RANK-RANKL-OPG expression after gingival mesenchymal stem cell hypoxia preconditioned application in an orthodontic tooth movement animal model

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ABSTRACT

Background: The expression of receptor activator of Nuclear Factor Kappa Beta (RANK) and its ligand (RANKL), as well as osteoprotegrin (OPG), in the alveolar bone (AB), may improve bone remodeling during orthodontic tooth movement (OTM). It is hypothesized that hypoxia-preconditioned gingival mesenchymal stem cells (GMSC) may be more effective than normoxia-preconditioned GMSC in this regard. This study aims to investigate the expression of RANK, RANKL, and OPG in the compression and tension sides of AB after allogeneic administration of GMSC that were normoxia or hypoxia-preconditioned in rabbits (*Oryctolagus cuniculus*) undergoing OTM.
Methods: Twenty-four healthy young male rabbits were divided into two groups: T1, which underwent OTM and received normoxia-preconditioned GMSC, and T2, which underwent OTM and received hypoxia-preconditioned GMSC. A ligature wire was attached to the mandibular first molar and connected to a 50 g/mm^2 closed coil spring, exerting force on the central incisor and left mandibular molar of the experimental animals. After 24 h of OTM, either normoxia- or hypoxia-preconditioned GMSC were injected into the gingiva of the samples in a single dose of 20 μl of phosphate-buffered saline (PBS). All samples were sacrificed on days 7, 14, and 28, and immunohistochemistry was performed to analyze the expression of RANK, RANKL, and OPG on the tension and compression sides.
Results: The expressions of RANK-RANKL-OPG in the alveolar bone of the compression and tension sides were significantly different during the 14-day period of OTM following allogeneic administration of GMSC that were normoxia or hypoxia-preconditioned ($p < 0.05$).
Conclusion: The expression of RANK-RANKL was significantly increased on the compression side of the alveolar bone during OTM after the administration of hypoxia-preconditioned allogeneic GMSC but not on the tension side. Conversely, RANKL-OPG expression was enhanced on the tension side but not on the compression side, as observed through immunohistochemical analysis in vivo.

1. Introduction

Malocclusion is characterized by abnormal tooth positioning, disharmony, and insufficient craniofacial bone growth.[1] Orthodontists play a crucial role in diagnosing malocclusion and treating patients for early orthodontic treatment to ensure comprehensive healthcare for patients. Additionally, orthodontic interventions and other oral health outcomes significantly affect the functional and emotional aspects of individuals' lives (OHRQoL).[2] Dental and oral health problems can have

a detrimental impact on an individual's overall well-being. Malocclusion, in particular, can significantly impair a person's quality of life.[3]

inflammatory pathways and responses that directly or indirectly affect OTM.[16] As a result, it is crucial to identify techniques that can enhance

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List abbreviations

AB	Alveolar bone
ALP	alkaline phosphatase
ANOVA	analysis of variance
BMSCs	Bone Marrow Mesenchymal Stem Cells
C	Celcius
CO_2	Carbon dioxide
$CoCl_2$	Cobalt Chloride (II)
Col-1	collagen type I
CXCR4	C-X-C motif chemokine receptors-4
GMSCs	Gingival Mesenchymal Stem Cells
HIF-1α	hypoxia inducible factor-1α
HIF-2α	hypoxia inducible factor-2α
hPDLSCs	human periodontal ligament cells
IHC	Immunohistochemistry
JNK	Jun amino-terminal kinases
KO	knockout
MMP-13	matrix metalloproteinase-13
mRNA	messenger ribonucleic acid
MSCs	Mesenchymal Stem Cells
NF-κB	Nuclear Factor Kappa Beta NF-κB
NFATc1	nuclear associated T-cell-1
Niti	Nickel Titanium
O_2	Oxygen
OHRQoL	oral health-related quality of life
OPG	Osteoprotegerin
OTM	Orthodontic Tooth Movement
PBS	Phosphate Buffer Saline
PDL	periodontal ligament
PDLSCs	Periodontal ligament stem cells
RANK	Receptor Activator Nuclear Factor Kappa Beta
RANKL	Receptor Activator Nuclear Factor Kappa Beta Ligand
rBMSCs	rat bone marrow mesenchymal stem cells
SAPK	phosphorylation via Stress-activated protein kinases
SC	Stem Cells
SD	Standard Deviation
SPSS	Statistical Software for Social Science
SS	Stainless Steel
T1	OTM and GMSCs normoxic-preconditioned
T2	OTM and GMSCs hypoxic-preconditioned
Tukey HSD	Tukey Honest Significant different
VEGF	Vascular Endothelial Growh factor

One of the most common dental procedures performed during adolescence is orthodontic mechanotherapy. However, advancements in technology and strategies have made it increasingly popular among adults as well. The goal of this treatment is to achieve a functional and stable occlusion while also ensuring optimal cosmetic outcomes.[4] Nevertheless, it's important to recognize that orthodontic treatment, particularly with fixed orthodontic appliances, often spans a period of at least a year and sometimes even several years. This extended treatment duration has the potential to impact not only tooth positioning but also overall oral health.[5] Orthodontic tooth movement (OTM) refers to the process of moving teeth within the jaw to enhance both aesthetics and stomatognathic function. This has prompted orthodontists to delve into the fundamentals of OTM with the aim of reducing and optimizing treatment time, thereby increasing patient satisfaction.[6]

