ORIGINAL ARTICLE

Osteogenesis and Osteoclastogenesis Regulation of the Midpalatal Area after Maxillary Suture Expansion Induced by Hyperbaric Oxygen Therapy (HBOT)

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ABSTRACT

Introduction: The junction between midpalatal suture is one of the easiest patterns for investigating the relationship between bone formation and resorption. Suture widening for the therapy of transverse maxillary deficiency, by elucidating molecular pathways from previous studies, which provide basic knowledge on bone homeostasis. The research purpose to analysed the regulation of osteogenesis and osteoclastogenesis through the ALP, TRAF-6, and midpalatal area after maxillary suture expansion, induced by the Hyperbaric Oxygen Therapy (HBOT) 2,4 ATA administration from day 8-14. **Methods:** The sample used 18 male Cavia cobaya aged 2-3 months, which were divided into three groups: Normal control K(O), negative control with expansion appliance K(-), and HBOT (P). After 14 days, Cavia cobaya were decapitated, followed by the horizontal cutting of the maxilla, and preparation into slides. Osteoclast and osteoblast number, as well as the ALP and TRAF-6 expressions, and the midpalatal areas were counted. **Results:** Statistical analysis using Kruskall Wallis showed significance in all groups ($p \leq 0.05$), while Pearson correlation test show the highest correlation between osteoblast and ALP ($r=0.951$), osteoblast and osteoclast ($r=0.848$), ALP and osteoclast ($pr=0.745$), as well as ALP and midpalatal ($r=0.704$). **Conclusion:** The treatment group demonstrated elevated levels of osteoclast and ALP expression, which collectively regulate the bone remodeling process, as well as osteoblasts, and the midpalatal area, with a decline in TRAF-6. Osteoblast has a role during the interaction of endothelial cells and osteoclasts in osteogenesis, while ALP is involved in the mineralization process of the bone matrix within the midpalatal area.

Keywords: Hyperbaric oxygen, Maxillary suture expansion, Osteoclast, Osteoblast, ALP

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INTRODUCTION

Orthodontic correction of maxillary constriction faces difficulties of facial development in transverse aspect regression during late childhood (1). In addition, a number of methods have been exploited to expand the maxillary, and this study used maxillary expansion appliances, which was proposed to attain certain expansion rates, including rapid (RME), as well as semi rapid or slow (SME) forms (1,2).

Caprioglio study confirmed the tendency for mechanical stress during the expansion process to result in dimensional differences in the midpalatal, based on radiographic, histologic and morphological studies. Furthermore, the multiple step healing process of mechanical separation involving bone cells encompasses the development and remodelling of connective tissue, and also new bones (3). The application of his technique in the treatment of maxillary transverse deficiency requires the use of molecular mechanisms illuminated from prior studies, which offer basic knowledge of bone homeostasis. There is limited information on the underlying process used for the modulation of bone formation and resorption during healing, although there are numerous reports on the application of tensional force to midpalatal suture, which which induces cartilaginous tissue replacement (4). Furthermore, the amount of remodeling appeared to be proportional to the sutures distance from and orientation to the applied force (5). Following the expansion of maxilla, The mesenchymal cells (MSCs) within the cartilaginous tissue are proliferating and differentiating into osteoblasts (4), and unlike the osteoblast family, Osteoclasts are multinucleated cells derived from hematopoietic stem cells (HSCs), which serve the function of bone resorption and remodelling (6). In addition, conducting the latter process in maxilla expansion focuses on bone cell balance (7), while the formation and resorption activity are the two major mechanisms of bone homeostasis (6).

