaya applied with Stichopus hermanii. *Significantly different.

## Discussion

The aim of this study is to investigate HSP-70 and ALP expression as Bone immune interaction Relapse Orthodontic with giving Nanopowder Stichopus hermanii application. In Relapse orthodontic, there are increasing HSP-70 as marker of bone survival to mechanical forces. Application af nanopowder Stichopus hermanii 3% in tension area, resulting decrease HSP-70 expression in relapse orthodontic. HSPs has known to have role in the regulation of cell function and defense against injury to the cell[20]. HSPs are one of the factors recognized that is enhanced by heat shock. HSP is a stress protein because it is not only enhanced by heat shock but also by ischemia, other pathological changes such as infections and inflammation, radiation, physical stress such as light, stress from enzymes, heavy-metal ion, arsenic, arsenic acid, methanol, active oxygen and stress from chemical and various amino acid derivatives[20]. The HSPs consist of multimember families based on the molecular weights of the proteins encoded, in example, HSP27, HSP60, HSP70, and HSP90. The HSP production is primarily mediated by heat shock transcription factors (HSFs) that interact with a specific regulatory element, heat shock element (HSE), present in the HSP gene promoters[21].

High-molecular weight HSPs such as HSP70 temporarily binds to proteins in immature state capable of helping the protein to mature and mediates attachments to polypeptides acting as molecular chaperones[20]. Study Araujo et al, 2007 showed that in culture experiment,

microarray revealed that only HSP70 increased when human PDL fibroblasts were exposed to mechanical stress[22]. HSP70, also named HSP72, is generally expressed at very low or undetectable levels in unstressed normal cells while it is highly expressed in many malignant tumors, consistent with the idea that it represents a prosurvival factor, playing an essential role as chaperone in protein folding[23]. Hsp70 inhibits the apoptotic pathways induced by ROS-producing agents such as TNFalpha, heat, cytotoxic drugs and radiation. Hsp70 may also mediate its anti-apoptotic effects by binding to mutant p53, causing a decrease in wild-type p53 (w.t. p53) or c-Myc, reducing the cellular levels of free c-Myc[24].

The induction of the HSP in higher organisms is regulated at the transcriptional and translational levels. The transcription of heat shock genes is regulated by the cis-acting heat shock element (HSE) in the promoter region and the trans-acting heat shock factor (HSF)[23].

Bone immune interaction involving HSP-70 and ALP are now well described. It has become well established that multiple soluble mediators of immune cell function including cytokines, chemokines, and growth factors regulate osteoblast and osteoclast activity, and cells related to osteoblasts, which form bone, are critical regulators of the hematopoietic stem cell (HSC) niche from which all blood and immune cells derive[25]. Chen research, 2015 showed that HSP-70 can increase the expression of Runx2, OSX, and ALP. These results suggest that HSP70 enhanced osteogenic differentiation of hMSCs through mediation of the transcription factors Runx2 and Osx[11]. In research using microarray pathway analysis and RT-PCR showed that the ERK signaling pathway is strongly related to HSP70-promoted osteogenesis, and blocking the ERK signaling pathway could decrease the effect of HSP70 on hMSC osteogenesis[11].

When the left first insisivus compressed towards the distal side during 14 days orthodontic tooth movement and appliance was released, relapse occured toward the mesial side. Rapid relapsee initially following 2 days appliance removal. Osteogenesis is needed for preventing relapse orthodontic movement to the original position before orthodontic treatment. Relapse orthodontic in this study showed there was increasing HSP-70 and ALP compare with K(-) group, but still more lower than P group. The role of HSP70 on ALP activity, an early marker of osteoblastic differentiation[26]. ALP hydrolyzes pyrophosphate to generate phosphate, which reacts with calcium to form hydroxyapatite and promote mineralization, and plays an important role in bone formation[11].

Some methods can stimulate bone remodeling to accelerate orthodontic tooth movement such as drug[27]. Nanopowder Stichopus hermanii is a natural drug that proposed to used for accelerating bone remodeling. The potency of extracts to be traditional medicine depends on their chemical compounds (secondary metabolites)[28]. In this research, Nanopowder Stichopus hermanii with their contain various active ingredient such as glycine, flavonoid chondroitin sulphate, cell growth factor, EPA DHA application in relapse orthodontic resulting decrease HSP-70 in P group compare with K(+) or relapse orthodontic group but still increase compare with K(-), also nanopowder Stichopus hermanii can increasing ALP compare with K(-) dan K(+) group.