Understanding the specific mechanisms involved in remodeling periodontal tissue is crucial for effectively targeting relevant cells and achieving optimal outcomes in orthodontic treatment.[7] This understanding enables researchers and clinicians to develop improved methods that specifically target cells involved in periodontal tissue remodeling, allowing for efficient, controlled, and safe OTM.[8] Orthodontic mechanical forces exerted on the tooth structure lead to both apposition and resorption of the alveolar bone, resulting in remodeling of the periodontal tissues.[9] However, the prolonged duration of orthodontic therapy increases the risk of caries, periodontal disease, and root resorption.[10–12] To expedite the process of orthodontic treatment, orthodontic mechanotherapy has gained significant attention. This approach combines surgical and nonsurgical interventions to facilitate OTM and reduce treatment duration.[13] However, surgical techniques such as corticotomy, which aim to accelerate OTM, may cause discomfort for about a week and pose potential postoperative complications.[14]

Force and vector analysis alone are insufficient to fully explain the process of OTM. The intricate interplay of factors, including differential attraction, gene expression, and activation of intercellular signaling pathways, presents a new paradigm for understanding OTM.[15] There are also pharmacological influences on signaling molecules, such as eicosanoids and prostanoids, which play a significant role in modulating

the rate of OTM while minimizing side effects and promoting periodontal tissue regeneration. Orthodontic mechanical stresses induce changes in the periodontal ligament (PDL) and alveolar bone, resulting in OTM.[17]

Researchers and orthodontists have been actively exploring innovative approaches to accelerate OTM in order to reduce treatment duration, orthodontic pain, discomfort, caries, periodontitis, and root resorption.[18] One potential avenue being investigated is the application of mesenchymal stem cells (MSCs) to enhance alveolar bone remodeling and optimize the rate of OTM. MSCs have the ability to influence macrophage polarization by releasing various biological and immunoregulatory substances, thereby facilitating bone remodeling.[19] A recent study has shown that human-derived stem cells can promote the development of osteoclasts in macrophage lines.[20] However, the extent of this effect is strongly dependent on factors such as cell clustering, cytokine stimulation levels, and the physical characteristics of the treated MSCs.[21] Furthermore, periodontal ligament stem cells (PDLSCs) have been shown to regulate macrophage polarization in response to mechanical stimulation, thereby promoting bone remodeling and facilitating OTM.[22]

Receptor activator of nuclear factor kappa-B Ligand (RANKL) is known to stimulate osteoclastogenesis and osteoclast maturation through various enzymatic pathways mediated by Receptor activator of nuclear factor kappa-B (RANK), which is the exclusive receptor present on osteoclast precursors. On the other hand, Osteoprotegrin (OPG) is a protein that acts as a decoy receptor for RANKL. Its function is to inhibit bone resorption by binding to RANKL and preventing its interaction with RANK.[23] The RANK-RANKL ratio showed a significant decrease, while the RANK-OPG ratio exhibited a substantial increase on the seventh day after the application of carbonate apatite scaffold and pulp stem cells on primary teeth.[24,25] Human gingiva has been found to contain gingival mesenchymal stem cells (GMSC), which possess regenerative, immunoregulatory, and immunomodulatory properties. In comparison to bone marrow-derived MSCs (BMSCs), gingiva serves as a favorable source of MSCs due to its abundant stem cell population and the ease of isolation through minimally invasive procedures. GMSCs

display similar phenotypic characteristics to BMSCs and are capable of cell differentiation and replenishment. Moreover, GMSCs exhibit higher proliferation and self-replication abilities compared to adult bone marrow stem cells.[26] GMSCs can differentiate into osteogenic, adipogenic, and chondrogenic cells.[27] Additionally, GMSCs have been shown to exert autocrine and paracrine effects.[28] The gingival tissue demonstrates remarkable regenerative capacity and faster wound healing compared to skin tissue, with gingival wounds being renewed and repaired within 7–14 days as opposed to 14–21 days for skin tissue. This indicates the presence of a stem cell pool and an environment conducive to tissue regeneration within the gingival tissue.[29–31] Under hypoxia conditions, MSCs exhibited enhanced skeletal muscle regeneration, increased blood flow, and vascular formation compared to MSCs maintained under normoxia conditions on the seventh day. Additionally, exposure to hypoxia upregulated the expression of C-X-C motif chemokine receptor 4 (CXCR4) in MSCs, which is essential for MSC migration in injured tissues.[32] Based on the preceding information, the objective of this study is to compare the expression of OPG, RANK, and RANKL in the compression and tension sides of the alveolar bone during OTM following the administration of allogeneic GMSC in vivo under hypoxic or normoxic conditions.

2. Materials and methods

2.1. Study design and ethical clearance

The study design employed in this research was a simple blind randomized, true-experimental design with a post-test only control group. The ethical clearance for conducting the animal study was granted by the Universitas Airlangga Research Ethical Committee, Surabaya, Indonesia, with appointment number 2. KE.017.02.2020.

2.2. Gingival mesenchymal stem cells isolation, culture, and preconditioning

The GMSC used in this study was isolated from the free margin gingiva of healthy young male white New Zealand rabbits (*Oryctolagus cuniculus*) obtained from the Stem Cell Research and Development Center at Universitas Airlangga in Surabaya. The GMSCs from the third passage of the laboratory cell stock were frozen for subsequent subculturing until reaching 90 % cell confluency in the fourth passage. Immunocytochemical characterization of the fourth passage GMSCs was performed, confirming positive expression of cluster differentiation (CD) markers 73, 90, and 105, and negative expression of CD45. Furthermore, alizarin red staining was used in previous work to evaluate the osteogenic potential of GMSCs from the fourth passage when co-cultured in osteogenic media.[33]

Before being used in the animal model, GMSCs undergo preconditioning in both normoxia and hypoxia conditions. For normoxia preconditioning, GMSCs are cultivated without the use of cobalt (II) chloride ($CoCl_2$) as a hypoxia mimicking agent (Sigma Aldrich, USA). On the other hand, for hypoxia preconditioning, GMSCs from the fourth passage are co-cultured with $CoCl_2$ on an M24 well culture plate for 24 h at 37 °C with 5 % CO_2 in an incubator (Esco Micro Pte. Ltd., Changi, Singapore). The expression of hypoxia-inducible factor 1-alpha (HIF-1α) (Cat no. ab6717, Abcam, MA, USA) is immunocytochemically evaluated in GMSCs during normoxia and hypoxia preconditioning to confirm the induction of hypoxia in GMSCs based on the previous study procedure.[34] Furthermore, GMSCs subjected to normoxia or hypoxia preconditioning are suitable for use in the OTM animal model.

