TRAP-6, OPG, and ALP Expression in Alveolar Bone on Orthodontic Tooth Movement Induced by Hyperbaric Oxygen Therapy and *Stichopus Hermanii*

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Abstract

Malocclusion remarkably prevails in 56% of the global population. This study has aims analyzing the HBOT method and S. hermanii gel incorporation in the alveolar bone remodeling advancement during the orthodontic tooth movement with TRAP-6, OPG, and ALP as biomarker. Thirty male guinea pigs, was randomized into five groups: Normal control group (NT), negative control group (NC) Helical springs were inserted during 14 days without any administration, Treatment group 1 (T1). Helical springs were inserted with S. hermanii gel administration at days 3 to 14, Treatment group 2 (T2) with HBOT treatment from 8 to 14 days at 2.4 ATA, Treatment group 3 (T3) with both administrations of S. Hermanii and HBOT treatment. The parameters stained through immunohistochemical (IHC) method. Positive expression of TRAP-6, OPG, and ALP was assessed semi-quantitatively according to the modified Kaemmerer method and were evaluated by using Kruskal Wallis and Mann Whitney tests ($P < 0.05$). The administration of combined treatments of S. Hermanii and HBOT was proven to increase the ALP and OPG expression and decrease the TRAP-6 expression, compared to NC and NT ($p \leq 0.05$).

The combined treatments did not affect the ALP expression significantly compared to the S. Hermanii-only group, which indicates that the elevated ALP expression was predominantly affected by the S. Hermanii components also OPG expression due to flavonoids, arginine, and the chondroitin sulfate components. the combined treatments did not lower the TRAP-6 significantly compared to the HBOT-only group, which indicates a predominant portion of oxygen exposure in the TRAP-6 decrease.

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Introduction

Malocclusion, an abnormal irregularity of the alignment or the interconnection of teeth during dental occlusion, can affect various oral functions, including chewing, swallowing, and speaking fluency, and could also generate temporomandibular dysfunction.[1] This abnormality remarkably prevails in 56% of the global population.[2] Malocclusion is corrected through orthodontic care to improve the dentofacial aesthetics and construct a normal occlusion, including corrections of crowded teeth, rotational deviation, and the tooth apical. These alterations require a protracted care period, from six months to two years of treatment.[3]

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The fundamentals of orthodontic care incorporate mechanical pressures to produce an orthodontic tooth movement.[3] Orthodontic tooth movement causes an area of tension and compression.[4] An orthodontic tooth movement would initiate remodeling in the tooth and the surrounding tissues, comprising dental pulp, gingiva, periodontal ligament, capillaries, innervations, cementum, and alveolar bones.[5]

Modifications in the cellular environment, cellular-to-matrix interchange, and gingival crevicular fluid (GCF) represent biomarkers in the periodontium state during orthodontic tooth movement.[6] Vascularization is an essential factor in tissue remodeling.[7] Vascular Endothelial Growth Factor (VEGF) is a collagen-synthesizing agent during endothelial growth[8], as collagen is the main constituent of the periodontal ligament.[9] The collagen fibers of the periodontal ligament in the tension area during the orthodontic tooth movement would be in perpetual contraction.

Transformation in the tension area would trigger cellular responses to stimulate osteogenesis in alveolar bones.[10]

During the alveolar bone remodeling, osteoblasts play a significant role in the bone remodeling process, which includes osteoblast-osteoclast interactions, systemic hormones regulation, and cytokines and growth hormone modulation.[4,11] Several parameters could be observed during this remodeling activity. Thrombin Receptor Activator Peptide 6 (TRAP-6) is a parameter representing the expression of osteoclasts receptor activator of nuclear factor κB (RANK) and its ligand, RANKL, a principal element in osteoclast differentiation of bone resorption. In contrast, osteoprotegerin (OPG), an osteoclast-inhibitory agent produced by osteoblasts, is another remodeling parameter that would compete with the RANKL. Thus, the RANKL and OPG regulate the turnover of the connective tissues.[11,12] Alkaline phosphatase (ALP) is a bone apposition remodeling parameter that induces osteoblast release during the bone remodeling process.[3]