HSP-70 with application nanopowder Stichopus hermanii was decreased compare with relapse orthodontic group means that mechanical relaps stress orthodontic with giving Nanopowder Stichopus hermanii implied reducing HSP-70 from participation in the folding of proteins by minimizing incorrect interactions within and between molecules, and also maintenance of proteins in their native folded states, and reducing repair or promotion of the degradation of misfolded proteins[29]. Reducing HSP-70 means function in cellular protection by modulating the engagement and/or progression of apoptosis induced by a variety of stress stimuli and their reduction chaperone function during physiological relapse conditions. In this research Stichopus hermanii in relapse orthodontic reducing HSP-70 that means Stichopus hermanii with active ingredient can reduce the injury effect to cell and their tolerance to mechanical stress. Reducing HSP-70 have function as a homeostatic mechanism to compensate during relapse orthodontic force as a result of circulatory disturbance[31].

HSP-70 with application Nanopowder Stichopus hermanii was little bit increase compare with K(-) showed that HSP-70 have mechanism in cell protection for homeostasis[12] when relapse mechanical stress induced even after applying Nanopowder Stichopus hermanii.

Increasing HSP-70 showed that cell still recognize relapse orthodontic with giving Nanopowder Stichopus hermanii as a mechanical stressor and induced cell response with increasing HSP-70. Nanopowder Stichopus hermanii with its contents can modulate immune response for cell.

Flavonoid in Nanopowder Stichopus hermanii implied to Hsp70 expression under baQ or specific silencing of Hsp70 prevented also the upregulation of IRE1α induced by ER stress, suggesting a functional link between Hsp70 and IRE1α, allowing the increased expression of the latter protein under conditions of ER stress that could be due to an increased translation of IRE1α or to a physical connection leading to an increased half-life of IRE1α.sal conditions and upon ER stress[23]. The other content of Stichopus hermanii, glycine have a cytoprotective effect to stress[32].

Flavonoid as Stichopus hermanii active content also resulted in a significant elevation of alkaline phosphatase (ALP), this result is parallel to the other study that showed flavonoid can increase ALP activity, collagen contents and osteoblast differentiation genes ALP, collagen, osteopontin (OPN), osteoprotegerin (OPG) and osteocalcin (OC) and bone morphogenetic protein (BMP) genes (BMP2, BMP4 and BMP7)[34]. Osteoblast differentiation is the primary event of bone formation. Bone ALP is a glycoprotein localized in the plasma membrane of osteoblastic cells, which is also one of the osteoblastic phenotype markers. ALP, and Collagen type 1 are early markers of osteoblast differentiation, while osteocalcin appear late, concomitantly with mineralization[33].

Chondroitin sulphate as a active content of Nanopowder Stichopus hermanii have potensial role in ALP. Pecchi, 2012 was investigated a potensial role of chondroitine sulphate in stimulated osteoblast showed that after 3 weeks of culture, cells formed a 3D membrane, showing a strong alkaline phosphatase activity and expressed genes characteristics of the osteoblastic phenotype [RUNX-2, COL1A1, bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OC)[37]. Nanopowder Stichopus hermanii consist of collagen as a higher concentration ingredient have also role in increasing ALP. Study of adult human osteoblastic cells were grown in a native type I collagen gel was showed that cultures expressed high alkaline phosphatase (ALP) levels[38].

The rate of bone remodeling define primarily by cells of the osteoblast lineage with ALP as one parameter and also bone formation are responsible for activating osteoclast[39]. In orthodontics attaining absolute anchorage is one of the greater tasks for successful outcome of the treatment[40]. Application Nanopowder Stichopus hermanii in relapse orthodontic have role in decreasing HSP-70 and increasing ALP compare with relapse group, while compare with normal group showed that increase HSP-70 and ALP means Nanopowder Stichopus hermanii might have protective role in cell stress and bone formation with different lineage. Nanopowder Stichopus hermanii with a higher concentration active content have more effect in bone formation than immune modulation. Bone immune interaction indirectly between HSP-70 and ALP in Nanopowder Stichopus hermanii application in relaps orthodontic. Nanopowder Stichopus hermanii have more potensial effect in bone formation rather than cell immune.

## Conclusions

There is corelation between decreasing HSP-70 and increasing ALP expression in tension area as bone immune interaction in osteogenesis relapse orthodontic after giving Nanopowder Stichopus hermanii application

## Declaration of interest

No conflict of interest for this article and the article is not funded or supported by any research grant.

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