2.3. Orthodontic tooth movement animal model and GMSC application

A 6-month-old healthy male New Zealand white rabbit (*O. cuniculus*) weighing approximately 3–4 kg (kg) was obtained from the animal cage at the Stem Cell Research and Development Center of Universitas Airlangga in Surabaya. To ensure proper hygiene, the cages were thoroughly cleaned and disinfected for one week prior to use as part of the preparations for the study. The male rabbits were acclimated to the laboratory cage environment for one week before the planned treatment was initiated. They were housed in standardized animal cages with consistent temperature and humidity. The rabbits were provided with standardized rabbit pellets and water following the animal laboratory protocol of the Stem Cell Research and Development Center at Universitas Airlangga in Surabaya, in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

T1: OTM + normoxia GMSC and T2: OTM + hypoxia GMSC were the two groups comprising 24 OTM animal models. Fixed orthodontic appliances were placed from the central incisor mandible to the left first molar mandible in the animal models. To transfer the first molar in the left portion of the mandible to the mesial, nickel-titanium (NiTi) and stainless steel (SS) ligature wires with a diameter of 0.009 inches were attached to an 8 mm closed coil spring (Ze Fang Technology Co., Ltd., Taichung City, Taiwan). The orthodontic mechanical force used to advance the molar to the mesial was set at 50 g/mm^2, which is considered a mild force when measured with a tension gauge (Morelli Orthodontics, Sao Paulo, Brazil).[35]

Following 24 h of orthodontic mechanical force application for OTM, GMSC normoxia or GMSC hypoxia preconditioning was administered to the periodontal tissue of the first molar in the mandible. A single dose of 20 µl containing 106 cell densities of GMSC in phosphate buffer saline (PBS) (OneMed, Sidoarjo, Indonesia) was delivered using a blunt microliter syringe (Hamilton Company, Model 203361). On observation days 7, 14, and 28, all samples were sacrificed by guillotine decapitation while receiving a mixture of 30 mg/kg ketamine (Sigma-Aldrich, catalog number K0551000) and 4 mg/kg xylazine (Sigma-Aldrich, catalog number X1126) intramuscularly. Additionally, the mandibles of each animal model were collected for immunohistochemistry analysis.

2.4. Immunohistochemical analysis

Formalin was applied to the mandible tissue, and samples were sliced with a thickness of 4 µm to create the paraffin block. Immunohistochemical staining was performed on slides. Anti-RANKL mouse monoclonal antibodies [C1] (ab239607) were used to detect RANKL expression at a dilution of 1:50 with PBS (Abcam, Cambridge, UK). A monoclonal mouse anti-RANK antibody [64C1385] (ab13918) was used to detect RANK expression at a dilution of 1:50 with PBS (Abcam, Cambridge, United Kingdom). Anti-OPG mouse monoclonal antibodies (E−10): sc-390518 (dilution 1:50 with PBS, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) were used for OPG detection. Bovine serum albumin (BSA) and 0.09 % sodium azide (NaN3) in PBS at pH 7.4 (ThermoFisher Scientific, Waltham, Massachusetts, USA) were used.

The mandible tissue was dehydrated and treated with 3 % hydrogen peroxide and distilled water for 5 min (OneMed, Sidoarjo, Indonesia). Subsequently, the mandibular tissue was pre-treated in citrate buffer at pH 6.0 and 350 W for 10 min. After washing with PBS, the tissue was incubated at 37 °C for 15 min and 120 min, respectively, with RANKL, RANK, and OPG monoclonal antibodies. Following another wash with PBS, the tissue was treated with avidin-biotin peroxidase (ThermoFisher Scientific, Waltham, Massachusetts, USA). The chromogenic visualization process was carried out using 3,3-diaminobenzidine (DAB) hydrochloride, followed by a 30-s staining with hematoxylin eosin. The slides were then passed through an ethanol series and xylene before being sealed with a glass cover infused with Canadian balm (Merck, Darmstadt, Germany). Positive expression of RANKL and OPG was detected in osteoblasts, while positive expression of RANK was observed in osteoclasts of alveolar bone tissue using a light inverted microscope (inverted TE 2000, Nikon, Tokyo, Japan) at 100x, 400x and 1000x magnification.

2.5. Data analysis

The study data were recapitulated and analyzed using mean and standard deviation in Statistical Software for Social Science (SPSS) version 20.0 (IBM Corporation, Illinois, Chicago, USA). One-way analysis of variance (ANOVA) and post-hoc Tukey Honest Significant Difference (HSD) were performed. The level of statistical significance was set at $p < 0.05$. Asterisks (*) indicate the degree of statistical significance (*$p < 0.05$), while "ns" represents non-significant results ($P > 0.05$).

3. Results

The study data were found to be normally distributed based on the one-sample Kolmogorov-Smirnov test, and the variances of all data were homogeneous according to the Levene test ($p > 0.05$). For the RANK, RANKL, and OPG variables on days 7, 14, and 28, an ANOVA test followed by Tukey HSD was conducted ($p < 0.05$). The expression of RANKL and OPG was observed in both the compression and tension sides of the OTM + GMSC normoxia group, as well as the OTM + GMSC hypoxia group, with a brown chromogen indicating osteoblasts, observed at magnifications of $400\times$ and 1000x using a light microscope (Figs. 1A–4A). Figs. 1B–4B present the mean and standard deviation (SD) of RANKL and OPG expressions on the compression and tension sides among the groups.