Oxygen also occupies a significant part during the alveolar remodeling process. In the previous study, the HBOT was proven to stimulate the growth and repair of vascularization, prevent inflammation, reduce free radicals, and amplify osteoblast activities during an orthodontic tooth movement. The hyperbaric oxygen therapy (HBOT) method is conducted through pure oxygen inhalation with increased pressure. The pressure is customarily applied at 1 to 3 or 1.5 to 2.4 atmosphere absolute (ATA) with 90 to 120 minutes of duration (Brahmanta, 2019; Brahmanta, 2016). In studies that have been carried out, exposure to hyperbaric oxygen can stimulate repair and growth of blood vessels, prevent the inflammatory phase, reduce free radicals, increase osteoblast activity in tooth movement.[6,13]

Besides the HBOT method, a natural sea biota of *Stichopus hermanii*, a golden sea cucumber, was also indicated to provide plentiful bioactive properties.[14] The bioactive constituents of the biota, including collagen, flavonoid, chondroitin sulfate, hyaluronate, and arginine, evinced to fasten the tooth movement for about 60% in the periodontal remodeling process during the orthodontic tooth movement.[10,15]

The integration of both methods was logically assumed to foster a better refinement in the periodontal remodeling activities. While the *Stichopus hermanii* gel would perform as local agents through topical preparation, the inhaled HBOT effects systemically. This combination was thought to synergically surpass the individual treatment limitations, enhance the effectivity, subtract the required dosing, and thus limit the adverse effects of the methods individually.[16]

Based on the reported potencies, we focus on analyzing the HBOT method and *Stichopus hermanii* gel incorporation in the alveolar bone remodeling advancement during the orthodontic tooth movement. TRAP-6, OPG, and ALP were observed as biomarker parameters.

Materials and methods

We conduct a true-experimental study with a post-test-only control group design. Ethical authorization was issued by the Ethics and Scientific Committee concerning experimental animal employment. The protocol was consented to by The Ethical Clearance of Health Experiment Committee, Faculty of Dentistry Hang Tuah University, under registration number 055/KEPK/I/2020.

Cavia cobaya, guinea pigs, were selected as study subjects. Thirty male guinea pigs in their marital age, aged two to three months, weighed 300 to 400 g, and were in their prime physical state were appointed. Simple random allocation was applied to randomize the subjects into five study groups:

(1) Normal control group (NT). No treatment was given during the 14 days of the treatment period, neither insertion of helical spring nor administration of *S. Hermanii* gel and HBOT treatment.
(2) Negative control group (NC). Helical springs were inserted during 14 days of the treatment period without any administration of gel and HBOT treatment.
(3) Treatment group 1 (T1). Helical springs were inserted during 14 days of the treatment period with the administration of gel at days 3 to 14, twice a day.
(4) Treatment group 2 (T2). Helical springs were inserted during 14 days of the treatment with the administration of HBOT treatment from 8 to 14 days at 2.4 ATA, three times 30 minutes each.
(5) Treatment group 3 (T3). Helical springs were

inserted during 14 days of the treatment period with both administrations of *S. Hermanii* gel at days 3 to 14 twice a day and HBOT treatment from 8 to 14 days at 2.4 ATA, three times 30 minutes each.

The selected *Cavia cobaya* were kept in a cage with sufficient light, air, and continuous feeding. The subjects were adapted for seven days to adequately adjust to the study environment and achieve their supreme general well-being before the experiments. Routine weighing of the study subjects was carried out to maintain the subjects' criteria.

***Stichopus hermanii* Ointment Preparation**

Golden sea cucumbers from Sumenep, Madura, were retrieved in wet condition. The internal viscera were removed, remaining the body walls. One kilogram of the body walls was cleansed, obtained, and contained in a plastic carrier to be further macerated using the laboratory blender with 71 Waring Commercial Model HGBTWT brand. The battered preparation was then freeze-dried in an Erlenmeyer at 85oC temperature with 5 M-Torr pressure for about 72 hours. The dried *S. Hermanii* was scaled and re-macerated into 31 g of powder.

