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PREVENTATIVE AND THERAPEUTIC EFFECTS OF BMSC EXOSOME ON
PERIODONTAL DISEASE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Physiology and Biophysics at Virginia Commonwealth University

by

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ABBREVIATIONS

AP-1: Activator protein 1

APC: Antigen-presenting cell

BV: Bone volume

CDC: Centers for Disease Control and Prevention

EDTA: Ethylenediaminetetraacetic acid

EV: Extracellular Vesicle

H&E: Hematoxylin and Eosin

HBMSC: Human bone marrow mesenchymal stem cell

IFN- γ : Interferon gamma

IL-10: Interleukin-10

IL-13: Interleukin-13

IL-1 β : Interleukin 1 beta

IL-4: Interleukin-4

IL-6: Interleukin-6

LPS: lipopolysaccharide

M1: M1 phenotype macrophage

M2: M2 phenotype macrophage

MHC: Major histocompatibility complex

Micro-CT: Micro-computed tomography

miRNA: microRNA

MSC: Mesenchymal stem cell

MV: Microvesicle

MVB: Multivesicular body

NF κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

OPG: Osteoprotegerin

PAMPs: Molecular pattern associated with pathogens

qPCR: Quantitative real-time polymerase chain reaction

RANK: Receptor activator of nuclear factor kappa-B

RANKL: Receptor activator of nuclear factor kappa-B ligand

ROI: Region of interest

RT: Reverse Transcription

TGF- β 1: Transform growth factor beta 1

TGM2: Transglutaminase 2

Th1: T helper type 1 cell

T_h17: T-helper 17 cells

Th2: T helper type 2 cell

TNF α : Tumor necrosis factor alpha

TV: Total volume

VOI: Volume of interest

Abstract

PREVENTATIVE AND THERAPEUTIC EFFECTS OF BMSC EXOSOME ON PERIODONTAL DISEASE

By Jin Ha Kim, BSc

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University

Virginia Commonwealth University, 2020

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Periodontitis is an inflammatory disease caused by the imbalance between host immune response and bacterial infection. Strategies to manage the uncontrolled, excessive immune response and to promote tissue healing are in great demand. Therapies based on mesenchymal stem cells (MSCs) are promising and the clinical effects of MSCs are largely mediated by their secretome, especially exosomes. Previously, we isolated and purified exosomes secreted by human bone marrow mesenchymal stem cells (hBMSC). These exosomes have proliferative, chemotactic, anti-inflammatory and anti-osteoclastic function in vitro. In this study, we had two hypotheses: 1) hBMSC exosomes would prevent periodontal inflammation and alveolar bone loss with immunomodulatory function; 2) hBMSC exosomes would enhance

periodontal tissue regeneration. To test these hypotheses, we used a rodent ligature-induced experimental periodontitis model. In the preventative experiment, rats were treated with hBMSC exosomes concurrently with ligature placement. In the regenerative experiment, rats were treated with hBMSC exosomes for 3 weeks and 6 weeks after the experimental periodontitis was induced. Our results show that, in the preventive study, hBMSC exosome treatment led to less disease progression as evidenced by increased alveolar bone volume, decreased linear bone loss, and less lymphocyte infiltration in the subepithelial area. However, through micro-CT and gene expression analyses, no significant differences were seen between the exosome treatment and control in the regenerative study at both time points. In summary, hBMSC exosomes can ameliorate the development of periodontitis via an immunomodulatory effect. However, more studies are needed in the future to investigate their regenerative potential in periodontal tissues.

CHAPTER 1: INTRODUCTION

1.1. PERIODONTAL DISEASE

Periodontal disease is one of the most prevalent inflammatory diseases (1). Periodontal disease refers to the inflammatory process in response to the overgrowth of dental plaque caused by dysbiosis of bacteria complex, which often results in alveolar bone loss, tooth mobility and finally loss of the affected tooth (2). According to Centers for Disease Control and Prevention (CDC), one out of every two American adults aged 30 and over has periodontal disease (3). Progression of periodontal disease can be unrecognized for a long time due to its slow and painless nature. Disease progression may have begun at younger age, but people often are first aware of the periodontal disease at older age when the irreversible damage has already been done. The prevalence of periodontal disease increases with age (4, 5). With more people living longer, the number of people developing periodontal disease will increase even more (6). In addition to increased prevalence in older age and detrimental effects on periodontal tissue, chronic periodontitis is associated with other systemic disease such as atherosclerosis, pneumonia, rheumatoid arthritis, adverse pregnancy outcome, diabetes and cancer (7-15). Periodontitis can also have a significant effect on the psychosocial well-being, as the disease causes the discomfort, food intake difficulty and changes in facial appearance. With physical impairment and psychological distress, periodontitis has a negative impact on life quality.

1.2 PATHOGENESIS OF PERIODONTAL DISEASE

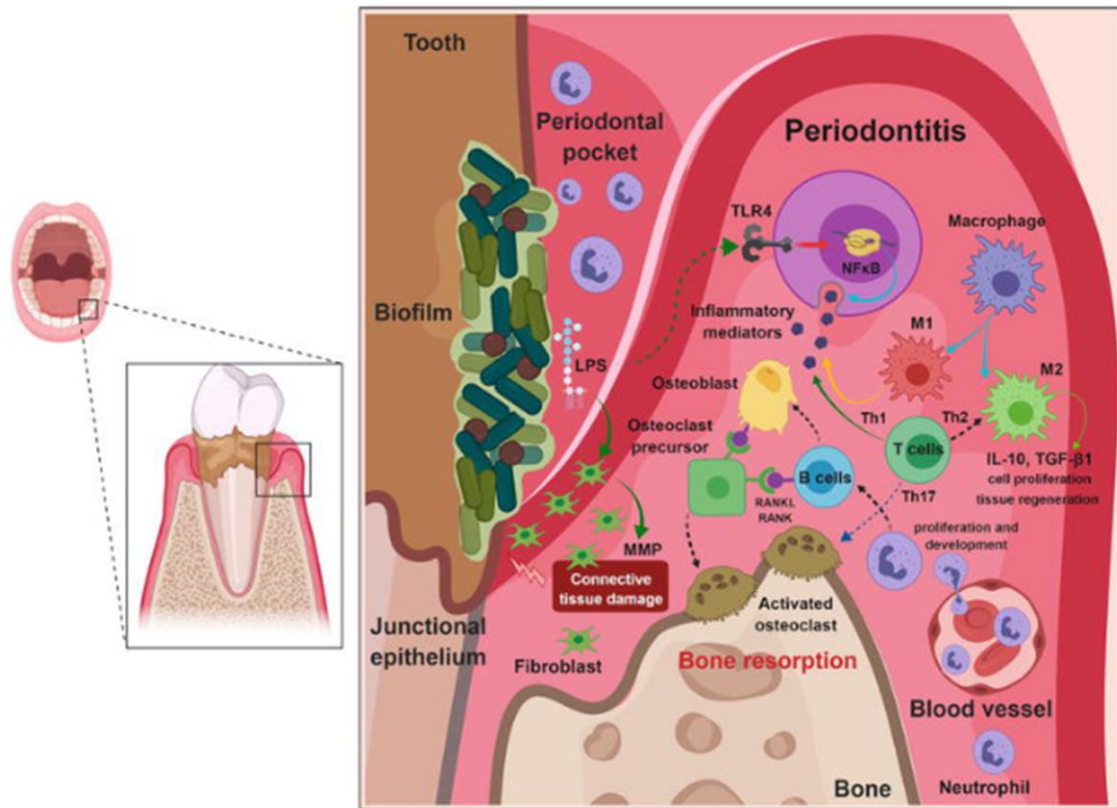
Host immune response plays a central role in the periodontal disease pathogenesis. In healthy condition, periodontal tissues are able to manage the bacterial infection with the controlled host immune response. When the balance between the control mechanism and bacterial infection is lost, disease progresses and it leads to the periodontal tissue damage.

Bacteria colonize plaque biofilms on the tooth surface and initiate inflammatory responses (16). Firm attachment of the plaque on the tooth surface makes it impossible for the host immune system to remove pathogens and continue to insult the periodontal tissues (17). Junctional epithelium is the first periodontal structure to encounter bacterial pathogens which can further cross the junctional epithelium and penetrate into the underlying connective tissue (18). Resident cells detect the molecular pattern associated with pathogens (PAMPs) such as lipopolysaccharide (LPS) and Toll-like receptors on the cells bind with PAMP causing the activation of proinflammatory transcription factors, such as NF κ B and AP-1 (19, 20), and the subsequent synthesize and release of inflammatory cytokines. Once the inflammation has been initiated, marginal periodontal ligament fibers are destroyed as a result of increased proteinase production and activity in the host immune system and therefore periodontal pockets are formed. Bacterial plaques spread apically along the root surface and deepen the pocket. The junctional epithelial cells are stimulated to proliferate and apically migrate along the root (21).

Gingival blood vessels below the junctional epithelium become inflamed. Neutrophils exit the post-capillary venules and migrate into the gingival sulcus (22). Neutrophils can recruit T-helper 17 cells (T_H17) to the infection site and more proinflammatory cytokines are produced. In addition, they promote the accumulation of B cells and plasma cells which express RANKL, a key osteoclastogenic cytokine, to induce bone resorption (23).

Macrophages polarize into two distinct phenotypes, M1 and M2. M1 macrophages are induced by the LPS of microorganism or by the Th1 related cytokines like IFN- γ , and secrete inflammatory cytokines such as TNF- α , IL-1 β , IL-6 (24). M1 macrophages can phagocytize microorganisms and promote inflammatory response, which contributes to the degradation of the collagen matrix in connective tissue (25). M2 macrophages are induced by the stimulation of Th2 related cytokines such as IL-4 and IL-13. They inhibit the inflammatory process by the secretion of anti-inflammatory cytokines including IL-10. Also, they can secrete TGF- β to promote cell proliferation and tissue regeneration (26). M1 rather than M2 macrophages predominate in the progression of chronic periodontitis (27).

Lymphocytes are activated by two types of signals and invade into periodontal tissue: one is the activation of antigen receptor of lymphocytes themselves and the other is the antigen-presenting cells, APC. Activated lymphocytes are the main source of RANKL, which induces osteoclastogenesis (28). RANKL/OPG/RANK system manages the balance of alveolar bone metabolism (29). RANKL binds to the receptor RANK on the surface of osteoclast or osteoclast precursor. The binding triggers



**Figure 1: Immune response during periodontal disease (description in the text).
Source: (Muñoz-Carrillo et al., 2019)**

osteoclasts to accumulate on the bone surface. OPG inhibits the biological functions of RANKL as a decoy ligand for RANK (30). Th1 lymphocytes also secrete inflammatory cytokines such as TNF- α and IL-1 β , which cooperates with Th17 lymphocytes in producing RANKL. Overall, lymphocytes have an important role in the progression of periodontitis and bone resorption (31, 32).

1.3 CURRENT TREATMENTS FOR PERIODONTAL DISEASE

Current treatments of periodontal disease are divided into non-surgical treatment and surgical treatment. Traditional treatments focus on the mechanical debridement procedures such as scaling and root planing and allow the periodontal tissue to restore itself (33). Complete elimination of bacterial biofilm with mechanical debridement procedures is usually unachievable because of the difficulty in accessing deep and tortuous pockets (34). Bacteria would recolonize within a week and infection will continue to thrive (35). Systemic antibiotics can be used along with the mechanical procedures in certain patients to improve the treatment efficacy. However, systemic antibiotics should be used with caution as unnecessary use can develop antibiotic resistance (36).

Periodontal surgical treatments focus on the elimination of periodontal pockets and the regeneration of periodontal apparatus. Pocket reduction surgery is a conservative procedure which reduces the soft tissue of periodontal pockets and creates new epithelial attachment with open access and debridement (37). The regeneration of periodontium can be accomplished with surgical treatments such as guided tissue regeneration and bone grafting. Although restoration to the original periodontal condition is not often achieved (38), periodontal surgical treatments can still be beneficial to people with severe periodontitis. In effort to overcome the limitations of current treatments, such as unpredictable outcome, sophisticated and complex procedures and high cost, researchers are searching for new therapies for periodontal regeneration.

1.4 REGENERATION POTENTIAL OF MESENCHYMAL STEM CELLS

Adult mesenchymal stem cells (MSCs) are capable of multilineage differentiation and self-renewal (39, 40). Bone marrow is the most common source for MSCs while they exist in almost all tissues. Human bone marrow stem cells (hBMSCs) have been widely used for tissue regeneration and cell-based therapies because of their therapeutic effects. MSCs have the ability to migrate to the inflammation site and the potential to differentiate into various cell types. Once activated, they can secrete anti-inflammatory molecules to suppress excessive inflammation and have immunomodulatory functions (41).

Past research has reported up to 20% of cementum and alveolar bone regeneration after MSC treatment in experimental defects in dogs (42). Also, positive clinical results were obtained in a small clinical trial (43). The amount of MSCs in the bone marrow is very low (44) while a large number of cells are required for clinical applications. Expansion *in vitro* is essential to obtain the adequate amount of cells. However, long-term culture and higher passages can lead to cell transformation and decrease in differentiation ability (45-47). While MSCs appear to have high regeneration potential, many challenges remain for their clinical application (48).