On day 28, the OTM + GMSC hypoxia group exhibited the highest level of RANKL expression. Conversely, on day 7, the OTM + GMSC normoxia group displayed the lowest level of RANKL expression on the compression side. There was a significant difference in RANKL expression on the compression side between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group on the observation day (Table 1). Additionally, on day 14, the PGO + GMSC hypoxia group had the highest RANKL expression, while the OTM + GMSC normoxia group exhibited the lowest RANKL expression on the tension side. A significant difference in RANKL expression on the tension side was observed between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group on the observation day (Table 2).

On day 14, the OTM + GMSC normoxia group exhibited the highest level of OPG expression. Conversely, on day 7, the OTM + GMSC hypoxia group displayed the lowest level of OPG expression on the compression side. There was a significant difference in OPG expression on the compression side between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group on the observation day (Table 3). On the other hand, OPG expression in the tensile area was reported to be highest in the OTM + GMSC hypoxia group on day 14, while it was lowest in the OTM + GMSC normoxia group on the same day. A significant difference in OPG expression on the tension side was observed between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group on the observation day (Table 4).

The immunohistochemical examination results revealed that the OTM + GMSC normoxia group exhibited RANK expression in both the compression and tension sides, whereas the OTM + GMSC hypoxia group displayed brown chromogen in osteoclasts observed at 400x and 1000x magnification under a light microscope (Fig. 5A and 6A). Figs. 5B and 6B present the mean and standard deviation (SD) of RANK expressions on the compression and tension sides across the groups. In this study, RANK expression in the stress region was found to be highest in the OTM + GMSC hypoxia group on day 14 and lowest in the OTM + GMSC normoxia group on day 28. On the day of observation, a significant difference in RANK expression on the compression side was observed between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group (Table 5). Additionally, RANK expression was highest in the OTM + GMSC normoxia group on days 14 and 28, and lowest in the OTM + GMSC hypoxia group on days 7 and 14. There was no significant difference in RANK expression on the tension side between the OTM + GMSC normoxia group and the OTM + GMSC hypoxia group on the observation day (Table 6).

4. Discussion

Osteoclasts play a crucial role in AB resorption, and the cytokines RANKL, RANK, and OPG are key regulators of OTM. OPG functions as an inhibitor of osteoclastogenesis, while RANKL promotes it. OPG acts as a soluble decoy receptor for RANKL, effectively blocking the interactions between RANKL and RANK, and thereby attenuating the impact of these proteins on osteoclastogenesis and bone resorption.[36–38] Previous studies have demonstrated that transferring the OPG gene to periodontal tissue can reduce alveolar bone resorption.[39] The balance between RANKL and OPG expression ratios also plays a role in bone remodeling processes. A higher ratio indicates an increased likelihood of bone resorption, while a lower ratio suggests a greater chance of bone formation.[40] In human periodontal ligament cells (hPDLCs), the RANKL/OPG ratio was found to be significantly elevated under hypoxic conditions. RANKL-mediated osteoclastogenesis plays a significant role in inflammatory bone resorption, particularly in conditions such as periodontitis.[41] Previous studies found that in hyperglycemic animal models under OTM, there was significantly enhanced RANKL expression. On the other hand, OPG expression was significantly decreased in hyperglycemic animal models under OTM.[42] Studies have identified activated T and B cells as biological sources of RANKL for bone resorption in periodontitis, with RANKL expressed in lymphocytes and macrophages. Hypoxia-induced hPDLCs have been shown to disrupt the balance of RANKL and OPG expression. Furthermore, under hypoxic
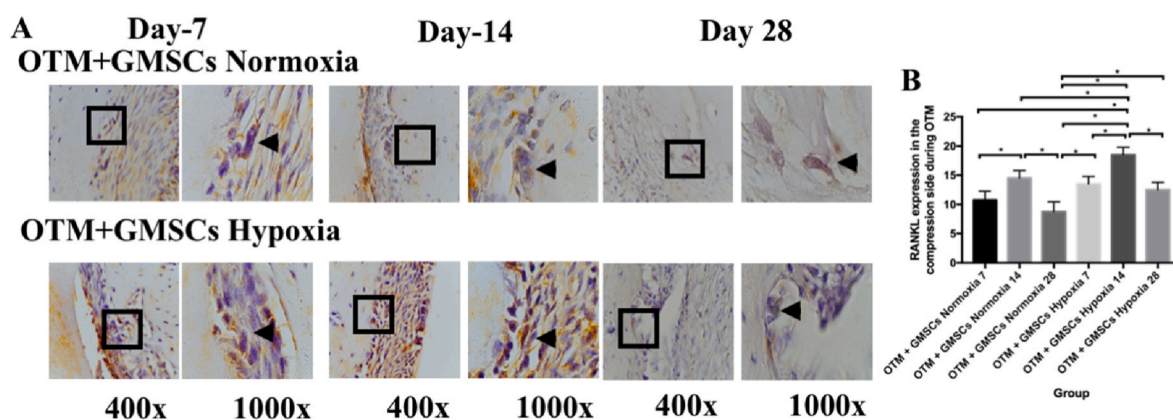

Fig. 1. (A) The positive RANKL expression in brown color (black square and arrow) in the osteoblast of compression side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of $400\times$ and 1000x under a light microscope (B) Average and standard deviation graphical bars of RANKL expression in each group. *significant difference between groups at $p < 0.05$.