Several pathways to assist in osteogenesis have been identified (6), although alkaline phosphatase (ALP)

function, calcium deposition and cell count were quantified for osteogenic differentiation of MSCs. Prior to this, osteoblasts are assumed to primarily stimulate an increase in the amount of MSCs (8). Furthermore, the differentiation of osteoclasts demands the presence of TNF family receptors, e.g., RANK, which specifically aggravates biochemical signalling through intracellular TNF receptor associated factors (TRAFs) recruitment, following the process of ligand binding and receptor oligomerization (9). Also, TNF-α-related apoptosis-inducing ligand (TRAIL) is associated with the induction of osteoclast differentiation through a TRAF6-dependent signalling pathway, leading to the consequent inhibition of RANK ligand (RANKL)-induced osteoclast differentiation (9). Controlling the quantity of resorption and remodelling of bone regeneration is indeed part of human bone physiology, although the balance between both processes is uncertain. Hence, there is a tendency of tipping, in favour of one over the other, which is dependent on the local microenvironment, including oxygenation (6).

HBOT is a medical treatment method characterized by the continuous inhalation of pure 100% oxygen, with ambient air pressure, which is slightly higher than atmospheric pressure, over a specified period (10). This technique increases the amount of dissolvable oxygen in a patient's blood serum, indicating the presence of a directly proportional relationship between ideal amount of gas dissolved in solution and the partial pressure. This association has been proven to confer beneficial effect for decompression illness, and is intended to reduce the injurious effects in wounded area. During HBOT, 100% oxygen is inspired from the chamber at pressure greater than 1 ATA (10, 11). In addition, there is often higher demand and increased utilization rates, during tissue repair and wound healing, as reduced or the total absence of the recuperation process has been linked with chronic hypoxia to the injury site (12).

The provision of HBOT daily, tends to adequately support oxygen supply and promote inflammatory development into proliferative process. This therapy is assumed to create a gradient between poorly and highly oxygenated tissues, respectively located in the center and the periphery, subsequently modulating the incidence of neovascularization (12, 13). Furthermore, the created conditions tend to increase the production of fibroblast and collagen in wounded area. Also, an increase in daily exposure is assumed to modify the rate differentiation in osteoblast, through an enhance in ALP activity, as well as the expression of type I collagen and Runx-2 mRNA, being indicative parameter at the early stages of culture (13, 14).

Prior investigations have shown preference for the use of HBOT in 2,4 ATA in 7 days, as against 5, because elevated vascularization of tissues is known to stimulate the remodeling process. Meanwhile, the subsequent insertion of orthodontic brackets up to day 10 had no significant positive effect, compared to the introduction of HBOT alone on day 7 (14). Wu et al, 2017, presented the ability to stimulate osteoblasts for routine HBO therapy, enhanced biomineralization, increased bone nodule formation, calcium deposition, as well as ALP activity, and subsequently provide cellular evidence for augmented bone regeneration. Literature review shows the presence of minimal studies related HBOT in osteoclastogenesis. The aim of this investigation, therefore, is to analyze the regulation of osteogenesis and osteoclastogenesis through ALP, TRAF-6, and midpalatal area, following the maxillary suture expansion induced by the administration of HBOT 2,4 ATA from day 8-14.

MATERIALS AND METHODS

The study involved the use of post-test only control groups, which were randomized, while the Ethics and scientific committee provided ethical approval, in relation to the use of experimental animal.

Animal preparation

This experiment required the use of eighteen male Cavia cobaya as samples, with the following criteria: male, aged 3-4 months, with body weight around 300-400 grams. Furthermore, the samples were grouped into 3 parts, encompassing the negative K(-), and positive control, which is specifically provided with maxillary expansion or K (+), and the treatment group, administered hyperbaric oxygen therapy (P) type Hype Animal chamber.

Preparation of Maxillary suture expansion and HBOT

Firstly, the Cavia cobaya was allowed to acclimatize for 7 days. Therefore, the K(+) and P group were treated with the helical spring as shown in fig. 1, to expand maxillary on day 3, up to the 14th day. In addition, the slow maxillary expansion type was selected for the research, and prior to the application, rubber separator with a tensile strength of 0.29 gr/cm2 was used in the left incisive up to the second day, in order to attain diastema. Furthermore, rubber separators with tensile strength of 0.48 gr/cm2 was replaced with helical spring on day 3. The subjects in the P group were confined in an animal chamber for the successful provision of HBOT with 2,4 ATA pure oxygen (100%) for 90 minutes (3 X 30 minutes), where 5 minutes interval was allowed for the intake of normal air (normobaric), after the 8th day, up to the 14th day, as seen as fig 2.