1.5 MSC DERIVED EXOSOMES

Exosomes are specific extracellular vesicles (EVs) secreted into extracellular fluid by all cells acting as messengers to communicate with other cells. Exosomes are nanoscale lipid bilayer EVs that carry lipids, mRNA, miRNA and proteins derived from the parental cell (49, 50). EVs are classified into three subtypes: exosomes, microvesicles (MVs), and apoptotic bodies (51, 52). Biogenesis process of exosomes

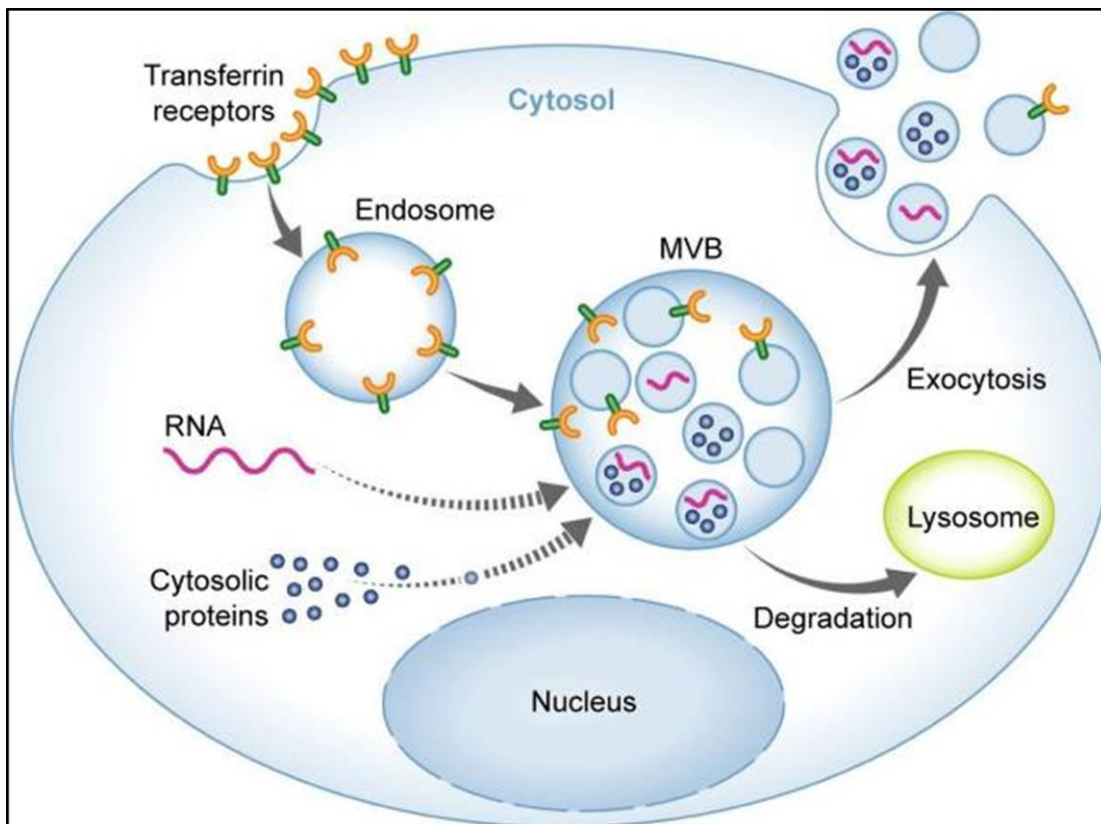


Figure 2: Endocytosis vesicle is formed from the plasma membrane which develops into late endosome. MVB is formed with inward budding in the cytosol from the endosome. Intraluminal vesicles in the MVB are released into extracellular space called exosomes. Source: (Schorey et al., 2015)

consists of four phases: initiation, endocytosis, multivesicular body (MVB) development, and release (53). Endosome is formed with endocytosis from the plasma membrane. Inward budding of endosome leads to the formation and accumulation of intraluminal vesicles inside the lumen developing MVB. MVB merges with the plasma membrane to release intraluminal vesicles into the extracellular space, which are called the exosomes (54).

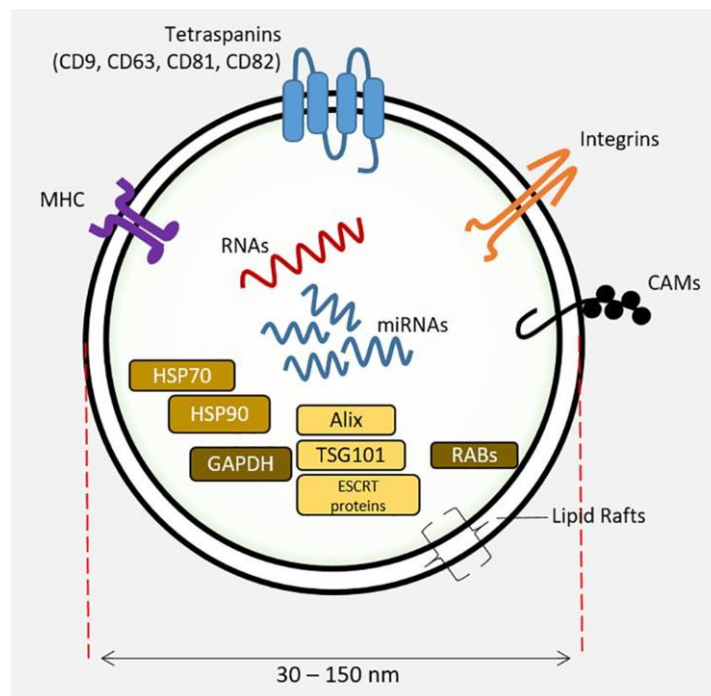


Figure 3: Exosomes are nanoscale extracellular vesicles containing specific proteins, RNAs and lipids. Proteins include HSP70, 90, GAPDH. Proteins involved in synthesis (Alix, ESCRT proteins, TGS101), and membrane associated or transmembrane proteins (RAB, Annexins, CAMs, Integrins, Tetraspanins, MHC I and II) and other cytosolic proteins. Source: (Cooper et al., 2020)

Exosomes contain plasma, cytosolic and nuclear proteins (55). Alix and TSG101 proteins are associated with exosome biogenesis, and RAB proteins, Annexins, integrins, major histocompatibility complex (MHC), tetraspanins are associated with membrane function (56). Tetraspanins such as CD9, CD81, CD82 and CD63 are involved in cellular interactions through binding with integrins and MHC (57). HSP70 and HSP90 are common cargo proteins (58). The associated pathway analysis of exosomal proteins revealed Integrin and mTOR signaling pathways, both of which are essential in osteoblast differentiation and bone formation (59).

Another abundant component of exosome cargo is miRNAs which regulate gene expression. miRNAs in exosomes represent the parental cell type and condition (50). Therefore, it is possible to therapeutically alter cell function through directing engineered miRNAs into exosomes (60, 61). Many studies proved that exosomes serve to convey signals from cell to cell (62-64). The transportation of miRNAs from parental cell to target cell results in the miRNA-mediated alteration in gene expression and cellular function. Thereby this is an important paracrine signaling mechanism (65), which can be used in the regenerative therapy. For example, exosomes have been reported to protect against acute kidney injury by altering cellular activities in epithelial/mesenchymal transition, matrix remodeling, apoptosis, differentiation, inflammation and repair process (66).

There are several advantages of using MSC exosomes in regenerative medicine instead of MSC as a whole cell. The small size of exosomes allows them to circulate throughout the body whereas MSCs are too large to pass beyond the capillary bed. A cell-derived lipoprotein coat of exosomes protects their cargo from degradation in the systemic circulation (49). Exosome delivery can avoid the transfer of mutated or damaged cells which might exhibit oncogenic potential and other noxious effects. Moreover, the use of exosomes can overcome the scarcity of MSCs because exosomes can be harvested multiple times from MSC culture (67). While more research is needed, it is certain that MSC exosomes are promising agents as stem cell-based, cell free regenerative therapy.

1.6 MSC DERIVED EXOSOMES IN BONE REGENERATION

MSC exosome-mediated regeneration has received great attention in the past decade. For instance, exosomes reduced the morphologic and functional damage of glycerol-induced acute kidney injury in SCID mice by inducing proliferation of tubular cells (68). BMSC-derived exosomes preserved retinal ganglion cell function and promoted axon regeneration through miRNA-dependent mechanisms (69). Treatment of MSC exosomes improved cardiac epicardial remodeling and increased left ventricular ejection fraction (70). The progression of hypoxic-induced pulmonary hypertension was significantly inhibited by intravenous MSC exosome injection (71).

In regard to bone regeneration, some studies demonstrated MSC exosomes have the ability to activate osteogenic differentiation, stimulate angiogenesis, and promote bone formation (72-75). miRNAs in hBMSC exosomes such as let-7a are a regulator of osteoblast differentiation (76). Many studies demonstrated hBMSC-derived exosomes carry miRNAs that contributes to bone regeneration (77-78). In addition to miRNAs, there are other important proteins mediating essential signaling pathways in bone regeneration. For example, the secretion factors from MSCs, including exosomes, can stimulate the expression of RUNX2, a transcription factor that regulates osteogenesis by promoting the differentiation of pluripotent stem cells into osteoblasts (79). hBMSC exosomes activates multiple signaling pathways such as Akt, Erk1/2 and STAT3 to induce angiogenic responses in fibroblasts (80). Wnt pathway plays important role in bone repair. hBMSC exosomes activates the Wnt3a- β -catenin pathway and induces angiogenic capacity of fibroblast (81). hBMSC exosomes also express high level of BMP9, transforming growth factor β 1 (TGF β 1), and vascular endothelial growth

factor (VEGF), and platelet-derived growth factor (PDGF) (82), all of which are important growth factors in osteogenic differentiation and angiogenesis (83-85).

Previous work in our lab demonstrated the effects of hBMSC exosomes on cell proliferation, migration, and cytokine production *in vitro*. The intensity of hMSC migration and proliferation increased as the exosomes dosage increased. From protein arrays, various cytokines and growth factors were enriched in the exosomes compare to the supernatant of the culture medium. The exosomes also showed anti-inflammatory effect, which suppressed IL-6 production in macrophage-like cells, and inhibited osteoclast differentiation *in vitro*. Many small RNAs, such as miR-486, 760, 6087, 7641, and 4792, are highly enriched in exosomes compared to total cell lysate.

1.7 SUMMARY

The host inflammatory response triggered by oral microbial biofilm on tooth surface causes periodontitis. Complications such as association with systemic disease, physical discomfort and negative psychosocial impact due to periodontal deformity can remarkably reduce the quality of life of patients. With the limitations of current treatments and the expected increase of disease prevalence, new innovative treatment which targets the uncontrolled periodontal inflammation is highly desired. While MSC is widely recognized, the applications of MSC in regenerative medicine have experienced several challenges. Exosomes secreted from MSCs have the advantages to overcome the limitations of whole cell delivery while retaining the beneficial regeneration potential of MSCs. MSC exosomes may serve as a novel stem cell-based, cell free regenerative therapy for treating periodontitis.

CHAPTER 2: SPECIFIC AIMS

Progressive periodontal inflammation leads to the destruction of periodontal tissues including alveolar bone. Recent studies demonstrated the outcome of stem cell exosome therapy in bone regeneration. However, the impact of MSC exosomes in the periodontal diseases is still not clear. The overall objective of this study is to explore the preventative and therapeutic potential of hBMSC exosomes therapy for periodontitis. The first hypothesis was that hBMSC exosomes could prevent periodontal inflammation and alveolar bone loss. The second hypothesis was that hBMSC exosomes may promote periodontal regeneration after the disease was established.

2.1 Specific Aims

The following aims were developed to test our main hypothesis:

Specific Aim 1: To determine if hBMSC exosomes would prevent periodontal inflammation and alveolar bone loss in ligation-induced periodontitis.

Sub-aim 1: To determine the effect of hBMSC exosomes on alveolar bone loss via Micro-CT analysis. Periodontitis was induced at rat maxillary molars by suture ligation and, concurrently, hBMSC exosomes were injected to gingival papillae every week. 3 weeks later, the left and right maxillae of the subjects were harvested, and

subsequently analyzed with the standardized micro-CT protocol. Bone volume and alveolar bone level were both quantified.

Sub-aim 2: To perform a histological analysis of periodontal tissue from the preventative model. Samples from the harvested maxillae were demineralized and sectioned. The slides were then stained with H&E to investigate the morphology and to qualitatively show the extent of prevention in disease progression.

Sub-aim 3: To perform a histomorphometric analysis of infiltrated lymphocytes in gingival subepithelial tissue to determine the effect of exosome on periodontal inflammation. Images of gingival tissue from H&E stained sections were captured. Lymphocytes in the gingival subepithelial tissue were quantified by specific characteristics of lymphocyte.