The 3.5% *S. Hermanii* gel was produced using a mixture of 0.35 g 100% *S. Hermanii* powder, 0.2 g CMC-Na powder, and 10 ml aquadest. The mixture was blended using a mortar and pestle on a hot plate.

Treatment Procedure

Bilateral incisive bands and helical springs were inserted using a plier for 14 days of the treatment period in the NC, T1, T2, and T3 groups. The insulin syringe was pre-blunted using scissors to prevent injury to the gingiva sulci of the *Cavia cobaya.*

S. Hermanii gel administration commenced three days following the insertion of the orthodontic devices and was released every two days during the 14 days of treatment. The natural gel was given in the morning and evening with a 0.025 ml dose each for the T1 and T3 groups.

The 2.4 ATA HBOT treatment was set out for seven days consecutively at 8 to 14 days of the treatment period for T2 and T3 groups without releasing the maxilla expansion. The treatment was executed in a multi-place chamber, three times 30 minutes of each treatment, with a 5-minute free-air interval between each exposure. The designated subjects were lodged in the multi-place chamber. The internal oxygen pressure raised gradually until reaching 2.4 ATA. A 100% oxygen flow was poured for 30 minutes, halted for 5 minutes for a free-air interval, and repeated three times for the same procedure. The sudden shift in the air pressure would trigger uneasiness and aural pain for the *Cavia cobaya.* The pain was managed through feeding and drinking, as the chewing mechanism would reduce the ongoing pain. Following the HBOT treatment, the air pressure was reconditioned to the initial pressure of 1 ATA. The *Cavia cobaya* was returned to the cage.

Figure 1. Administration of *S. Hermanii* gel and HBOT treatment.

All study groups were immolated on day 14 through the Overdose of Chemical Anaesthetic method using a ketamine-acepromazine agent. The subjects were then decapitated and sampled for the maxilla and teeth. The maxilla was fixated in a 10% buffered solution of formalin and Ethylene Diamine Tetra Acid (EDTA), then deparaffinized and stained through immunohistochemical (IHC) method using TRAP-6, OPG, and ALP monoclonal antibodies. Positive expression of TRAP-6, OPG, and ALP was assessed semi-quantitatively according to the modified Kaemmerer method using the CX22 Binocular, Olympus brand, with ×400 enlargement. The Immunoreactive Score (IRS) was quantified, through the multiplication of positive cells score percentage with the intracellular staining score intensity produced **(Table 1)**.

Point score	Percentage of Stained	Intensity of Staining
0	No cells with positive reaction	No color reaction
1	≤10% cells with positive reaction	Low intensity of color reaction
2	11%-50% cells with positive reaction	Average intensity of color reaction
3	51%-80% cells with positive reaction	Intense color reaction
4	80% cells with positive reaction	
Scoring points: A × N		

Table 1. IHC scoring interpretation.

The positive score percentage was classified from 0 to 3, with (0) represents no positive cells, (1) represents positive cells <30 %, (2) positive cells 30-60%, and (3) positive cells >60%. Moreover, the intracellular staining score intensity was also classified from 0 to 3, with (0) representing no color reaction, (1) low intensity of the color reaction, (2) medium intensity, and (3) profound intensity. Positive cells with anti-TRAP-6, OPG, and ALP were indicated as brown coloring. The data were evaluated by using Kruskal Wallis and Mann Whitney tests ($P < 0.05$).

Results

This study was aiming to the observation of the enhancement of orthodontic tooth movement following the administration of 3.5% *Stichopus hermanii* gel and 2.4 ATA HBOT using TRAP-6, OPG, and ALP as parameters. The expression of TRAP-6, OPG, and ALP was retrieved through quantification of each parameter using 400× enlarged IHC preparation of alveolar bones in the tension area (Fig 1, 2, 3, 4, 5).

The obtained outcomes were analyzed as ordinal data using descriptive and analytic statistics. Kruskal-Wallis and Man-Whitney tests were utilized with a 95% significance rate, $p<0.05$, employing Statistical Package for the Social Sciences (SPSS) program version 22.0. IHC analysis of TRAP-6, OPG, and ALP expression was scored as the following (Table 1).