The Cavia cobaya were subsequently euthanized, using a lethal dose combination of ketamine and acepromazine 1:1 (0.05 mg/ kg bw), followed by the decapitation of maxilla.

Procedures for producing cell slide and study of osteoblast, osteoclast, midpalatal area, ALP and TRAF-6 expression

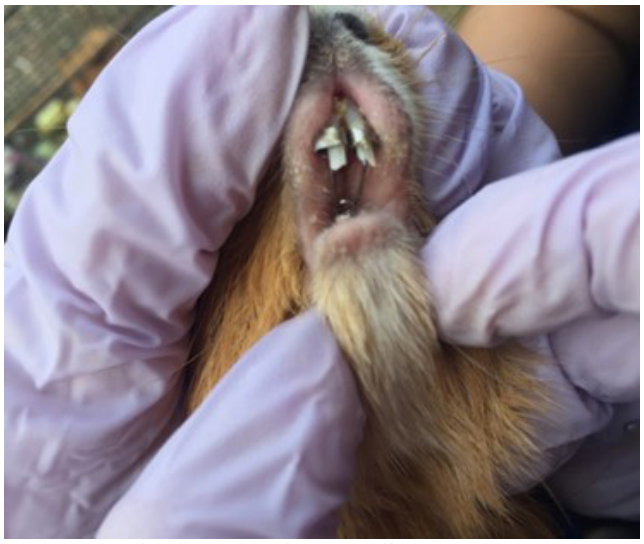

Figure 1: Helical spring (slow maxillary expansion) was applied in *Cavia cobaya* maxilla

A transverse cut of the maxilla specimens were retained in a solution of 10% buffer formaline, prior to the mounting of slides. These were then colored using Hematoxyllin eosin for the evaluation of osteoblast, osteoclast and the midpalatal area. Subsequently, the expression of ALP and TRAF-6 were then examined using immunohistochemistry method with ALP (abcam) monoclonal antibodies, and TRAF-6 product (abcam) polyclonal antibodies, before the commencement of microscopic observations. In addition, the histological slide of osteoclast, osteoblast, and midpalatal area were examined with a light microscope, where the Olympus type CX 31 and 40X magnification was applied. The photographs were then obtained to measure the ALP and TRAF-6 expressions observed at 400x, and each section was also evaluated for size, as records were replicated three times in the view areas.

This study was performed at the laboratory of Biochemistry, Medical Faculty, as well as the Laboratory of Oral Biology, Hang Tuah University. Furthermore,

Figure 2: *Cavia coba* in the Hype animal chamber of HBOT

the following instrumentation were used: weighing scale and rubber separators for the Cavia cobaya, as well as separating plier, maxillary expansion, cotton pellet, pinset, , small sized coil, surgical scissors, and the animal chamber required for the process.

Data obtained from calculating the number of cell were tabulated and statistically analyzed using descriptive test. This was followed by Kruskall Wallis and Pearson correlation tests, to identify the association between osteoclast, TRAF-6, osteoblast, ALP, and midpalatal area induced by HBOT, after maxillary suture expansion. In addition, both used a 0.05 level of significance.

RESULTS

Osteoclast number, osteoblast and midpalatal area were evaluated in HE colourization and ALP, as well as TRAF-6 expression, using Immunohistochemistry, after administering HBOT 2,4 ATA from day 8-14 to the treatment groups. The results are shown in the fig. 3 and fig. 4.