Specific Aim 2: To determine if hBMSC exosomes would promote periodontal regeneration in ligation-induced periodontitis.

Sub-aim 1: To perform Micro-CT analysis of bone volume and alveolar bone level to determine the effect of hBMSC exosomes on periodontal bone regeneration. Periodontitis was induced at rat maxillary molars by suture ligating for 3 weeks. After that, the sutures were removed and weekly exosome injection was started for 3 or 6 weeks. The left and right maxillae were harvested, and subsequently analyzed with the standardized micro-CT protocol. Bone volume and alveolar bone level were both quantified.

Sub-aim 2: To determine the gene expression profiles of periodontal tissues during inflammation resolution and tissue regeneration. Gingival papillae were dissected from formalin-fixed maxillary samples and RNA was extracted. RT-qPCR was performed to analyze the expression of a panel of genes related to inflammation activation and resolution.

2.2 SIGNIFICANCE OF THE STUDY

The purpose of these studies was to establish the therapeutic effects of hBMSC exosomes in periodontitis with possible further development of an innovative and less invasive regeneration therapy. Current periodontal therapies focus mostly on the debridement of bacterial pathogens. However, the chronic inflammatory response in the hosts is correlated with the susceptibility and severity of the disease. Therefore, safe, effective, and efficient therapies to control the excessive inflammatory response can be the key of successful treatment for periodontitis. Our previous studies suggested the regenerative effects of hBMSC exosomes in calvarial defects. It would be exciting if exosome therapy can also be applied to periodontal regeneration and provide a better alternative treatment modality which is more time and cost effective and efficacious. Our study may lead to novel preventive and therapeutic strategies which would benefit patients with periodontal disease globally.

CHAPTER 3: METHODS

3.1. SPECIFIC AIM 1: TO DETERMINE IF hBMSC EXOSOMES WOULD PREVENT PERIODONTAL INFLAMMATION AND ALVEOLAR BONE LOSS IN LIGATION-INDUCED PERIODONTITIS

Studies in Aim 1 sought to determine if hBMSC exosomes would prevent periodontal inflammation and alveolar bone loss in ligation induced periodontitis model in rats. hBMSC exosomes are known to be proliferative, chemotactic, anti-inflammatory, and anti-resorptive from *in vitro* studies. With our ultimate goal to develop a more effective clinical therapy for periodontal defects, *in vivo* studies are the reasonable next step in progression. We first tested if hBMSC exosomes can exert immunomodulatory function to prevent periodontal inflammation and alveolar bone loss.

20 Male Sprague-Dawley rats (250-300g) were divided into 2 groups: exosome treatment and control. The rats were anesthetized with isoflurane. Buprenorphine was administered pre-surgically for pain management. 3-0 silk sutures were placed into the gingival sulci around the maxillary left second molar (Figure 4). The sutures were left in place for three weeks to induce experimental periodontitis. Both groups received weekly injections concurrently with suture placement. Injections were administered locally at two palatal gingival tissue sites: interdental papillae between 1st and 2nd molars and 2nd and 3rd molars of the left maxilla. Exosome treatment group received 1.6 μ g

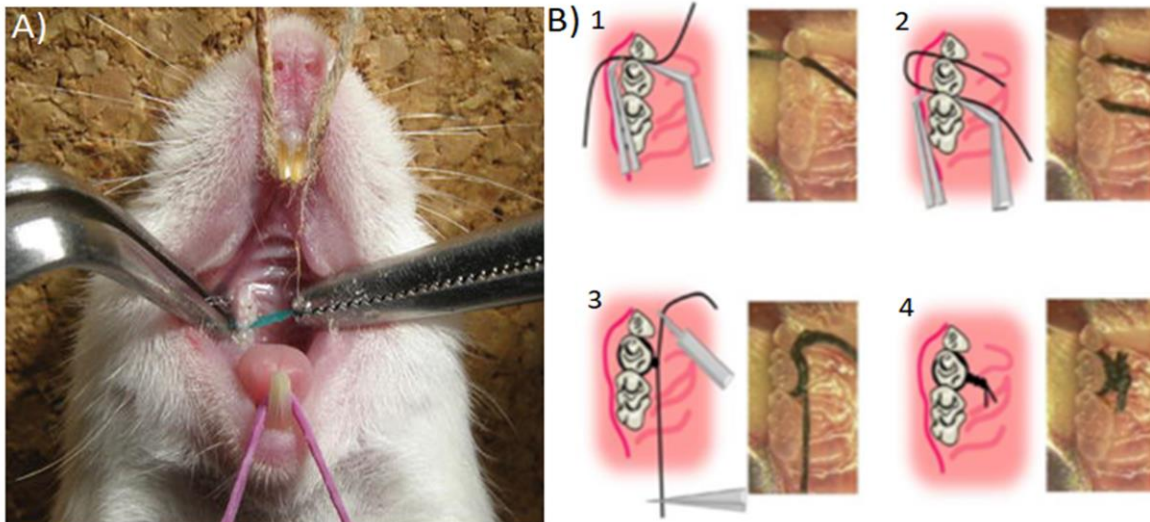


Figure 4: (A) Rats were laid down on the back and both upper and lower jaws were immobilized by fixing a string. (B) 3-0 suture was placed around the maxillary left second molar. Suture was first passed through interdendum between second and third molar apically. Same suture was passed through another interdendum between first and second molar apically. Suture was looped around the neck of the second molar and tied firmly using a triple-knot. Excess suture was cut. Source: A) (Muraoka et al., 2018), B) (Abe & Hajishengallis, 2013)

exosomes on weekly injection day: 2.5 μL of exosomes per injection site with 0.33 $\mu\text{g}/\mu\text{L}$ concentration. Exosomes were harvested from hBMSC culture media through sequential ultracentrifugation and quantified by measuring the total protein content through BCA assay. Same volume of saline was injected to the control group. After 3 weeks, rats were euthanized with CO_2 and the maxillae were harvested and fixed in 10% formalin for further analysis (Figure 5).

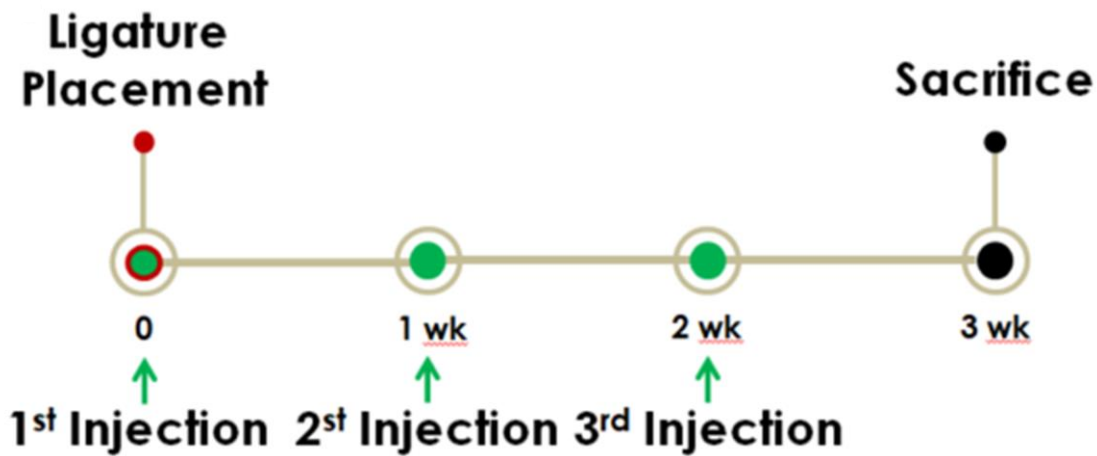


Figure 5: First injection was done on the same day of ligature placement (red). Injections (green) were given three times weekly. After 3 weeks, rats were euthanized with CO₂.

3.1.1. AIM 1.1: TO DETERMINE THE EFFECT OF hBMSC EXOSOMES ON ALVEOLAR BONE LOSS VIA MICRO-CT ANALYSIS

The fixed left and right maxillae were scanned by a micro-CT machine, Bruker SkyScan 1173. Obtained images were reconstructed using NRecon Reconstruction software. Reconstructed images were opened in DATAVIEWER software to align each sample with standard parameters shown in Figure 6. Transverse plane of each aligned samples was saved for volumetric analysis. Sagittal plane of each aligned samples was saved for alveolar bone level analysis.

For volumetric analysis, the transverse plane images of samples were opened in CTA software. Region of interest (ROI) was established in 121 transverse plane layers

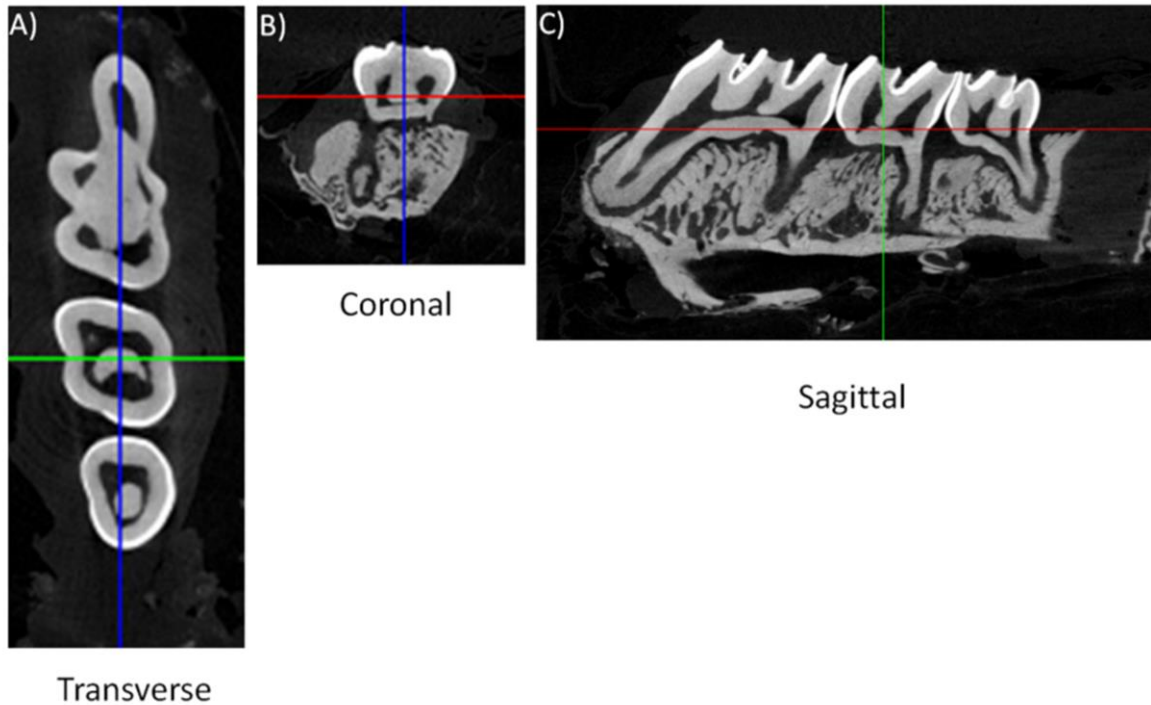


Figure 6: Three planes of the sample can be viewed in DATAVIEWER software. (A) First, the mesial end of first molar and the distal end of the third molar were aligned along with the vertical blue line from the transverse plane at the cemento enamel junction (CEJ) of the second molar. (B) Second, the buccal CEJ and the palatal CEJ of the second molar were aligned along with the horizontal red line from the coronal plane. (C) Third, the mesial CEJ and the distal CEJ of the second molar were aligned along with the horizontal red line from the sagittal plane.

starting from the top layer which is the CEJ of the second molar. Two ROIs, one was the area between the first molar and second molar and the other was the area between the second molar and third molar, were traced by hand using computer mouse in all 121 layers as shown in Figure 7. Once the ROI drawing is complete, bone volume was quantified with setting threshold within the software. Threshold was found to be the lowest acceptable pixel brightness of the bone area. Values above are counted as solid