Fig. 2. (A) The positive RANKL expression in brown color (black square and arrow) in the osteoblast of the tension side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of 400× and 1000x under a light microscope (B) Average and standard deviation graphical bars of RANKL expression in each group. *significant difference between groups at p < 0.05.
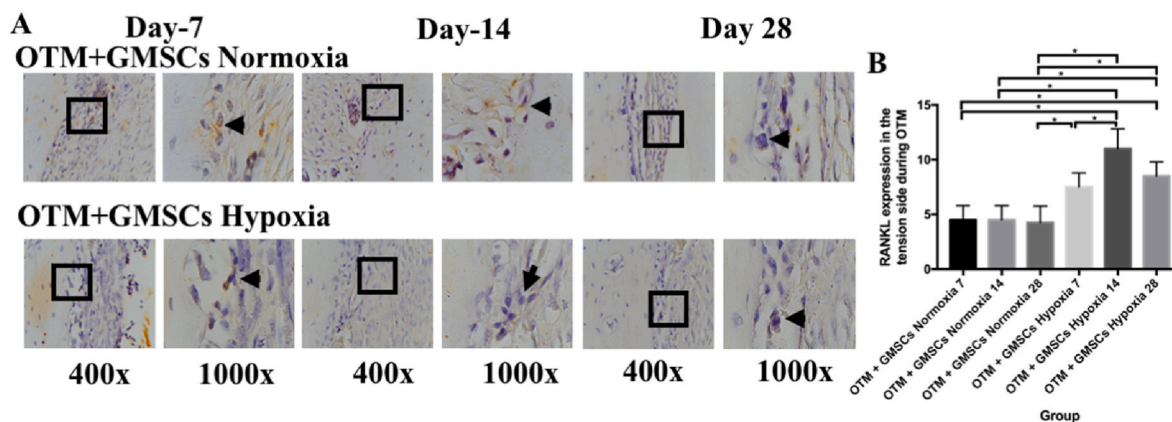

Fig. 3. (A) The positive OPG expression in brown color (black square and arrow) in the osteoblast of compression side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of 400× and 1000x under a light microscope (B) Average and standard deviation graphical bars of RANKL expression in each group. *significant difference between groups at p < 0.05.
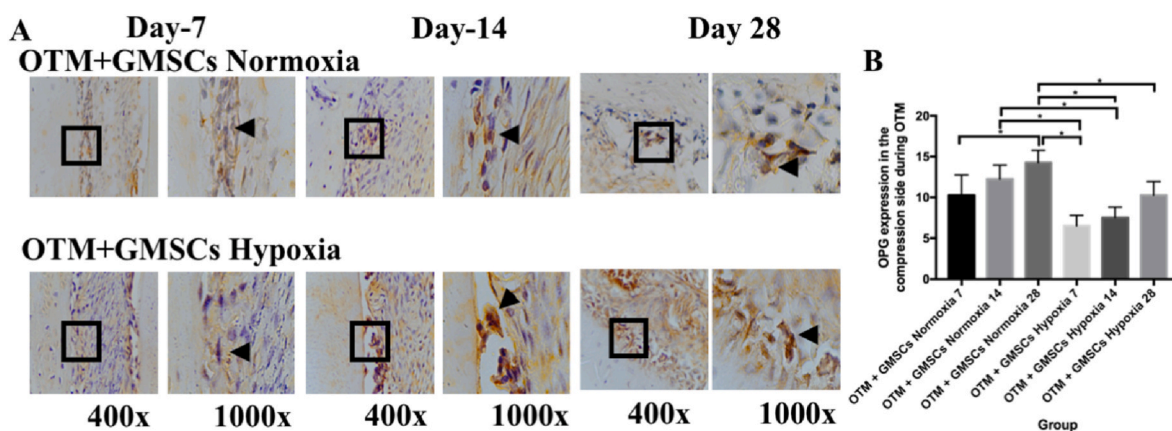

Fig. 4. (A) The positive OPG expression in brown color (black square and arrow) in the osteoblast of the tension side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of 400× and 1000x under a light microscope. (B) Average and standard deviation graphical bars of RANKL expression in each group. *significant difference between groups at p < 0.05.
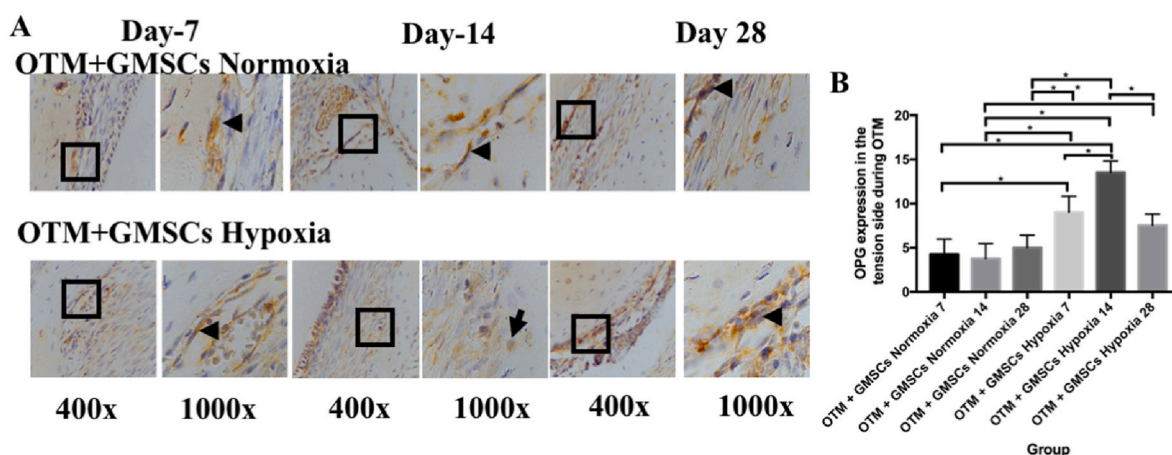
conditions, hPDLCs themselves become a cellular source of both RANKL and OPG. The expression of RANKL and OPG in hPDLCs under hypoxia is a crucial pathogenic mechanism in alveolar bone resorption.[41]