Descriptive analysis showed the distribution and summary of data, subsequently clarifying the results

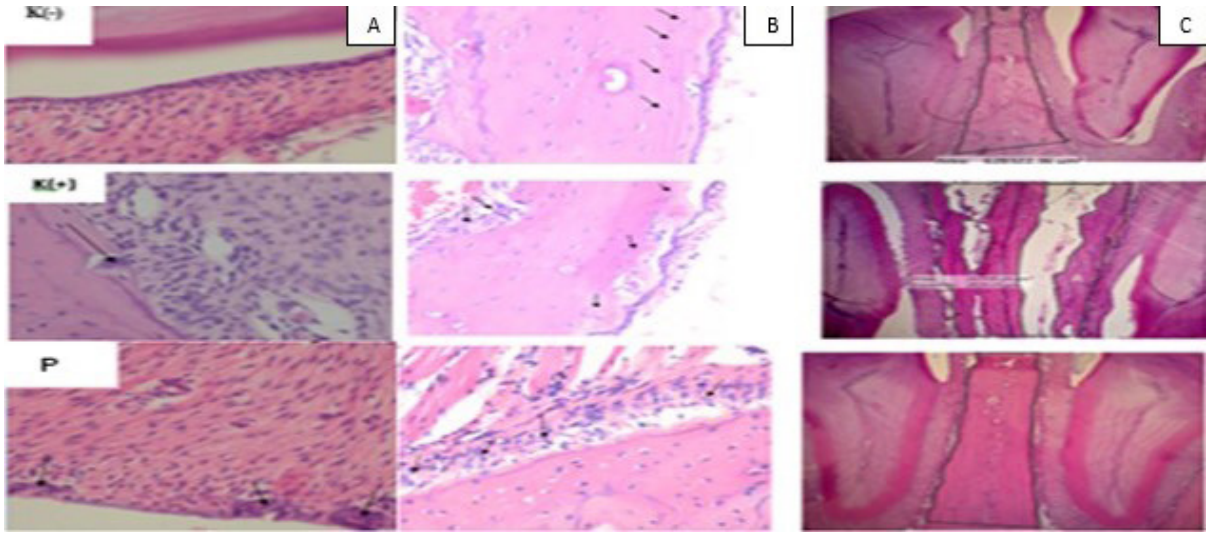

Figure 3: Hematoxyllin Eosin colouration of osteoblast (A), osteoclast (B) and midpalatal area (C) of Cavia cobaya in K(-) group, K(+) group, and P group with 40x magnification after maxillary expansion

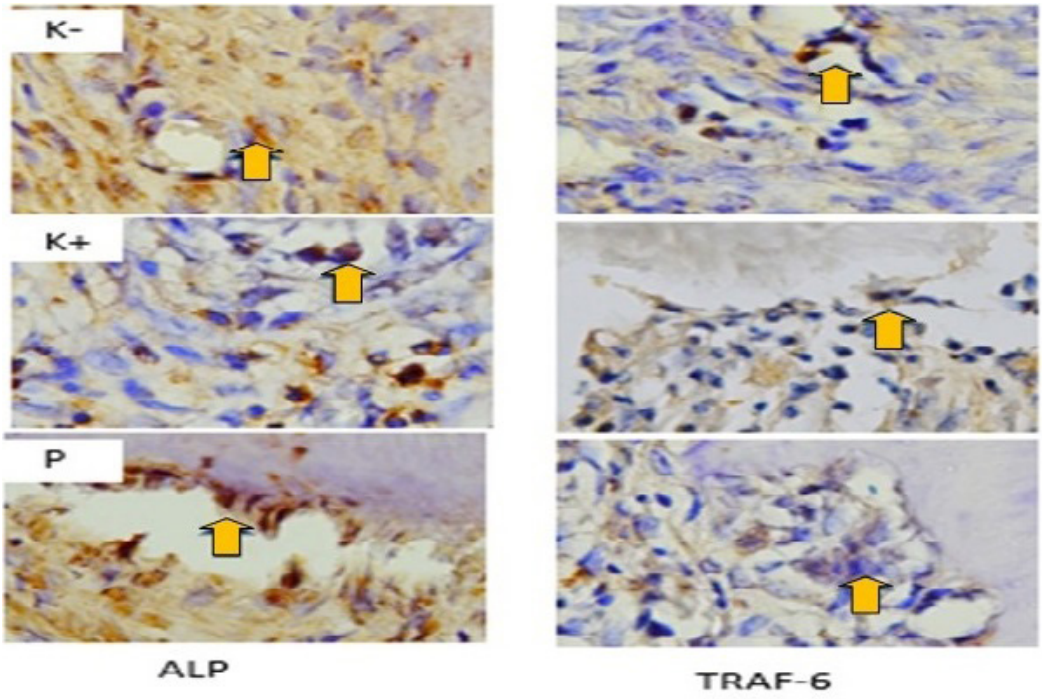

Figure 4: Immunohistochemistry ALP and TRAF, in K(-) group, K(+) group, and P group with 400x magnification after maxillary expansion

presentation. Therefore, Kruskall Wallis and Pearson correlation tests were conducted using analytic statistics at a significant level of 95% (p=0,05), with the help of SPSS software.