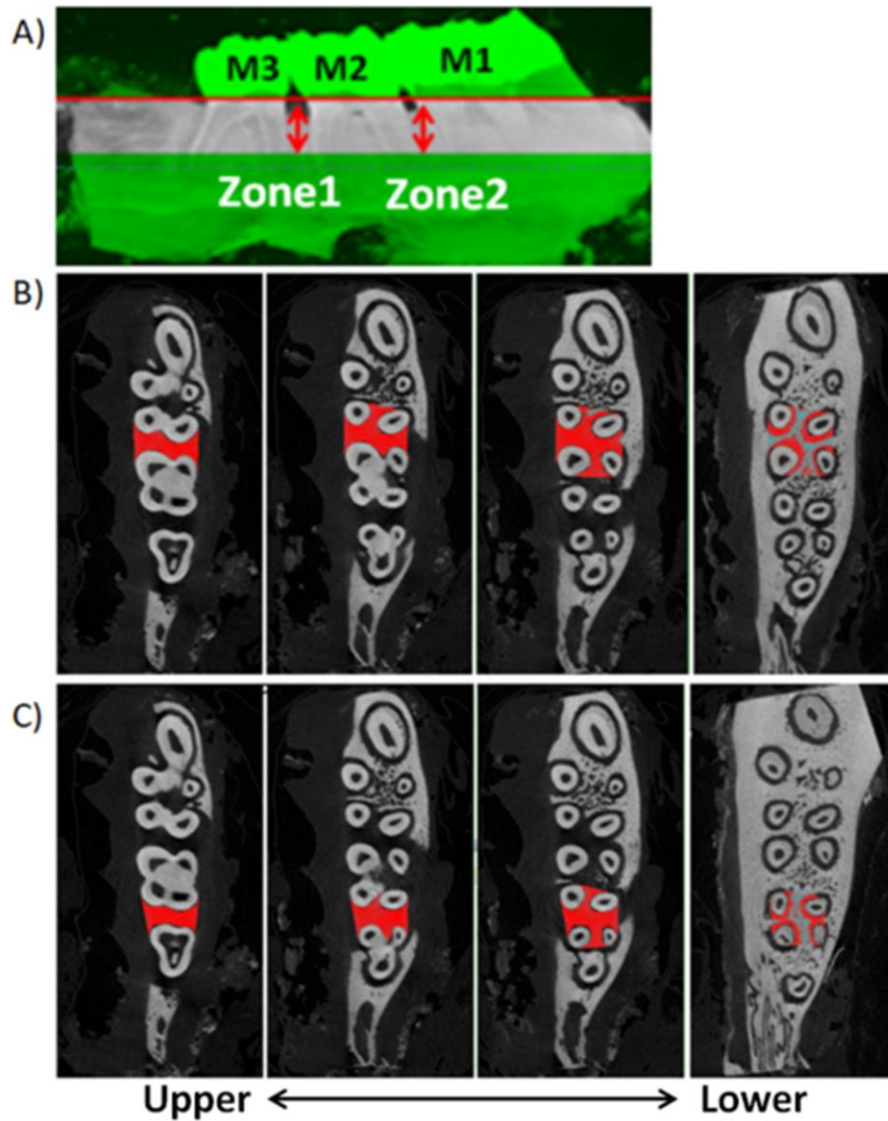


Figure 7: (A) Bone volume was measured in two different area, zone 1 and zone 2. Zone 1 is the area between the first molar and the second molar. Zone 2 is the area between the second molar and the third molar. ROI was drawn in the transverse plane with the straight tangent line from four roots in each zone as the connecting boundaries of the ROI. (B) The process of drawing the ROI of zone 1 was repeated approximately every 5 layers. (C) The process of drawing the ROI of zone 2 was repeated approximately every 5 layers.

bone. Quantitative analysis provided the value of tissue volume (TV), bone volume (BV), and a ratio of the two (BV/TV) that represents the percentage of total bone within the ROI.

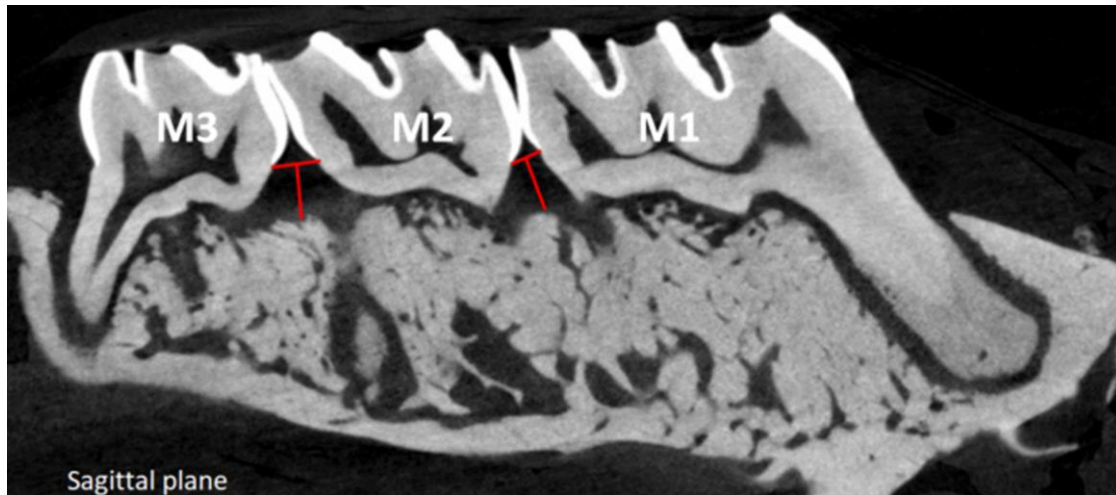


Figure 8: CEJ of the first molar (M1) and the second molar (M2) were connected with the straight line. From the midpoint of this line, a perpendicular straight line was drawn to alveolar bone crest (ABC). This perpendicular line was measured for the alveolar bone level analysis. Same measurement was done between the M2 and the third molar (M3).

For alveolar bone level analysis, the sagittal plane images of samples were opened in CTAn software. Among those sagittal plane images, one or two sagittal plane images that the crowns of the first molar, second molar, and third molar were contacted to each other were selected. These images were opened in ImageJ software. The CEJ midpoints between the first molar and second molar and also between the second molar and third molar were located using line tools. From the midpoint, a perpendicular line

was drawn to the alveolar bone crest (ABC) as shown in Figure 8. This perpendicular line was measured to represent the linear bone loss measurement for alveolar bone level analysis.

3.1.2. AIM 1.2: TO PERFORM HISTOLOGICAL ANALYSIS OF PERIODONTAL TISSUE FROM THE PREVENTATIVE MODEL

After micro-CT analysis, maxillae samples were decalcified in a 14% EDTA solution that was pH balanced to 7.2-7.4 with 10N NaOH. The samples were placed in the solution with a stir bar to ensure constant movement and placed in a cold room for approximately 16 days. Once the samples became soft, they were rinsed four times with H₂O and the samples were temporarily placed back into formalin until ready for embedding and sectioning. With help from the CMMC (Cancer Mouse Models Core, Massey Cancer Center, Richmond, VA), the samples were embedded in paraffin blocks and sectioned in the sagittal plane from the palatal side so that the roots of the molars are visible. The sections were placed on slides and stained with Hematoxylin and Eosin (H&E). The H&E stain helped visualize tissue morphology and allowed for qualitative analysis of the samples. Images were captured using Phenochart, a slide visualizer supplied by PerkinElmer.

3.1.3. AIM 1.3: TO PERFORM A HISTOMORPHOMETRIC ANALYSIS OF INFILTRATED LYMPHOCYTES IN GINGIVAL SUBEPITHELIAL TISSUE TO DETERMINE THE EFFECT OF EXOSOME ON PERIODONTAL INFLAMMATION

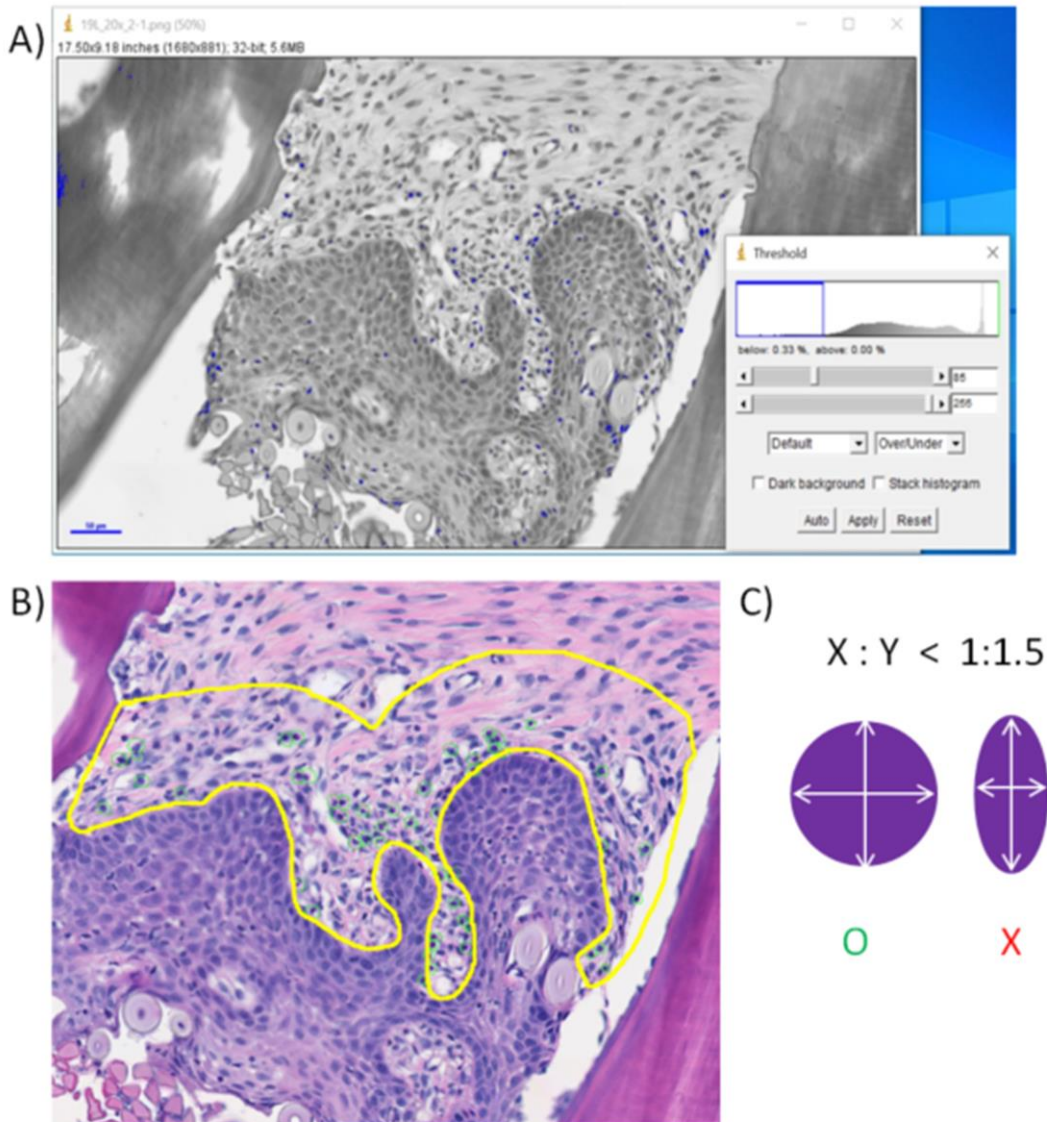


Figure 9: (A) 20x resolution images of the periodontal tissue between M1 and M2 were changed into grayscale image. Threshold was set at the nucleus of epithelial layer. Cells which had higher intensity nucleus than the epithelial layer were shown in blue. (B) From the original 20x resolution images, 50 μ m depth of subepithelial tissue area, ROI, were drawn in yellow line. (C) The longest diameter of nucleus which is above threshold within ROI was measured. Another diameter which is the perpendicular to the first diameter was also measured. If the ratio of those two diameters were smaller than 1 :1.5, the cell was counted as the infiltrated lymphocytes.

After obtained the histology images of samples, infiltrated lymphocytes in gingival subepithelial tissue were quantified. Images of the periodontal tissue between the first molar and the second molar with 20x magnification were captured from the Phenochart software. The images were opened in ImageJ software and the infiltrated lymphocytes were counted based on the criteria as shown in Figure 9. The first criterion was the cells should have a higher nuclear staining than that of gingival epithelial cells. The second criterion was that the cells should have smaller nucleus than the epithelial cell nucleus. The third criterion was that the ratio of two perpendicular diameters of nucleus than 1.5. Cells matched these criteria within the ROI, which includes a subepithelial area 50µm from the basal cell layer, were counted as the infiltrated lymphocytes.

3.2. SPECIFIC AIM 2: TO DETERMINE IF hBMSC EXOSOMES WOULD PROMOTE PERIODONTAL REGENERATION IN LIGATION-INDUCED PERIODONTITIS

Studies in Aim 2 sought to determine if hBMSC exosomes would promote healing in the ligation induced periodontitis. This regenerative model, an extension of the preventive study, simulates the clinical situation when patients come to the clinics with existing periodontitis. The hypothesis was that the hBMSC exosomes have osteogenic potential and promote periodontal regeneration.