Descriptive Analysis

ALP expression was assessed using antibody staining from Bioss Antibodies, while TRAP-6 and OPG expression was assessed using antibody staining from antibodies.online.com. The results obtained were analyzed semi-quantitatively using the presented scoring system in Table 1.

The TRAP-6, OPG, and ALP expression IHC score analysis presented a variable mean between each study group. The results showed increases in ALP and OPG expression in all the treatment groups (T1, T2, T3) compared to the control group (NC). However, the TRAP-6 expression merely showed a minimum decline in the T1 and T3 groups.

The Shapiro-Wilk normality test indicated an abnormal distribution of the outcomes ($p\leq0.05$). Thus, the results obtained were semiquantitative data and should be analyzed using a non-parametric statistic, the Kruskal-Wallis Test.

		ALP			TRAP-6			OPG		
Study Groups	N	Mean	Median	*p*-value	Mean	Median	*p*-value	Mean	Median	*p*-value
NT	6	1	1	0.00	1.33±0.5	1	0.00	1	1	0.00
NC	6	1.5±0.5	1.5		8.83±0.8	9		2.7±0.7	2.5	
T1	6	3.33±0.8	3.5		6±0.9	6		7.5±1.04	7.5	
T2	6	5.5±1.05	5.5		4.67±0.9	4.5		6.3±0.5	6	
T3	6	6.5±1.5	6.5		7±0.9	7		4.5±0.5	4.5	

Table 2. Mean and median of TRAP-6, OPG, and ALP expression as parameters in alveolar bone remodeling following administration of *Sticophus hermanii* gel and HBOT treatment during the orthodontic tooth movement.

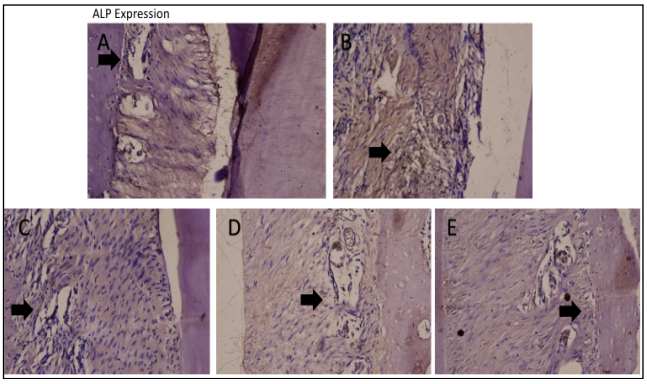

Figure 2. Immunohistochemical figures of ALP expressions osteoblast cells of the alveolar bone tissue. A. NT groups, B.NC groups, C. T1 groups, D. T2 groups, E. T3 groups with 400X enlargement.

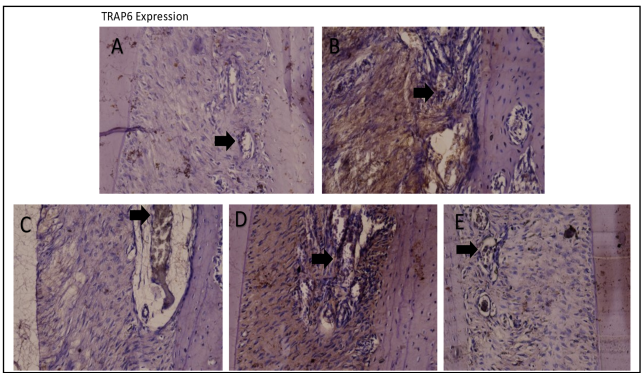

Figure 3. Immunohistochemical figures of TRAP6 expressions osteoclast cells of the alveolar bone tissue. A. NT groups, B.NC groups, C. T1 groups, D. T2 groups, E. T3 groups with

400X enlargement.