Figure 5 demonstrated a linear increment from K(-) to P, with the exception of TRAF-6 expression. Figure 5 showed the propensity for HBOT to increase osteoclast, osteoblast number, ALP, TRAF-6 expression and midpalatal area, following the maxillary suture expansion.

Prior to the conduction of hypothesis test, each group was tested for normalty, using Shapiro – Wilk test. This approach was used because the samples were less than 50, and the results obtained showed a normally distributed data. The results of the Kruskall Wallis test

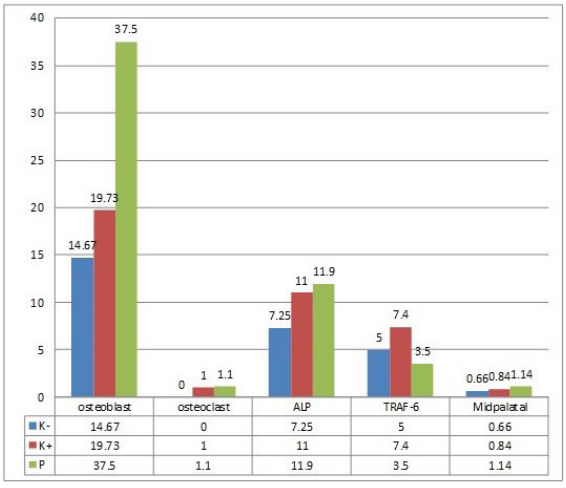

Figure 5: Bar diagram mean of osteoclast, osteoblast, ALP, TRAF-6, and midpalatal after maxillary expansion

showed substantial difference in the sample at p≤0.05 between all groups, after maxillary suture expansion treatment. Pearson correlation test in table 1 showed the highest correlation between osteoblast and ALP (r=0.951), osteoblast and osteoclast (r=0.848), ALP and osteoclast (r=0.745), as well as ALP and midpalatal (r=0.704).

Table I: Pearson Correlations between ALP, TRAF-6, osteoblast, osteoclast, midpalatal after maxillary suture expansion induced by HBOT

		ALP	TRAF6	osb	osc	midpalatal
ALP	Pearson Correlation	1	-.485*	.951**	.745**	.704**
	Sig. (2-tailed)		.041	.000	.000	.001
TRAF6	Pearson Correlation	-.485*	1	-.481*	-.340	-.438
	Sig. (2-tailed)	.041		.043	.167	.069
osteoblast	Pearson Correlation	.951**	-.481*	1	.848**	.570*
	Sig. (2-tailed)	.000	.043		.000	.014
osteoclast	Pearson Correlation	.745**	-.340	.848**	1	.515*
	Sig. (2-tailed)	.000	.167	.000		.029
midpalatal	Pearson Correlation	.704**	-.438	.570*	.515*	1
	Sig. (2-tailed)	.001	.069	.014	.029	

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

Cavia cobaya were selected because of some advantages possessed over other animals. These include the relative inexpensive nature, ease of acquiring large samples, effortless maintenance over a long period of time, relatively less stressful preparation by histology, and has also the highest amount of antibodies needed for molecular and cell biology techniques (15). Furthermore, the use of expansion for 11 days was obtained from the extrapolation of Cavia cobaya and human life span, using a comparison of 5 against 70 years, hence an equivalent of 154 days or 5 months in humans was achieved (16). The results showed the highest mean of osteoclast and osteoblast, ALP and midpalatal area in the treatment group. This outcomewas as a result

of the treatment capacity to increase angiogenesis and neovascularization, which is closely associated with the midpalatal bone formation process, and consequently leading to elevated osteoblastic activity (14). Furthermore, osteoblast has been affiliated with bone component synthesis, particularly for the proteoglycan, I-collagen, and glycoprotein types, including osteonectin (Brahmanta, 2019), though MSCs were analyzed for osteogenic differentiation by monitoring alkaline phosphatase (ALP), calcium deposition, and cell number (8). With the exception of TRAF-6, a decline in expression was observed after the induction of HBOT, based on the indirect role played in osteoclastogenesis,, and directly through the TRAIL signaling pathway (9).