Hypoxia has been shown to impact the synthesis of OPG and RANKL, and it significantly enhances osteogenic differentiation in rat bone marrow mesenchymal stem cells (rBMSCs). This effect is demonstrated by increased bone volume following transplantation of hypoxia-preconditioned BMSCs. Histological examination comparing hypoxia

Table 1
The average ± SD, normality test and different test between RANKL expression groups in the compression side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.7783	0.9332	0.8156	0.0001*
OTM + GMSC Normoxia	10.75 ± 1.50[b]	14.5 ± 1.29[a]	8.75 ± 1.71[b]	
Normality test	0.2242	0.9719	0.85	
OTM + GMSC Hypoxia	13.50 ± 1.29[b]	18.50 ± 1.29[a]	12.50 ± 1.29[b]	
Homogenity Test	0.9719	0.9719	0.9719	
p		0.0001*		

*information: significant at $p < 0.05$ between groups, and between observation days. Different [ab]superscripts indicate differences between groups (based on the Tukey HSD test).

Table 2
The average ± SD, normality test and different test between RANKL expression groups in the tension side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.5276	0.6296	0.9754	0.0001*
OTM + GMSC Normoxia	4.5 ± 1.29[a]	4.5 ± 1.29[a]	4.25 ± 1.50[a]	
Normality test	0.9719	0.9719	0.2242	
OTM + GMSC Hypoxia	7.5 ± 1.29[a]	11.0 ± 1.83[b]	8.5 ± 1.29[a]	
Homogenity Test	0.9719	0.7143	0.9719	
p		0.05897		

*information: significant at $p < 0.05$ between groups, and between observation days. Different [ab]superscripts indicate differences between groups (based on the Tukey HSD test).

Table 3
The average ± SD, normality test and different test between OPG expression groups in the compression side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.5777	0.1269	0.9709	0.399
OTM + GMSC Normoxia	10.25 ± 2.50[b]	12.25 ± 1.71[ab]	14.25 ± 1.50[a]	
Normality test	0.9109	0.85	0.2242	
OTM + GMSC Hypoxia	6.50 ± 1.29[b]	7.50 ± 1.29[b]	10.25 ± 1.71[a]	
Homogenity Test	0.9719	0.9719	0.85	
p		0.002*		

*information: significant at $p < 0.05$ between groups, and between observation days. Different [ab]superscripts indicate differences between groups (based on the Tukey HSD test).

Table 4
The average ± SD, normality test and different test between OPG expression groups in the tension side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.9774	0.1269	0.8278	0.00001*
OTM + GMSC Normoxia	4.25 ± 1.70[a]	3.75 ± 1.29[a]	5 ± 1.29[a]	
Normality test	0.85	0.85	0.1612	
OTM + GMSC Hypoxia	9.00 ± 1.83[b]	13.5 ± 1.29[a]	7.5 ± 1.29[b]	
Homogenity Test	0.7143	0.9719	0.85	
p		0.0146*		

*information: significant at $p < 0.05$ between groups, and between observation days. Different [ab]superscripts indicate differences between groups (based on the Tukey HSD test).

BMSCs to normoxia BMSCs revealed reduced cellular death, increased bone production, and enhanced trabecular bone in the hypoxia BMSC group. Immunological analysis of hypoxic BMSCs demonstrated elevated expression of HIF-1α and beta-catenin, as well as increased levels of VEGF, alkaline phosphatase (ALP), osteocalcin, and collagen type I (Col-1).[43] Additionally, transfer of MSCs to the periodontal ligament increases the amount of OTM by upregulating the expression of RANKL messenger ribonucleic acid (mRNA).[44]

Constant hypoxia at 1 % O_2 has been found to reduce osteoclast development and resorption without affecting cell survival. It has been demonstrated that constant hypoxia inhibits RANKL-induced osteoclastogenesis by phosphorylating nuclear-associated T-cell-1 (NFATc1) through stress-activated protein kinases (SAPK) and Jun amino-terminal kinases (JNK).[45] Previous research has shown that hypoxia alters the expression of RANK and RANKL and enhances RANKL-induced cell migration through the PI3K-Akt–HIF–1 signaling pathway.[46] The Wnt and OPG-RANKL-RANK signaling pathways are two crucial systems that collaborate to regulate bone resorption and remodeling, with the bone formation differentiation factor Runt-related transcription factor-2 (Runx2) connecting these two signaling networks. Excessive expression of Runx2 leads to an increase in RANKL and a decrease in OPG and beta-catenin, resulting in a reduction in bone mass and bone volume in mice. Hypoxia has dual effects on bone remodeling. It significantly enhances the activity of HIF-1, promoting matrix formation while reducing the levels of Runx2 and Wnt. However, in the presence of pro-inflammatory cytokines, hypoxia activates HIF-2, leading to increased production of VEGF, matrix metalloproteinase-13 (MMP-13), and collagen, all of which are associated with extracellular matrix degradation.[47]