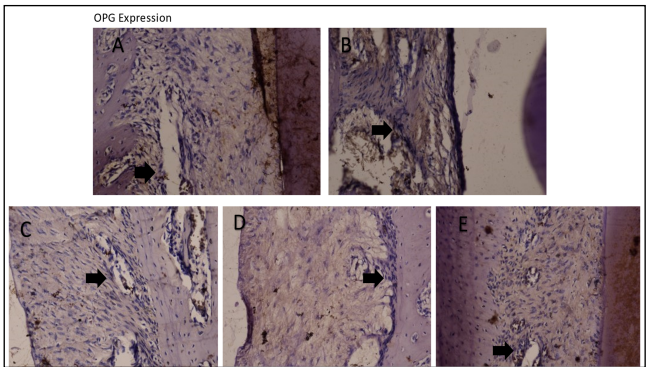

Figure 4. Immunohistochemical figures of OPG expressions osteoblast cells of the alveolar bone tissue. A. NT groups, B.NC groups, C. T1 groups, D. T2 groups, E. T3 groups with 400X enlargement.

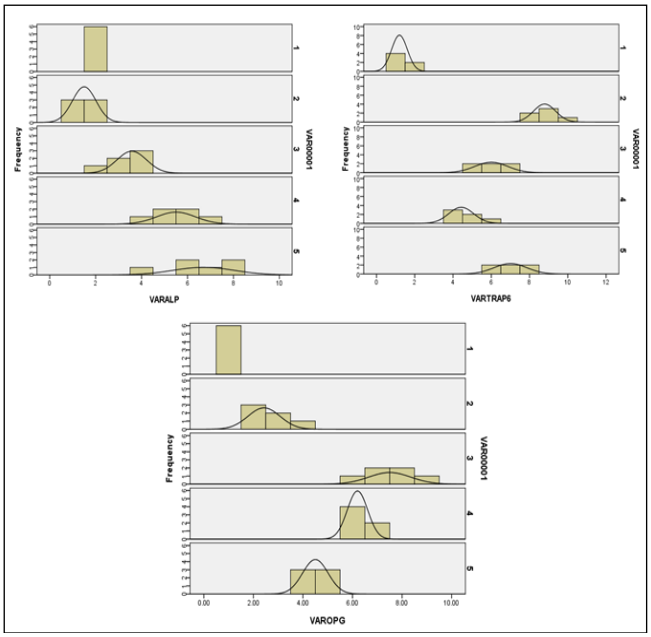

Figure 5. Histogram output of Kruskal-Wallis' variability differences on parameters ALP, TRAP-6, and OPG expressions.

The histogram shape and distribution between each parameter were found diversified, along with their median results. Hence, these statistical results could only be accounted for mean analysis and not for median analysis.

Comparative Analysis

The Kruskal-Wallis' analysis presented a significant difference in the TRAP-6 (p=0.00), ALP (p=0.00), and OPG (p=0.00) expressions in the alveolar bone during the enhanced orthodontic tooth movement employing administration of *Stichopus hermanii* gel and HBOT treatment.

The Kruskal-Wallis analysis results of TRAP-6, OPG, and ALP expression indicated particular significant differences between matched study groups. Despite the insignificant comparison of ALP expression between the negative control and the normal control group, the ALP expression in all the treatment groups exhibited a significant increase compared to the normal and negative control groups (p≤0.05).

No significant difference was found between the HBOT-only group and the combined treatments group, however significant increase in ALP expression was found between the *S. Hermanii*-only group and both the HBOT-only and the combined treatments groups.

Matched Study Groups	ALP	TRAP-6	OPG
NT vs NC	0.056	0.003*	0.002*
NT vs T1	0.007*	0.003*	0.002*
NT vs T2	0.002*	0.003*	0.002*
NT vs T3	0.002*	0.003*	0.002*
NC vs T1	0.006*	0.003*	0.004*
NC vs T2	0.003*	0.003*	0.003*
NC vs T3	0.003*	0.008*	0.006*
T1 vs T2	0.007*	0.031*	0.042*
T1 vs T3	0.007*	0.096	0.003*
T2 vs T3	0.189	0.006*	0.003*

*p≤0.05

Table 2. Mann-Whitney analysis results on TRAP-6, ALP, and OPG expressions in alveolar bone during the enhanced orthodontic tooth movement employing administration of *Stichopus hermanii* gel and HBOT treatment.