After the expansion was treated in K(+), lesser new bones were formed in the midpalatal, compared to the K(-) and P. Also, orthodontic forces hinder the growth of periodontal ligament on the dental root, and this decreases the tissues' blood perfusion. This condition is followed by hypoxia, which affects cell proliferation or induces apoptosis, in accordance with the oxygen gradient. Recently, there has been a considerable increase in interest on Hypoxia pathways and hypoxia-inducing transcription factors HIF-1α and HIF-2α causing bone remodeling and bone disease (17). The combination of upregulated tissue proliferation rates with angiogenesis facilitates the assumption that hypoxia fundamentally contributes to the bone remodeling processes during orthodontic treatment (18). In addition, acute exposure has been affiliated with an increase in the ability for mature osteoclasts to resorb bone (17). Meanwhile, maxillary expansion further causes the deformation of blood vessels, as well as the irregularity of tissues around the tooth, leading to the modification of cell metabolism, which result from hypoxic conditions and the decreased level of nutrition (19). In addition, the development and activity of human osteoclasts derived from PBMCs can also be stimulated during inflammation, particularly in areas without effective stromal cell support, and also in pure populations of CD14+ monocytes. This result indicates that the osteoclastogenic hypoxia response is an intrinsic property of this cell lineage (13). Furthermore, it is also speculated that smallest new bone formed as a result of helical spring used as slow maxillary expansion is less forceful in creating skeletal effect (20). These treatment approaches led to some changes at dental and transversal level, which involved both skeletal and dentoalveolar during expansion (21).

The ossification process is initiated at the suture margins, as well as the bone islands (including acellular masses and inconsistently calcified tissues) situated in the middle of the sutural gap of K(+), as seen in figure 3 (20, 22). The subsequent formation of spicules occurs in several places along the suture, which increases in quantity, with maturation (20). This further leads to the formation of numerous scalloped areas that are closely attached to one another, and separated by connective tissue in some areas. Simultaneously, there is an increase in interdigitation, followed by early fusion in the posterior region of the suture and subsequent development of ossification from the posterior to the anterior. Conversely, there is also an incidence of cortical bone resorption in the sutural ends, as well as cancellous bone formation (23, 24, 25).

The entire process is terminated with the formation of new bones, as the osteoid matrix experiences mineralization, which is evident in the margins, and also within the suture center. This manifestation has also previously been detected with the appearance of trabecular fishbone. In addition, collagen were also identified in a transversal orientation on the newly-formed bone. This is related to the suture long axis, which is characterized by a longitudinal orientation, assumed to be associated with the response to mechanical forces (26). Therefore, the the amount of expansion obtained after the separation of midpalatal suture, possibly influences the healing time, which extends through several following months (21).

The P group administered the helical spring with HBOT showed an increase in the number of osteoblast, ALP and midpalatal area, compared to the K(+). This indicates the propensity for HBOT to accelerate the formation of new bone in the midpalatal, marked with the replacement of spongiosa with compact bone. In addition, there is also a possibility of increased vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). These are the general angiogenic aspects affiliated with angiogenesis and neovascularization, which is highly associated with elevated osteoblastic activity, known to be essential in the process of forming new bones (14). Moreover, over 30 new vessel growths infiltrated the wound, subsequently achieving about 85% pO□'s of control tissue. Therefore, the proposed mechanisms of HBOT action include: (1). Transport of oxygen to poorly or absently vascularized body sites, including slow healing wounds. These actions are the product of a physical relation between the concentration of pressure and gas in a liquid, as the HOBT treatment conveys a significant amount of pure oxygen to blood plasma; (2). The hyperbaric and normoxic cyclic cycles produce a stress reaction by continuously ondulating the number of reactive oxygen species (ROS) in the tissues. Various growth factors including those involved in angiogenesis propagation often inhibit signal transduction pathways (27).