52 Male Sprague-Dawley rats (250-300g) were divided into 5 groups: baseline (8 rats), 3-week exosome treatment (11 rats), 3-week control (11 rats), 6-week exosome treatment (11 rats), and 6-week control (11 rats). The rats were anesthetized with

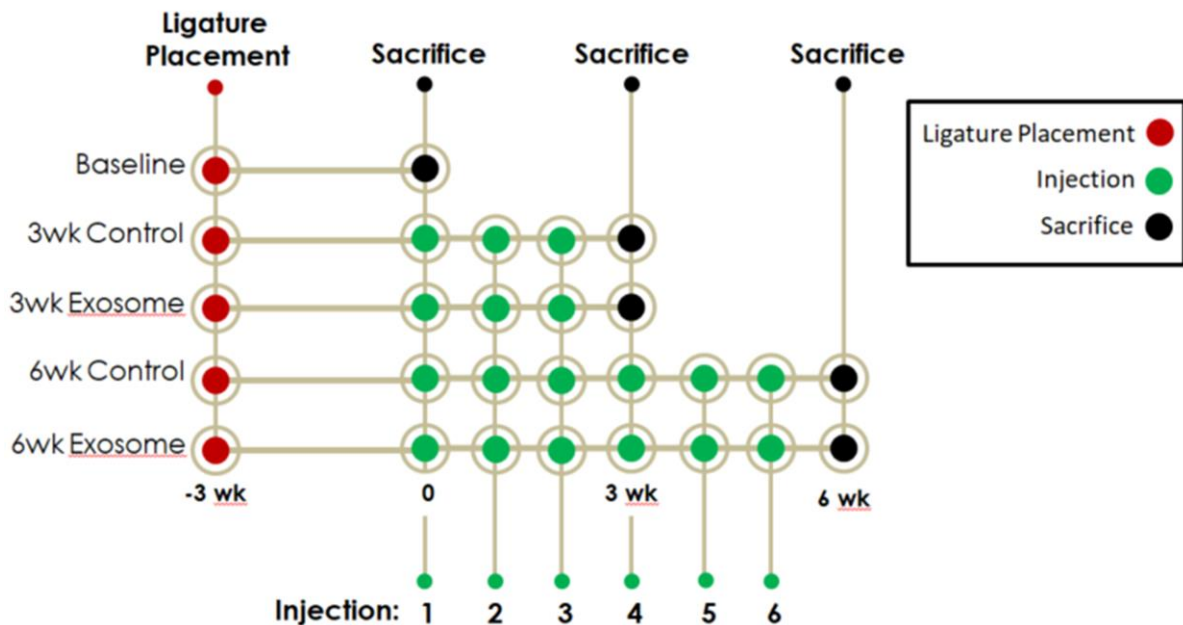


Figure 10: Ligature was placed for 3 weeks on the left second molar. Ligature was removed after 3 weeks and baseline group was sacrificed at this time point. Other groups received injection at the time of ligature removal. 3 week groups are sacrificed after 3 weeks of injections. 6 week groups are sacrificed after 6 weeks of injections.

isoflurane. Buprenorphine was administered pre-surgically for pain management. 3-0 silk sutures were placed into the gingival sulci around the maxillary left second molar as shown in Figure 4. The sutures were left in place for three weeks to induce experimental periodontitis. After 3 weeks, rats in the baseline group were euthanized with CO₂ and the maxillae were harvested and fixed in 10% formalin for further analysis. For all other groups, sutures were removed and the first injections were given at the same time. Injections were administered locally at two palatal gingival tissue sites: interdental papillae between 1st and 2nd molars and between 2nd and 3rd molars of the left maxilla. Exosome treatment group received a total of 1.7 µg exosomes on weekly injection day: 2.5 µL of exosomes per injection site with 0.35 µg/µL concentration.

Exosomes were harvested from hBMSC culture media through sequential ultracentrifugation and quantified by measuring the total protein content through BCA assay. Same volume of saline was injected to the control group. 3 weeks after the first injection, rats from both 3-week exosome treatment and 3-week control groups were euthanized with CO₂ and the maxillae were harvested and fixed in 10% formalin for further analysis. 6 weeks after the first injection, rats from the 6-week exosome treatment and 6-week control groups were euthanized with CO₂ and the maxillae were harvested and fixed in 10% formalin for further analysis.

3.2.1. AIM 2.1: TO PERFORM A MICRO-CT ANALYSIS OF BONE VOLUME AND ALVEOLAR BONE LEVEL TO DETERMINE THE EFFECT OF hBMSC EXOSOMES ON PERIODONTAL BONE REGENERATION

Both left and right maxillae were scanned using a micro-CT machine, Bruker SkyScan 1173. Obtained images were reconstructed using NRecon Reconstruction software. Reconstructed images were opened in DATAVIEWER software to align each sample with standard parameters shown in Figure 6. Transverse plane of each aligned samples was saved for volumetric analysis. Sagittal plane of each aligned samples was saved for alveolar bone level analysis.

For volumetric analysis, the transverse plane images of samples were opened in CTAn software. Region of interest (ROI) was established in 121 transverse plane layers starting from the top layer which was the CEJ of second molar. ROI was the area between 8 roots of the molars: two distal roots of the first molar, four roots of the second molar, and two mesial roots of the third molar. ROI was traced by hand using computer

mouse in all 121 layers as shown in Figure 11. Once the ROI drawing was complete, bone volume was quantified with setting threshold within the software. Threshold was found to be the lowest acceptable pixel brightness of the bone area. Values above were counted as solid bone. Quantitative analysis provided the value of tissue volume (TV), bone volume (BV), and a ratio of the two (BV/TV) that represents the percentage of total bone within the ROI.

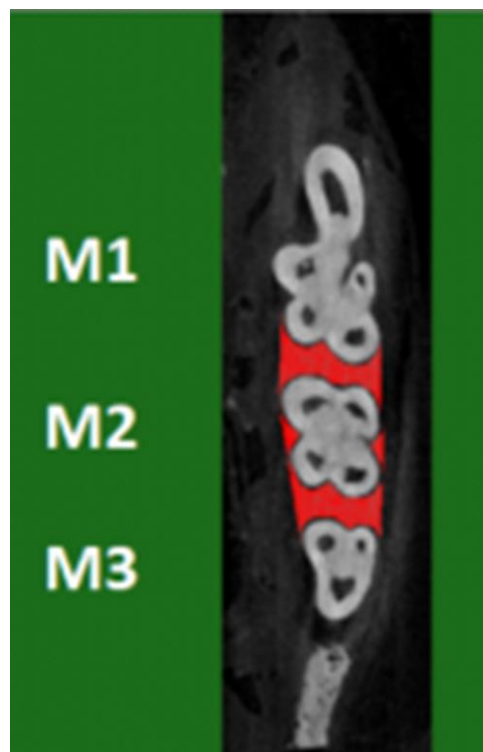


Figure 11: (A) Bone volume is measured in the area between 8 roots; 2 distal roots of M1, 4 roots of M2, and 2 mesial roots of M3. ROI was drawn in the transverse plane with the straight tangent line from each root as the connecting boundaries of the ROI. The process of drawing the ROI was repeated approximately every 5 layers.

For alveolar bone level analysis, the sagittal plane images of samples were opened in CTAn software. Among those sagittal plane images, one sagittal plane image

of the midpoint of the second molar was selected. Then, 5 images of every 10th sagittal plane image toward both buccal and palatal side from the selected midpoint image were selected as well. Total 11 sagittal plane images were selected from each sample. The distance between the CEJ of M2 and the ABC in all 11 images were measured in Image J software to represent the linear bone loss (Figure 12).

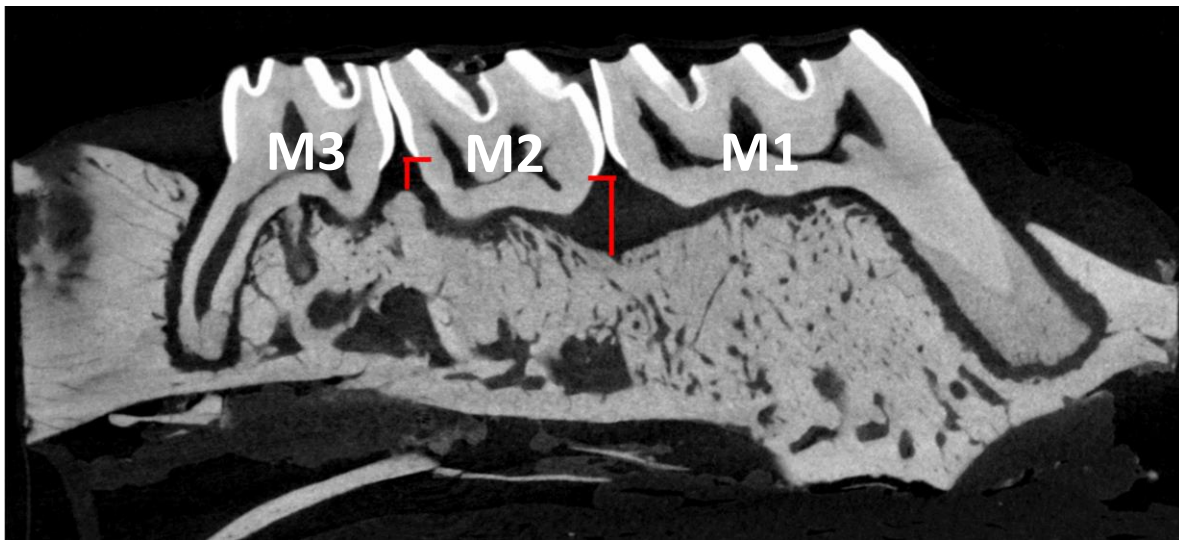


Figure 12: Horizontal line was drawn at the mesial CEJ of the second molar (M2). From the horizontal line, a perpendicular straight line was drawn to alveolar bone crest (ABC) between the first molar (M1) and M2. This perpendicular line was measured for the alveolar bone level analysis. Same measurement was done from the distal CEJ of M2 and the alveolar crest between the M2 and the third molar (M3).

3.2.2 AIM 2.2: TO DETERMINE THE GENE EXPRESSION PROFILES OF PERIODONTAL TISSUES DURING INFLAMMATION RESOLUTION AND TISSUE REGENERATION

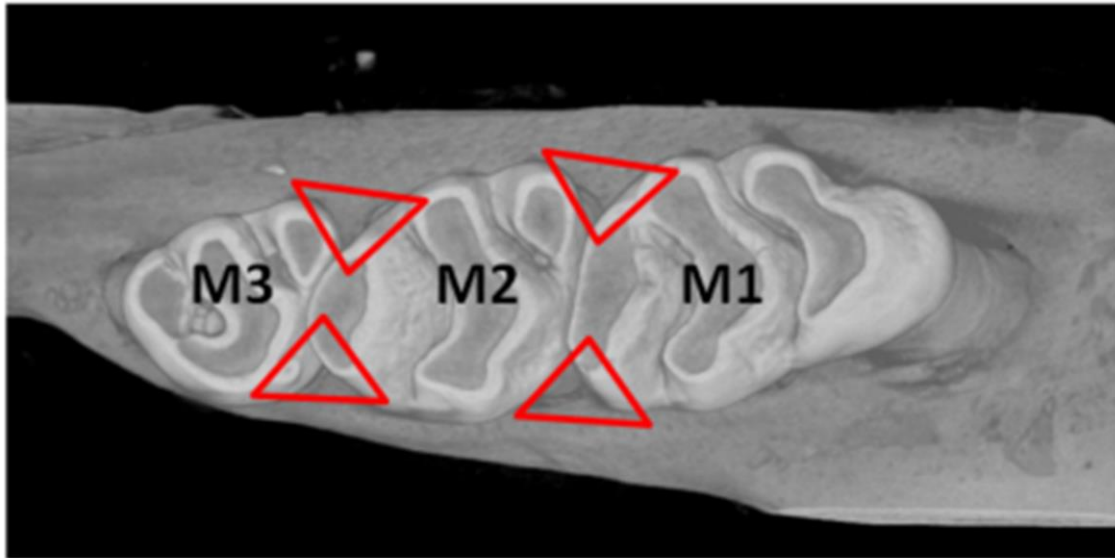


Figure 13: Periodontal Tissue was collected for the gene expression profile analysis. Red triangles show where the periodontal tissue was collected from; between M1 and M2, and M2 and M3. Collected samples were placed in 10% formalin until RNA is extracted.

Periodontal tissues between the first and second molar, and the second and third molar were collected with B12 surgical scalpel blade (Figure 13) from the baseline group (4), 3-week exosome treatment (5), 3-week control (5), 6-week exosome treatment (5) and 6-week control (5). RNA from the periodontal tissue sample was extracted with *Quick-RNA*[™] FFPE kit (Zymo Research). The tissue samples were digested overnight with twice amount of Proteinase K as suggested from the manual at 55°C. After multiple RNA isolation steps following the manual, RNA was eluted in 15µl DNase/RNase-Free Water. Concentration of the RNA was measured with NanoDrop[™] One/One^C Microvolume UV-Vis Spectrophotometer (Thermo Scientific).

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA from the extracted RNA. The volume contained ~200ng of RNA of each sample was calculated. 2.9 µl of RT master mix was prepared on ice by adding, 1.0 µl of 10X RT Buffer, 0.4 µl of 25X dNTP Mix (100mM), 1 µl of 10X RT Random Primers, and 0.5 µl of MultiScribe™ Reverse Transcriptase. Calculated volume of the RNA samples were added along with the RT master mix. If the RNA sample volume needed was larger than 7.1 µl which was the maximum amount allowed, only 7.1 µl of the RNA sample was added. Total amount all together should be 10 µl. If the total amount was smaller than 10 µl, Nuclease-free water was added to make the total amount 10 µl. Reverse Transcription (RT) reaction was performed at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA were stored at -20°C.