Ferroptosis is a distinct form of programmed cell death that is associated with RANKL-driven osteoclast differentiation and the iron depletion response mediated by ferritinophagy. In normoxia, an iron deficiency response characterized by increased transferrin receptor 1 and reduced ferritin, followed by RANKL activation, has been linked to downregulation of aconitase activity. However, this downregulation was not observed under hypoxia. In hypoxia, HIF-1α downregulates ferritinophagy and autophagy flux, as well as decreases autophagosome formation. The involvement of ferroptosis in osteoclasts has been demonstrated both in vitro and in vivo, suggesting that targeting HIF-1α and ferritin to induce ferroptosis in osteoclasts could be a potential therapeutic approach for pathological bone resorption.[48] Osteocytes and osteoblasts, known to be the primary sources of RANKL during mechanically-induced OTM, produce additional RANKL in response to pro-inflammatory signaling molecules released during OTM.[49] This increased RANKL binds to the RANK receptor on osteoclasts and osteoclast progenitor cells, leading to enhanced bone resorption.[17] Conversely, OPG functions as a decoy receptor for RANKL and inhibits bone resorption.[24] This balance between bone resorption and deposition is influenced by OTM, and medication use can also have an impact.[17,50] HIF-1α plays a crucial regulatory role in the molecular mechanisms of OTM. Recent studies suggest that mechanical strain or bacterial lipopolysaccharide may contribute to the stabilization of this transcription factor during periodontitis.[51–54] HIF-1α is known to be stabilized by hypoxia, but emerging research indicates that this stabilization also occurs under normal conditions.[55] Additionally, VEGF and cyclooxygenase 2 (COX-2), due to their significant effects on vascularization and inflammation during OTM, have the potential to influence osteoclastogenesis by increasing the RANKL/OPG ratio, which is necessary
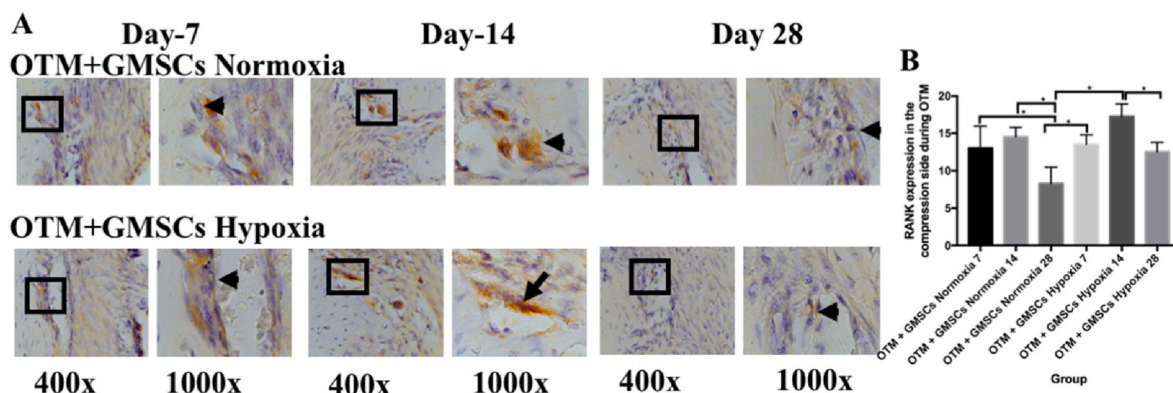
Fig. 5. (A) The positive RANK expression in brown color (black square and arrow) in the osteoclast of compression side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of 400× and 1000x under a light microscope (B) Average and standard deviation graphical bars of RANKL expression in each group. *significant difference between groups at p < 0.05.
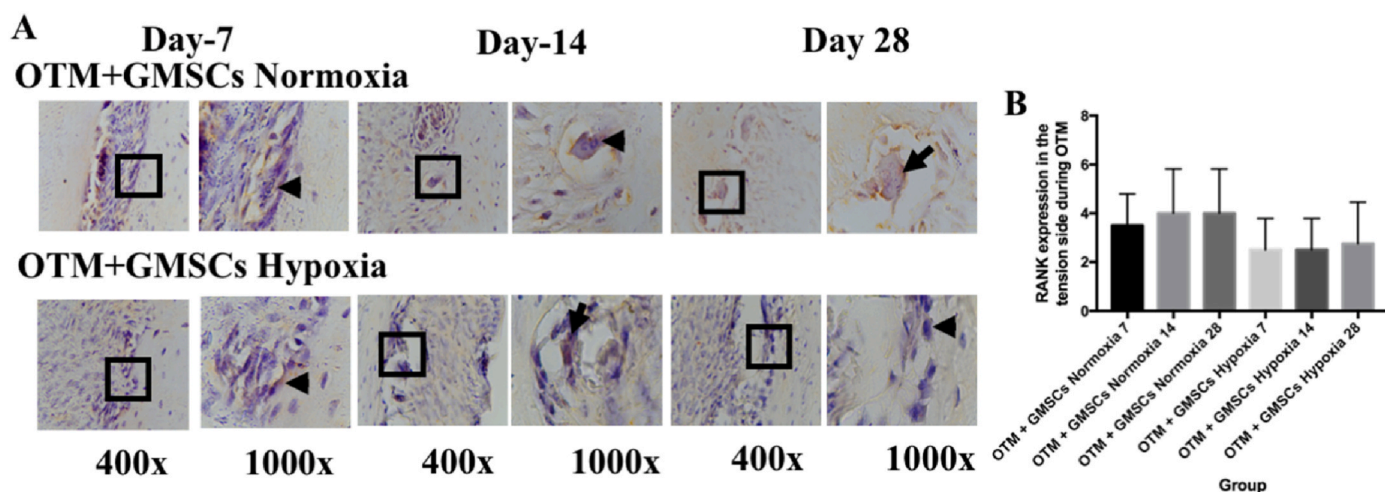

Fig. 6. (A) The positive RANK expression in brown color (black square and arrow) in the osteoclast of the tension side alveolar bone under orthodontic tooth movement after application of GMSC normoxia or hypoxia preconditioning with magnifications of 400× and 1000x under a light microscope. (B) Average and standard deviation graphical bars of the RANKL expression in each group.