A decline in pro-inflammation mediators hampers the development and consequent activity of osteoblast proliferates, which differentiate into mature forms, therefore increasing the amount of bone remodelling, the formation of new bone in the midpalatal, as well as density elevation was also reported. Surprisingly, the treatment with HBOT also augments the osteoclast number, although this was not higher than the outcome for osteoblast. The result was related to the hypoxic conditions (17), and an increase in the resistance of

HBOT to RANKL-induced osteoclast formation was also reported. In addition, the early stage of therapy affected the production of mononuclear and multinuclear osteoclasts, indicating a difference in reaction based on the level of precursor growth in which monocytes were further formed along the lineage. This is due to a higher degree of tolerance to the anti-osteoclastic effect of HBO relative to less willing to commit cells. (28). This research, therefore, established the occurrence of a decline in TRAF-6 expression, alongside the increase in osteoclast number.

The result of Kruskall Wallis test showed the presence of significance differences between all groups, based on the osteoblast and osteoblast number, ALP, TRAF-6 and midpalatal area, during maxillary expansion. In fact, the HBOT appears to become more resistant to osteoclast formation caused by RANKL (28), this also means the stimulation of in vitro proliferation and differentiation of human osteoblasts. Meanwhile, increased biomineralization of HBO is characterized by an increase in the formation of bone nodules, calcium deposition and ALP (13,29), and the therapy significantly promotes osteoblast proliferation and the progression of the cell cycle 3 days after therapy. This also facilitated the increased the mRNA expression of fibroblast growth factor (FGF)-2, and also the Akt protein levels, phosphorylated ERK, p70S6K, nuclear factor (NF)-κB, phosphorylated c-Jun N-terminal kinase (JNK) and protein kinase C (PKC)α (30). Therefore, the resulting osteogenesis is observed to be higher than osteoclastogenesis, following the promotion of the midpalatal area, and the bone remodelling cycle is also increased with HBOT.

Pearson correlation test showed the presence of a strong positive correlation between osteoblast and ALP (P=0.951), osteoblast and osteoclast (p=0.848), ALP and osteoclast (p=0.745), ALP and midpalatal (p=704) in the treatment group. In addition, osteoblast and ALP were confirmed to regulate the process of osteogenesis, leading to the formation of midpalatal area, which requires at least three months for completing the mineralization process in the expanded suture (31). This component, alongside osteoclast are correlated, both physically and biochemically, and there are numerous factors involved in the interaction with the endothelial cells during osteogenesis and repair, including BMP-2, OP-1 (32,33). Conversely, higher ALP expression was associated with the midpalatal area, creating a sinergystic effect. This is due to the ability to function as an osteoblast differentiation marker, mainly involved in the mineralization process of midpalatal bone matrix (31). Hence, an increase in ALP reflects the functional activities of osteoblastics in osteogenesis and remodelling of the expanded suture. There was also a correlation with osteoclast and thus ALP downregulation and osteocalcin is consistent with reduced mineralization, leading to modest changes in osteoclast, which is the expression of osteoclastogenic factors (34).

CONCLUSION

In conclusion the result and discussion show the ability for HBOT in 2,4 ATA provided from day 8-14 to initiate an upsurge in the number of osteoclast and osteoblast, as well as ALP expression, and midpalatal area, although a decline was observed with TRAF=6. In addition, a strong positive correlation was established between osteoblast and ALP, osteoblast and osteoclast, ALP and osteoclast, and also ALP and midpalatal, in the treatment group, after maxillary suture expansion. The osteoblast number and ALP expression have been associated with the regulation of bone remodeling, based on the role played in the interaction between the endothelial cells and osteoclasts. This incidence was observed during both osteogenesis and repairs, characterized by the mineralization of bone matrix in the midpalatal area.

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