Target-specific preamplification was performed on the cDNA generated from the extracted RNA. 5 µl of cDNA was preamplified in a volume of 30 µl containing 1x PerfeCTa® SYBR® Green FastMix® (Quantabio) and 34 primer pairs (Supplementary Table 1), 40nM of each primer. Preamplification was performed at 95°C for 3 minutes followed by 20 cycles of amplification at 95°C for 20 seconds, 60°C for 3 minutes, and 72°C for 20 seconds and a final additional incubation at 72°C for 10 minutes. Preamplified cDNA samples were stored at -20°C. Preamplified cDNA samples were diluted 1:10 with water before performing the Quantitative real-time PCR (qPCR).

qPCR was performed in the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) using 10 µl reactions containing 5 µl of 1x PerfeCTa® SYBR® Green FastMix® (Quantabio), 400nM of each primer and 2 µl preamplified and diluted cDNA.

The same primers were used in the target specific preamplification reaction. qPCR was performed at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute, followed by a melting curve ranging from 60°C to 95°C, 2s per 0.3°C increment. The PCR products were used to run gel electrophoresis in order to validate the primer specificity. $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression. For each animal, the result from left side was normalized to that from the right side and the fold change was presented.

3.3 STATISTICAL ANALYSIS

All the calculation was done in Excel spreadsheet. The data were analyzed using unpaired t-test and one-way analysis of variance (ANOVA), followed by Turkey multiple-comparison tests. P-values less than 0.05 were considered statistical significance. Graphs for each data were prepared using Graph Pad Prism 7.02 (Graph Pad Software, CA, USA).

CHAPTER 4: RESULTS

4.1. SPECIFIC AIM 1: TO DETERMINE IF hBMSC EXOSOMES WOULD PREVENT PERIODONTAL INFLAMMATION AND ALVEOLAR BONE LOSS IN LIGATION-INDUCED PERIODONTITIS

Maxillae samples were analyzed by standardized micro-CT protocols and statistical regression. Tissue morphologies were confirmed by histological analysis. Infiltrated lymphocytes in the subepithelial tissue were quantified by histomorphometric analysis.

4.1.1. AIM 1.1: TO DETERMINE THE EFFECT OF hBMSC EXOSOMES ON ALVEOLAR BONE LOSS VIA MICRO-CT ANALYSIS

In the preventative study, periodontitis was induced on the left maxilla and the right maxilla remained healthy. The alveolar bone loss analysis was normalized to the healthy side of the same animal. Bone volume (BV) was divided by the tissue volume (TV) to calculate the percentage of the bone volume in the ROI (BV/TV). BV/TV was then normalized to the healthy side BV/TV. The normalized BV/TV in the exosome treatment group (0.68, $p < 0.05$) was significantly higher than the control group that was 0.56, showing significantly less bone loss in the exosome treatment group (Figure 14). Alveolar bone level (ABL) was measured by the distance from the CEJ to the ABC in

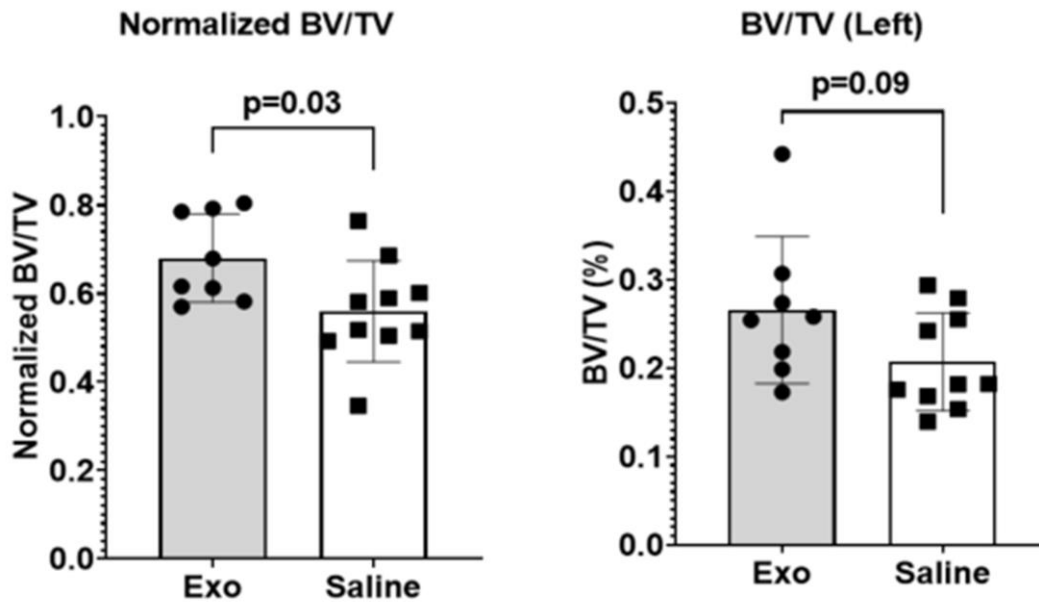


Figure 14: The volumetric measurements of the exosome treatment group were significantly larger than the control group in both the disease side data and the normalized data. Alveolar bone loss was significantly reduced in the exosome treatment group.

the interproximal space between molars (Figure 8). If the distance is longer, the ABL is lower. In opposite, if the distance is shorter, the ABL is higher which means less bone loss (Figure 15). ABL was only measured in the interproximal space because it was where the most bone loss occurred due to ligature placement as shown in Figure 16. The linear distance measurement of the disease side was also normalized to the healthy side. The normalized linear measurement in the exosome treatment is smaller than the control group (1.34 vs. 1.67, $p=0.06$), also indicating less bone loss in the exosome treatment group (Figure 17).

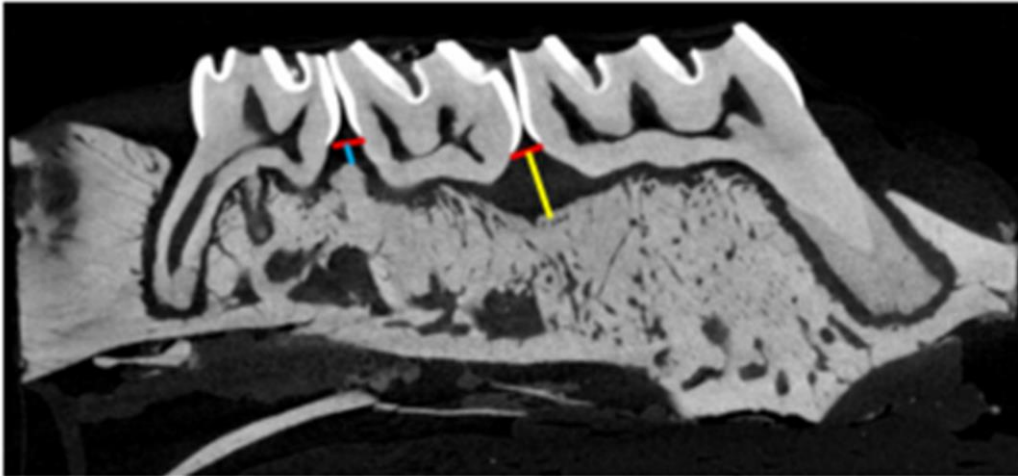


Figure 15: ABL is higher when the linear distance measurement from CEJ to ABC shown in blue line is smaller. Higher ABL means less alveolar bone loss. ABL is lower when the linear distance measurement from CEJ to ABC shown in yellow line is larger. Lower ABL means more alveolar bone loss.

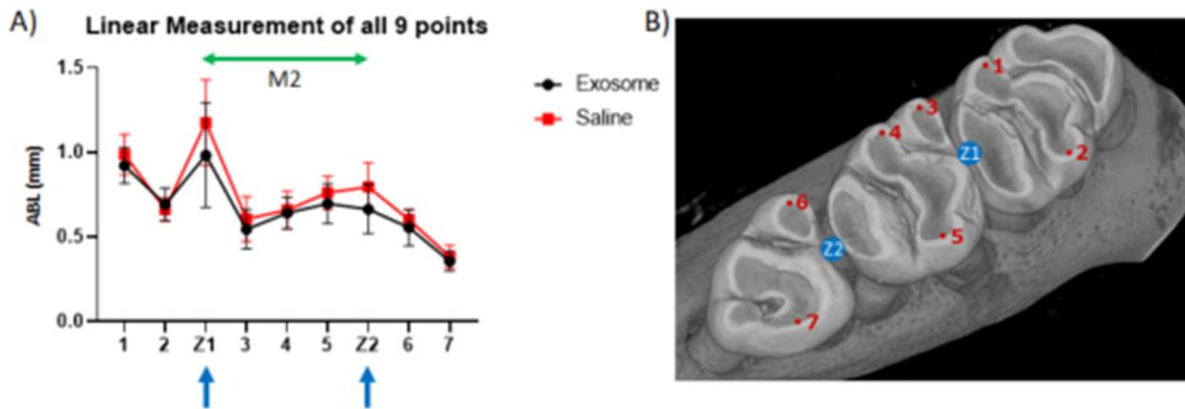


Figure 16: (B) Linear distance measurements from CEJ to ABC were done in 9 different locations. (A) Results showed that the most bone loss happens in the interproximal space, Z1 and Z2. The green arrow showed the data points at M2.

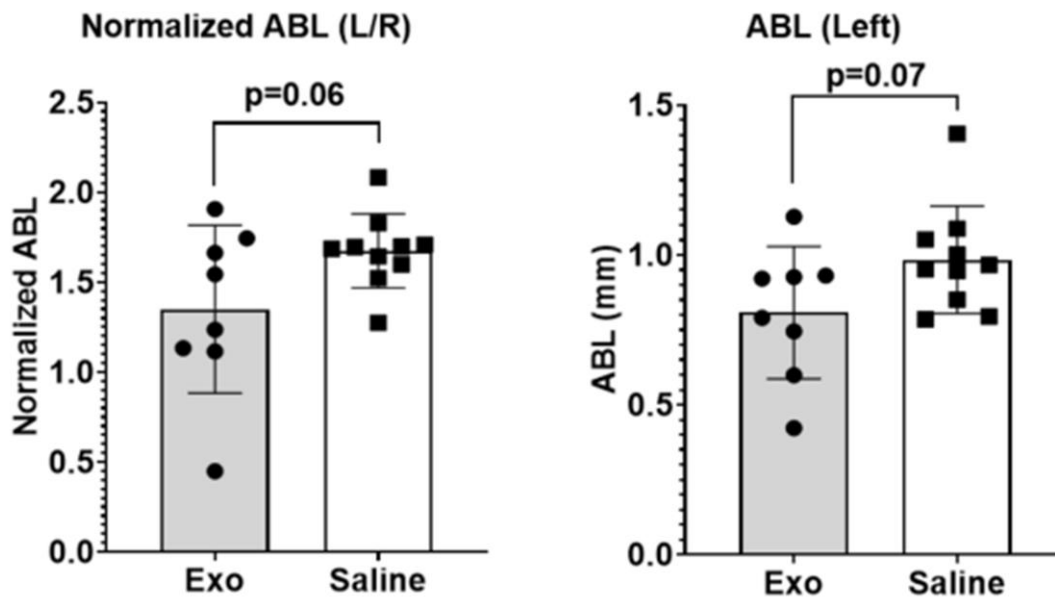


Figure 17: The linear distance measurements of the exosome treatment group were significantly smaller than the control group in both disease side data and the normalized data, denoting significantly higher ABL. Alveolar bone loss was significantly reduced in the exosome treatment group.

4.1.2. AIM 1.2: TO PERFORM HISTOLOGICAL ANALYSIS OF PERIODONTAL TISSUE FROM THE PREVENTATIVE MODEL

A histological analysis was used to provide insight of the effects exosomes may have on tissue morphology. H&E stained histology images of the control group exhibited prominent epithelial hyperplasia. In contrast, the exosome treatment group showed less increased epithelial cell proliferation (Figure18). Also, there was more severe apical migration of junctional epithelium observed in the control group than the exosome treatment group (Figure19).