The TRAP-6 expression in all the treatment groups manifested significant decreases compared to the negative control group (p≤0.05). Within the matched treatment groups, the most substantial decline compared to the negative control group was found in the HBOT-only treatment group, while the *S. Hermanii*-only and the combined treatment groups merely present a slight decrease. Nonetheless, no significant difference was found between the *S. Hermanii*-only and the combined treatment groups.

All the treatment groups revealed significant increases in the OPG expression compared to both the normal control and the negative control groups (p≤0.05). Curiously, the OPG expression in the *S. Hermanii*-only and the HBOT-only groups was found higher than in the combined treatments group.

Discussion

The combined treatments of 3.5% *S. Hermanii* gel on day 3 to 14 and 2.4 ATA HBOT treatment on day 8 to 14 was employed to analyze the TRAP-6, ALP, and OPG expressions in alveolar bone during an enhanced orthodontic tooth movement. *Cavia cobaya* was appointed as the study subject.

Orthodontic tooth movement relies on coordinated tissue resorption and formation in the surrounding bones and periodontal ligaments. Tooth loading causes local hypoxia and fluid flow, initiating an aseptic inflammatory cascade and culminating in osteoclast resorption in areas of compression and osteoblast deposition in areas of tension. Compression and tension are associated with particular signaling factors, establishing local gradients to regulate the remodeling of the bone and periodontal ligament for tooth movement.[17] When the pressure is applied sufficiently to the teeth, periodontal remodeling would occur. Vascularization and oxygen pressure contributed an equal significant role in the process of remodeling.[13]

Periodontal ligaments hold a valuable function in tooth movement orthodontically, due to the capacity of these tissues in responding to sustained mechanical forces. Thus, alveolar bone remodeling could occur and facilitate orthodontic tooth movement.[4,8,10] Alveolar bone remodeling mainly relies on the activity of the two predominant cells in the bone tissue, the osteoclast and osteoblast. Osteoclasts are nucleated cells that are designated to dissolve the bone matrix, while osteoblasts are the osteogenic ones. Both of these cells maneuver accordingly through mechanosensory functions.[18]

The application of orthodontic mechanical pressure peaked the osteoclasts count per bone surface unit up to 50 hours following the pressure application.[4] This concept is in corresponds with the increase of TRAP-6 expression presented in this study following the orthodontic mechanical pressure exertion. When activated, TRAP-6 would synthesize lysine 63-linked polyubiquitin and activate IκB kinase, which would phosphorylate IκBα, an NF-κB inhibitor, and diminish through degradation. Degradation of IκBα would release the NF-κB from the IκBα/NF-κB complex and promote the penetration of the NF-κB to the cell nucleus, thus activating the gene expression required in osteoclasts formation. It could be assumed that TRAP-6 is responsible as a major adapter molecule for the RANK signaling pathway associated with osteoclastogenesis.[19]

The outcomes of this study also illustrated the orthodontic mechanical pressure influence on the osteoblasts count escalation. These results are in concordance with a previous study that outlined an mRNA expression and ALP activity alteration of the type I collagen in osteoblasts or cementoblasts during the implementation of orthodontic mechanical pressure. The study also reported an OPG expression elevation as a result of orthodontic mechanical pressure.[20]