Table 5
The average ± SD, normality test and different test between RANK expression groups in the compression side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.9163	0.925	0.6534	0.0001*
OTM + GMSC Normoxia	13 ± 2.94[a]	14.50 ± 1.29[a]	8.25 ± 2.22[b]	
Normality test	0.7335	0.9719	0.7982	
OTM + GMSC Hypoxia	13.50 ± 1.29[b]	17.25 ± 1.71[a]	12.50 ± 1.29[b]	
Homogenity Test	0.9719	0.85	0.9719	
p		0.004*		

*information: significant at p < 0.05 between groups, and between observation days. Different [ab]superscripts indicate differences between groups (based on the Tukey HSD test).

for osteoclast differentiation.[56,57]

GMSC have been identified as crucial cells in maintaining periodontal homeostasis due to their ability to form cementum-like complexes and PDL, as well as their immunomodulatory activity.[58–61] Various mechanical and biological methods have been employed to

Table 6
The average ± SD, normality test and different test between RANK expression groups in the tension side.

Group	Day			p
	7	14	28	
	Average ± SD			
Normality test	0.8568	0.8006	0.4763	0.8876
OTM + GMSC Normoxia	3.5 ± 1.29	4 ± 1.82	4 ± 1.82	
Normality test	0.9719	0.7143	0.7143	
OTM + GMSC Hypoxia	2.5 ± 1.29	2.50 ± 1.29	2.75 ± 1.71	
Homogenity Test	0.9719	0.9719	0.85	
p		0.0652		

induce appropriate progenitor cell populations at sites of OTM resorption. However, these techniques primarily focus on indirectly stimulating cellular and mechanical activation, which is influenced by factors such as frequency, duration, and mechanical force of the stimulation. On the other hand, direct transplantation of stem cells into the PDL was demonstrated to directly increase cellular and molecular activity, which was an effective strategy for delaying the resorption process.[44]

GMSCs may have crucial roles in PDL organization and alveolar bone remodeling, in addition to their therapeutic effects on void resorption.[62–64] Previous studies investigating the impact of hypoxia on

the expression of RANKL and OPG in hPDLCs in vitro have shown that hypoxia significantly increases RANKL expression and levels of soluble RANKL protein, while reducing OPG mRNA expression and protein levels.[43] These findings are consistent with other research conducted on cultured mouse BMSCs, which also demonstrates that hypoxia can regulate the expression of OPG and RANKL mRNA in rBMMSCs and significantly enhance their osteogenic differentiation.[41]

OPG acts as a decoy receptor for RANKL and is released by osteoblasts and fibroblasts in the PDL. Its role is to protect bone from excessive resorption by binding to RANKL and preventing it from interacting with RANK receptors on osteoclast precursor cells. This interaction inhibits the differentiation and fusion of osteoclasts, which are responsible for bone absorption. The ratio of RANKL expression to OPG, rather than the absolute expression of RANKL, is crucial in determining osteoclast activity in conditions such as OTM and periodontitis. Controlling osteoclastogenesis is necessary for OTM, while balancing osteoclast activity is important for both OTM and periodontitis.[65,66] Studies on periapical and periodontal lesions have shown that an increase in the RANKL/OPG ratio is a significant predictor of osteolytic activity in the development of periodontitis, whereas a decrease in the ratio is associated with inactive lesions.[66] The RANKL/OPG system plays a crucial role in the etiology of both OTM and periodontitis.[67]

The decreased RANKL/OPG expression ratio in HIF-1α knockout animals, as well as the altered RANKL gene expression during OTM, may be attributed to changes in macrophage invasion or the impact of myeloid HIF-1α deletion on periodontal ligament fibroblasts. RANKL binds to the RANK receptor on osteoclast precursor cells, promoting their differentiation and fusion into bone-absorbing osteoclasts. Higher levels of RANKL expression are associated with increased bone resorption, which explains the observed increases in OTM velocity, bone density, and periodontal bone level trend.[68] The increased RANKL gene expression is also correlated with higher levels of Acp5 and a trend toward Ctsk gene expression, indicating enhanced osteoclast activity. Acp5 codes for tartrate-resistant acid phosphatase (TRAP), while Ctsk is involved in cleaving telopeptides within collagen type I fibers, and matrix metalloproteinase-9 (MMP-9) is known to indicate osteoclast activity. Cathepsin K, an osteoclast-secreted protease, activates MMP-9, allowing for the degradation of organic components in the bone during resorption.[17,69,70]

According to studies on the role of HIF-1α in bone growth and remodeling, HIF-1α activation reduces osteoclastogenesis while increasing the resorptive activity of mature osteoclasts.[71] In mice, the deletion of HIF-1α results in lower trabecular bone volume due to its involvement in osteoclast activation.[72,73] Conversely, overexpression of HIF-1α has been shown to increase osteoclastogenesis while decreasing osteoblast differentiation.[74] Initially, there was no difference in alveolar bone density between HIF-1α knockout mice and wild-type mice. However, myeloid HIF-1α knockout mice exhibited lower bone densities and experienced faster OTM compared to wild-type mice, indicating a bone-protective role of myeloid HIF-1α. These mice also showed increased osteoclast activity and RANKL expression, as well as consistent ALP and Runx2 expression even after OTM.[68,75] To further investigate the allogeneic use of hypoxia-preconditioned GMSC in orthodontic treatment patients and reduce the duration of orthodontic therapy, randomized clinical studies with longer observation periods and diverse analysis techniques are needed. Therefore, one limitation of this study is its current experimental animal phase, which includes a limited observation period and assessment procedures.

5. Conclusion

It can be inferred that the expression of RANK-RANKL was significantly increased on the compression side of the alveolar bone during OTM after the administration of hypoxia-preconditioned allogeneic GMSC, while no significant changes were observed on the tension side. In contrast, immunohistochemical analysis showed an enhancement of

RANKL-OPG expression on the tension side but not on the compression side. Further studies are necessary to investigate these findings using longer observation periods and different experimental evaluation methodologies.

Contribution authors

The author declares that all authors participated in this research and publication.

Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its Submission.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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