Control

Exosome

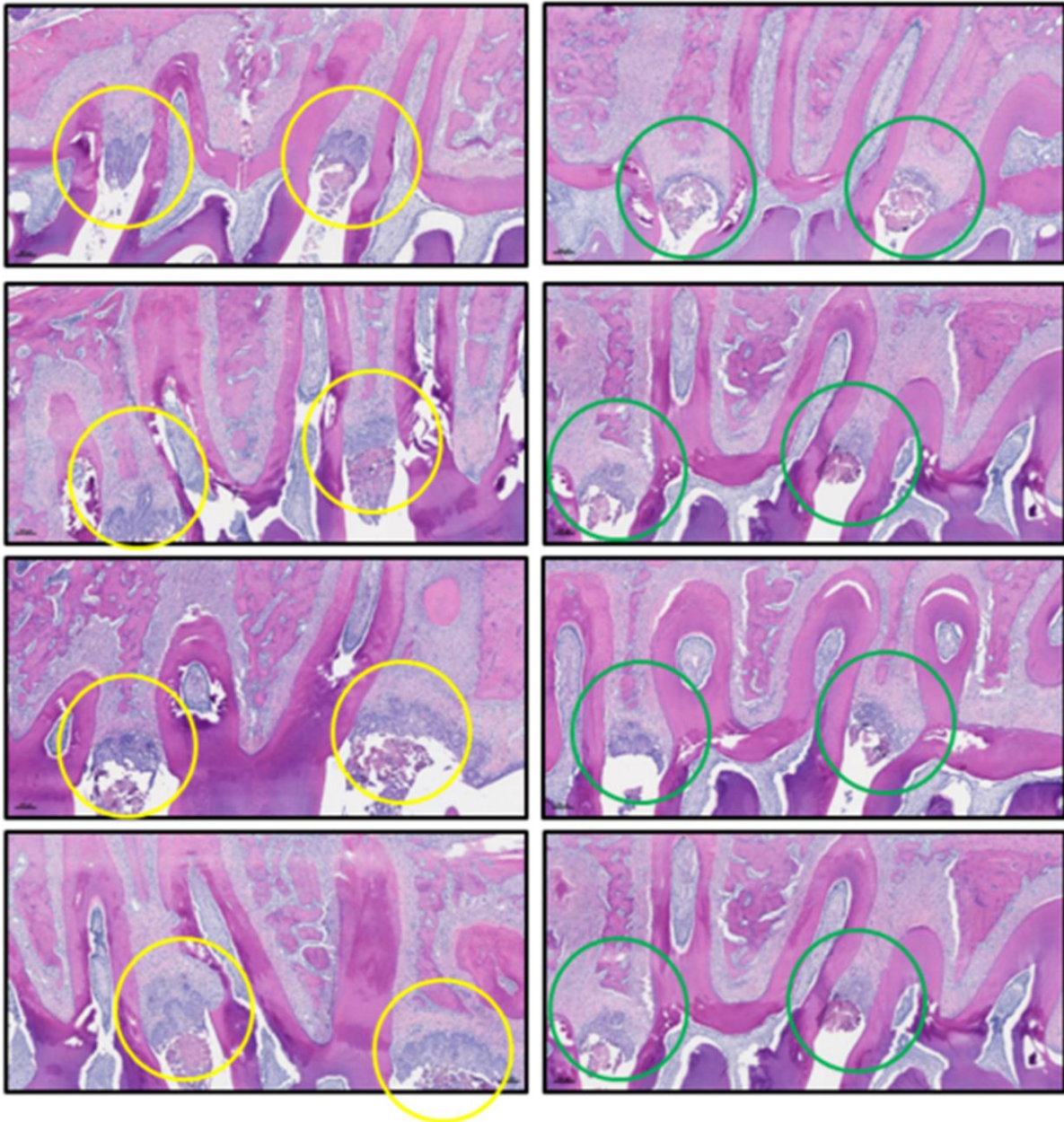


Figure 18: H&E stained histology sections showed that there were more increased epithelial cell proliferation in the control group than the exosome treatment group. The epithelial layer of control group is marked with yellow circle. The epithelial layer of exosome treatment group is marked with green circle.

Control

Exosome



Figure 19: H&E stained histology sections showed that there were more severe apical migration of junctional epithelium (JE) in the control group than the exosome treatment group. CEJ is marked with black line. Apical migration of JE of the control group is marked with yellow arrow. Apical migration of JE of the exosome group is marked with green arrow.

4.1.3. AIM 1.3: TO PERFORM A HISTOMORPHOMETRIC ANALYSIS OF INFILTRATED LYMPHOCYTES IN GINGIVAL SUBEPITHELIAL TISSUE TO DETERMINE THE EFFECT OF EXOSOME ON PERIODONTAL INFLAMMATION

Infiltrated lymphocytes in the subepithelial tissue were quantified from the 20x H&E stained histology images. Lymphocytes are mostly small in size. Their nucleus appears as dense purple and monochromatic round shape. These characteristics made it clear to identify infiltrated lymphocytes in subepithelial tissue from the H&E stained histology images (Figure 20). In average, there are 2135 infiltrated lymphocytes/mm² in the control group. Significantly less amount of the infiltrated lymphocytes was found in the exosome group, shown in Figure 21 (1686, p<0.05).

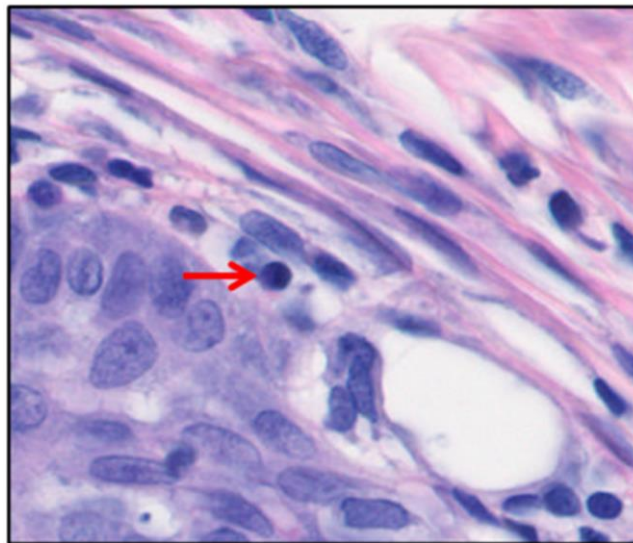


Figure 20: Red arrow indicates infiltrated lymphocyte. Lymphocytes have round dark nucleus surrounded by a narrow rim of cytoplasm. There are clear distinction between cytoplasm and nucleus in the color intensity. Most of the lymphocytes are small in size.

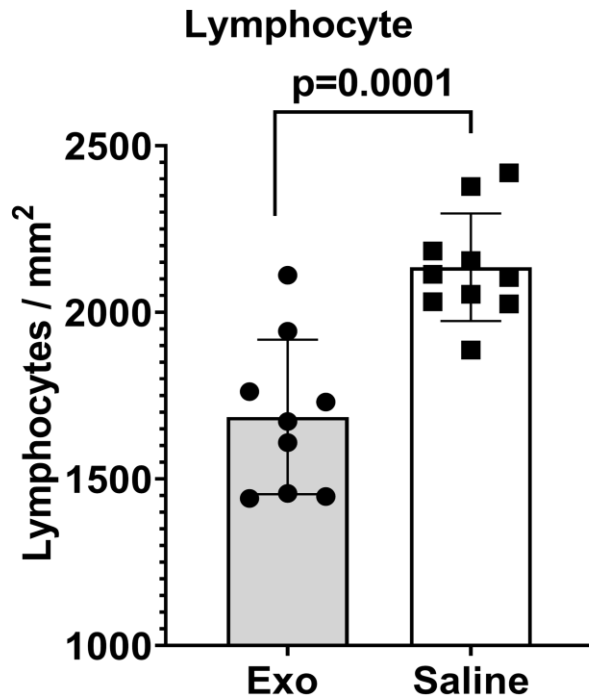


Figure 21: There are significantly less infiltrated lymphocytes in the subepithelial tissue from the exosome treatment group than the control group ($p < 0.05$).

4.2. SPECIFIC AIM 2: TO DETERMINE IF hBMSC EXOSOMES WOULD PROMOTE PERIODONTAL REGENERATION IN LIGATION INDUCED PERIODONTITIS

Maxillae samples were analyzed by standardized micro-CT protocols and statistical regression. Macrophage related gene expression was analyzed in the periodontal tissue to create a gene expression profile during bone regeneration.

4.2.1. AIM 2.1: TO PERFORM A MICRO-CT ANALYSIS OF BONE VOLUME AND ALVEOLAR BONE LEVEL TO DETERMINE THE EFFECT OF hBMSC EXOSOMES ON PERIODONTAL BONE REGENERATION

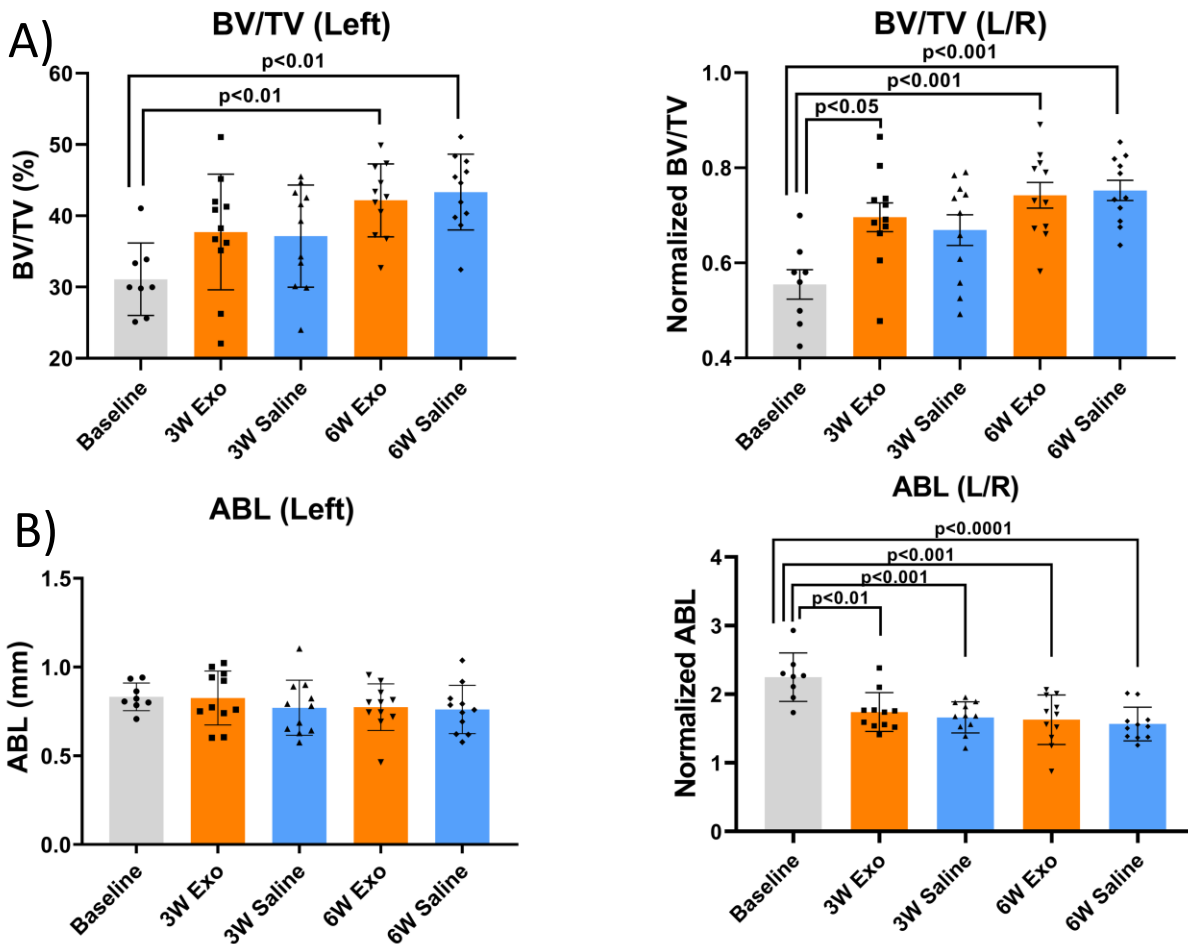


Figure 22: (A) Baseline group has significantly less normalized BV/TV than other groups: 3-week exosome treatment group, 6-week exosome treatment group, and 6-week control group. (B) Baseline group has significantly longer distance from CEJ to ABC, meaning lower alveolar bone level (ABL). P-values were indicated on the graph.

In the regenerative study, periodontitis was induced on the left maxilla and the right maxilla remained healthy. The alveolar bone loss analysis was normalized to the healthy side of the same animal. For bone volume measurement, the differences between the exosome treatment group and the control group at both 3-week and 6-week didn't reach statistical significance. Similarly there was no significant difference between the exosome group and the control group at both 3-week and 6-week in the alveolar bone level analysis. However, the baseline group had significantly less bone volume than the 3-week exosome treatment group ($p < 0.05$) and both 6-week groups ($p < 0.001$). Also, there was significantly lower alveolar bone level (after normalization to the right side) at the baseline compared to all other four groups showing the ligature placement successfully induced periodontitis. The increasing bone volume and alveolar bone level showed a natural healing progression with time. (Figure 22).

4.2.2 AIM 2.2: TO DETERMINE THE GENE EXPRESSION PROFILES OF PERIODONTAL TISSUES DURING INFLAMMATION RESOLUTION AND TISSUE REGENERATION

Periodontal tissues from the regenerative model were collected. cDNA was generated from the extracted RNA from the collected periodontal tissues. RT-qPCR was done to determine the expression of genes related to inflammation activation and resolution. A panel of 34 genes was selected and primers targeting these genes were designed (supplementary table 1). The transition of macrophages from M1 phase to M2 phase is a keystone step in the healing process. Due to time limitation, the expression of one gene from each M1 and M2 gene pool were analyzed. Each gene (TNF α and TGM2) was selected after the validation of the specificity and efficiency of primers by gel electrophoresis (Figure 23) and RT-qPCR. Both TNF α and TGM2 gene expression

didn't show any significant trend during the bone regeneration process (Figure 24) due to the small number of sample size (n=4 or 5).