The administration of *S. Hermanii* gel in this study aimed to promote tooth movements using a natural marine pharmaceutical agent. A foregoing study applying 3% of *S. Hermanii* gel on the gingiva sulci was proving a 60% acceleration of tooth movement through the hastening of bone remodeling.[10,18] The bioactive components contained in the golden sea cucumbers include lectin, sterol, saponin/triterpene glycoside, protein, collagen, mucopolysaccharide, glycosaminoglycans (GAGs), chondroitin sulphate-E, chondroitin sulfate fucosylated, amino acids, fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA], vitamins (thiamine, riboflavin, niacin, ascorbic acid, alpha-tocopherol), carotenoids, minerals (iron, magnesium, calcium, zinc, chromium), polyphenol, flavonoid, superoxide dismutase (SOD), omega-3, omega-6, omega-9, and cellular growth factor. The highest constituent contained in *S. Hermanii* is protein, composed of 86% of the extract, while 80% of the protein consists of collagen.[10,21,22,23] In Prameswari (2017) study, the administration of 3% *S. Hermanii* nanopowder was proven to alleviate the ALP expression during an orthodontic relapse. Flavonoid was assumed to be the bioactive component in the golden sea cucumbers affecting the ALP expression. Flavonoid was able to significantly inflate the ALP activity and ALP differentiation gene expression along with the increase of collagen gene expression, osteopontin (OPN), osteoprotegerin (OPG), osteocalcin (OCN), bone morphogenetic protein (BMP).[24]

Flavonoid works through pro-inflammatory mediator inhibitory activity. This anti-inflammatory capacity of the flavonoids would inhibit the cyclooxygenase (COX) and lipoxygenase (LOX),

thus limiting the inflammation duration, promoting the proliferation capacity of the Transforming Growth Factor-β (TGF-β), and accelerating the proliferation phase. Flavonoids also influence bone density and stimulate the osteoblast maneuver.[25] Flavonoid promotes osteoblast differentiation through signaling pathways of ERK and JNK, which are superfamilies of Mitogen-Activated Protein Kinase (MAPK). ERK has two isoforms of ERK1 from the MAPK3 pathway and ERK2 from the MAPK1 pathway, which is both expressed in the osteoblasts. The JNK pathway, which is a novel signaling pathway in osteoblasts differentiation induction through ALP control, is also induced by flavonoids.[26]

The arginine contained in the golden sea cucumbers further alleviates the ALP expression due to the nitrite oxide (NO) synthetase reinforcement capability, which was proven to inhibit bone resorptions.[27] The chondroitin sulfate contents, a GAGs, were demonstrated to enhance ALP expression through the BMP-4 elevation, which induces osteoblasts differentiation in the adenovirus-infected calvarial murine cells.[28,29,30]

The results of this study illustrated a declined TRAP-6 expression in groups given mechanical pressure and *S. Hermanii* extract. *S. Hermanii* nanopowder administration was able to inhibit osteoclastogenesis due to its bioactive compounds. EPA and DHA are the suspected osteoclastogenesis inhibitory agents, which perform directly in the osteoclast-specific TRAP-6 gene.[31] Flavonoid was previously known as an osteoclast inhibitor through RANKL inhibition and TRAP-6 activity limitation. The decrease in TRAP-6 expression was proportional to the formed osteoclast counts.[32] The suppression of osteoclastic differentiation and formation induced by flavonoid quercetin is mediated through the inhibition of NF-κB and AP-1 activation.[33] In the 3% *S. Hermanii* nanopowder administration, the decline in TRAP-6 expression was accompanied by bone appositions.[10]

Studies on OPG expression following golden sea cucumber extract reported an escalation in the parameter counts, followed by a RANKL decrease in the RANK/RANKL/OPG system. This increase was assumed due to the flavonoids and quercetin constituents in the golden sea cucumber extract. Srivastava (2013), in corresponds, described flavonoids as bone-protecting agents, which is beneficial in osteoblastic differentiation, thus increasing the OPG and decreasing the RANKL.

The HBOT exposure in this study resulted in the OPG and ALP expression escalation and TRAP-6 expression decline ($p \leq 0.05$). The HBOT treatment is popular for its bone formation enhancement. An oxygen exposure at 2.4 ATA, 97.9% O_2, and 90 minutes per day could increase osteoblast differentiation and proliferation and as well as bone formation determined by increased alkaline phosphatase activity and expression of type I collagen and Runx-2 mRNA during the early stages of culture. The HBOT reparative action in osteonecrotic bone loss through a mechanism to stimulate angiogenesis Oxygen hyperbaric may also improve surgical outcomes through a direct beneficial effect on osteoblast differentiation generating a larger bone mass available for reconstruction.[35] The HBOT treatment was also proven to increase the OPG expression in the bone formation process. Mulawarmanti, 2020 examining the effect of the HBOT treatment, pressure, and hyperoxia on RANKL-induced osteoclast formation in RAW 264.7 cells and human peripheral blood monocytes (PBMC), detailed that OPG is produced by osteoblasts and other cell types, including peripheral blood lymphocytes. OPG, the soluble decoy receptor for RANKL, inhibits RANKL binding to the receptor activator of NF-kB (RANK) and prevents osteoclastogenesis and bone resorption.[37,38] RANK/RANKL/OPG ratios and the level of other inflammatory cytokines, such as TNF, constitute critical mediators of osteoclastogenesis in diabetes with periodontal disease (Mulawarmanti, 2020). Daily exposure to HBOT (2.4 ATA, 97% O2, 90 min), hyperbaric pressure (2.4 ATA, 8.8% O2, 90 min), or normobaric hyperoxia (1 ATA, 95% O2, 90 min) significantly decreased RANKL-induced osteoclast formation and bone resorption in normoxic conditions. HBO had a more pronounced anti-osteoclastic effect than hyperoxia or pressure alone and also directly inhibited osteoclast formation and resorption in hypoxic conditions, a hallmark of many osteolytic skeletal disorders. The suppressive action of HBOT was at least in part mediated through a reduction in RANK, NFATc1, and Dc-STAMP expression and inhibition of hypoxia-induced HIF-1α mRNA and protein expression, thus this existing data provide mechanistic evidence supporting the HBOT as an adjunctive therapy to

prevent osteoclast formation and bone loss associated with low oxygen partial pressure.[39] To be assumed, exposure to the HBOT decreases the TRAP-6 expression in this study.

Administration of combined treatments of *S. Hermanii* gel and HBOT treatment was acclaimed to increase the ALP and OPG expression and decrease TRAP-6 expression compared to the normal control and negative control groups ($p \leq 0.05$). These findings are sourced from the interaction between the mechanisms of the two treatments. The local work of bioactive constituents of *S. Hermanii* gel works in synergy with the systemic HBOT. Both the *S. Hermanii* gel and the HBOT treatment have a direct impact on all the bone parameters. Increased ALP and OPG expression were believed due to the flavonoids, arginine, and chondroitin sulfate components in the *S. Hermanii* gel, in interaction with the HBOT. In parallel, the decreased TRAP-6 expression was due to the integration of EPA, DHA, and flavonoid compounds with the HBOT procedure. The advantages of the combined treatments are primarily focused on the treatment effectivity enhancement and limitation of adverse effects. The distinct treatment mechanisms create a synergic effect, thus reducing the required dosing of each treatment and limiting the unfavorable consequences.[40]

Administration of the combined treatments did not affect the ALP expression significantly compared to the *S. Hermanii*-only group, which could indicate that the elevated ALP expression was predominantly affected by the *S. Hermanii* component rather than the HBOT. The greatest increase in OPG expression on *S. Hermanii*-only group. On the other hand, the combined treatments did not lower the TRAP-6 significantly compared to the HBOT-only group, which indicates a predominant portion of oxygen exposure in the TRAP-6 decrease. The combined treatments were only significant in synergy in increasing the OPG expression.

Conclusions

The administration of combined treatments of *S. Hermanii* gel and HBOT was proven to increase the ALP and OPG expression and decrease the TRAP-6 expression, compared to the normal control and the negative control group ($p \leq 0.05$). The combined treatments did not affect the ALP expression significantly compared to the *S. Hermanii*-only group, which indicates that the elevated ALP expression was predominantly affected by the *S. Hermanii* components rather than the HBOT and also OPG expression due to flavonoids, arginine, and the chondroitin sulfate components in the S. Hermanii gel. the combined treatments did not lower the TRAP-6 significantly compared to the HBOT-only group, which indicates a predominant portion of oxygen exposure in the TRAP-6 decrease, rather than the *S. Hermanii* components.

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Declaration of Interest

The authors report no conflict of interest.

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