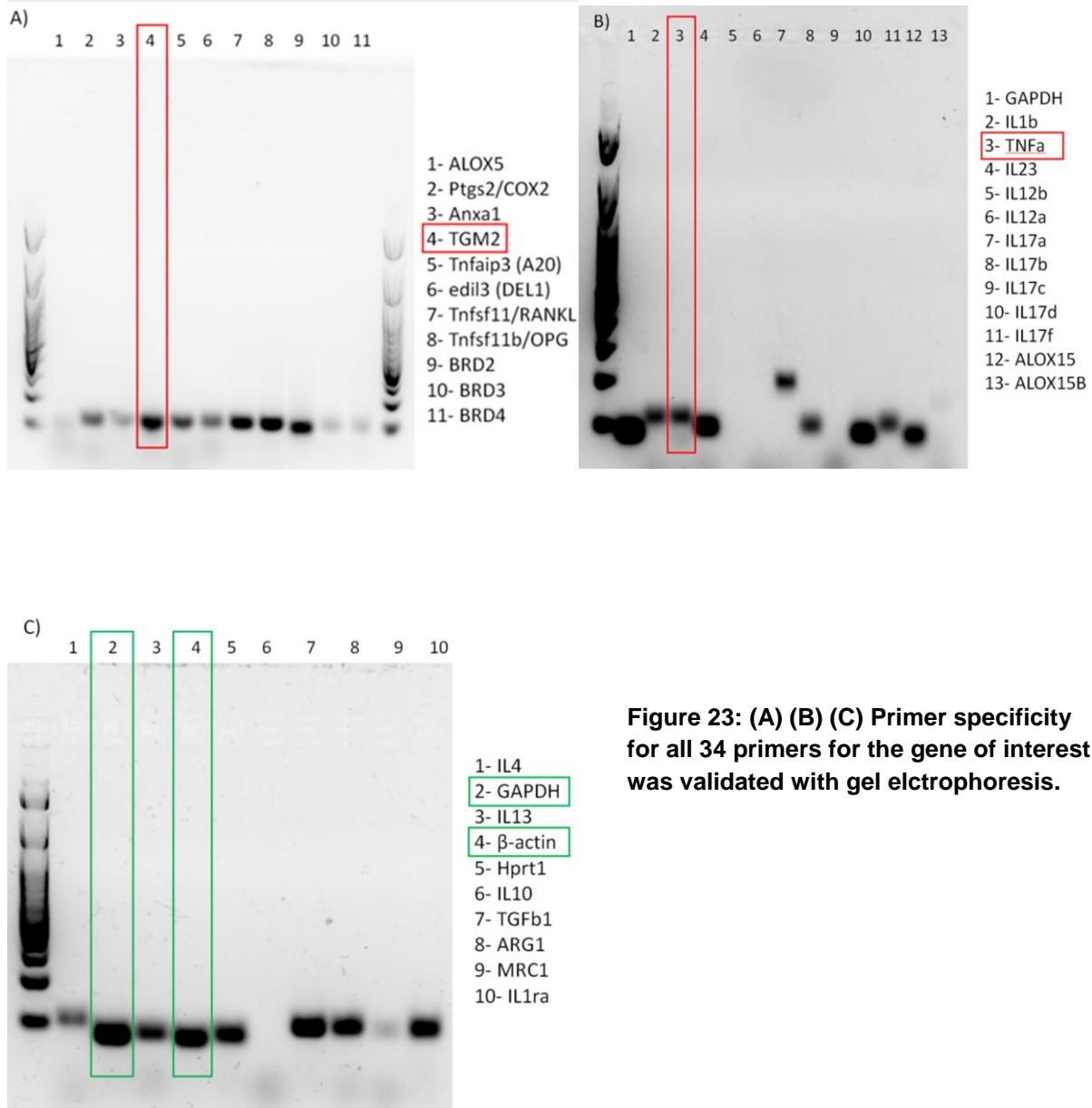


Figure 23: (A) (B) (C) Primer specificity for all 34 primers for the gene of interest was validated with gel electrophoresis.

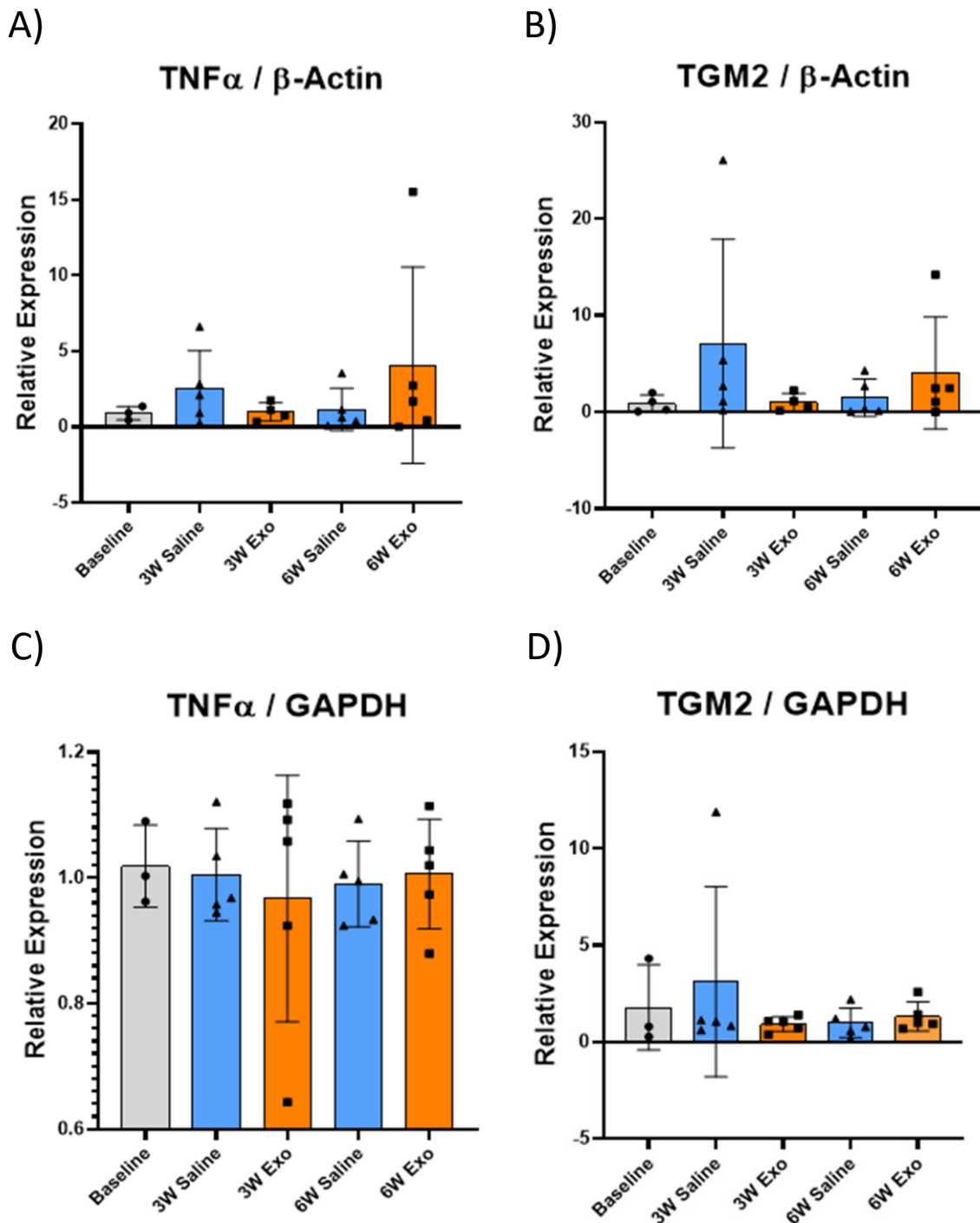


Figure 24: $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression. (A) TNF α expression was normalized to β -Actin, (B) TGM2 expression was normalized to β -Actin, (C) TNF α expression was normalized to GAPDH, (D) TGM2 expression was normalized to GAPDH. (A)(B)(C)(D) Disease side was normalized to the healthy side.

CHAPTER 5: DISCUSSION

In the past decades, exosome therapy has received a lot of attention due to its ability to regulate cell and tissue function in the regeneration process. Studies have shown that exosomes are osteogenic and anti-resorptive in bone disease (88-90). Periodontal disease is caused by the accumulation of bacteria which activates the inflammatory response. The continued presence of bacteria results in the destruction of periodontal tissue including alveolar bone (91). In this study, we hypothesized that hBMSC exosomes prevent alveolar bone loss and have regenerative potential in diseased periodontium.

In the preventative study, we found that hBMSC exosomes reduced periodontal inflammation and alveolar bone loss. We were able to set up a standard Micro-CT protocol for rat maxillae samples, and therefore reliable and reproducible data were generated. Bone volume and alveolar bone level analysis showed the reduction of the alveolar bone loss after the exosome treatment. This suggests that the exosomes have a preventative effect on the alveolar bone, which is similar to what was shown in other degenerative bone diseases (88). In healthy periodontium, junctional epithelium is attached onto enamel and protects the tooth surface. With the invasion of bacterial pathogenic components, junctional epithelium is detached from the enamel and migrates down to the root, which creates pockets (92). Also, the epithelial tissue interacts with bacterial pathogens through TLRs producing cytokines and proteinases which lead to epithelial proliferation and migration into the inflamed area (93, 94).

Imbalanced immune response to the consistent bacterial insults leads to the destruction of periodontal tissue, which is associated with the infiltration of lymphocytes into the connective tissue (95). We observed lesser extent of epithelial cell proliferation and apical migration and reduced amount of infiltrated lymphocytes in the subepithelial tissue after the exosome treatment. Overall, this preventative study demonstrated the possibility of exosomes to be a protective agent for periodontal disease with the ability of regulating inflammatory response.

Histomorphometric analysis was used instead of immunohistochemistry to quantify the infiltrated lymphocytes in this study because we encountered technical difficulties finding antibodies for lymphocytes. We can continue to try different antibodies to detect and visualize the lymphocytes in the future study to confirm the findings in our current study.

In the regenerative model, our data didn't support that hBMSC exosomes promote the alveolar bone regeneration. Many factors may be associated with the results. For example, the dosage of exosomes should be further explored. We observed the efficacy of the exosomes in the preventive study. The main difference between the two models is the timepoint of the injection of hBMSC exosomes. Administering of hBMSC exosomes starting at the healthy stage or the early stage of immune response may have been enough to suppress the inflammatory response and subsequently prevent alveolar bone destruction, however, higher exosome concentration may be needed to enhance the tissue repair in the presence of severe periodontitis. The fact that the regenerative effect of exosomes is dose dependent has also been shown in other bone diseases (96-98). Also, different methods to deliver the exosomes can be tested in the

future. In this study, we used subgingival injection because it is easy and less invasive. However, the amount of exosomes that can be delivered through this method is limited due to the small injection volume (~2 µl/site). Excessive liquid volume will lead to tissue necrosis. It is also not clear how long the exosomes can stay at the disease site after the injection. Other studies also indicated the importance of delivery strategy. Chew et al. demonstrated that exosome loaded in collagen sponge promoted regeneration potential (98).

The self-regeneration of periodontium was appreciated in our regenerative model, which was shown by the increased BV/TV values at 6-week comparing to baseline. This phenomenon has also been documented in literature (99, 100). To explore the important genes that are involved in the inflammation resolution and tissue regeneration process, we designed a panel of primers targeting genes involved in the transition of macrophages (M1-M2), key enzymes in the metabolism of lipidic inflammation resolution products, cytokines and growth factors. Periodontal tissue was collected from the formalin fixed maxillae sample to extract RNA. RNA integrity was assessed prior to further process. In order to obtain reliable data from the downstream research process, RIN number usually should be above 7. Our extracted RNA showed RIN numbers between 2 and 3, indicating that formalin fixation broke down RNA molecules and modified RNA. The poor RNA quality might contribute to the large variance that we saw in the PCR results. Other studies suggested that using formalin free fixative, such as PAXgene Tissue System (101) and cryopreservation (102), can improve the RNA quality to enhance the comparability and reliability of RT-PCR experiments. Even

though our preliminary gene expression results didn't lead us to any conclusion yet, we've demonstrated the feasibility of our strategy for future studies.

CHAPTER 6: CONCLUSION

Current traditional periodontal disease treatments focus mainly on mechanical debridement. Adjunctive treatments such as systemic antibiotics are associated with serious complications and antibiotic resistance. It is important to direct route of our journey in developing future preferential therapy to harness the host immune system modulation strategy since the destruction of periodontal tissues was done directly by host immune response. Cell-based treatments using MSCs are thought to be an alternative novel therapy due to its pleiotropic effects. Problem of scarcity and possible oncogenic potential with utilizing MSCs can be solved with hBMSC exosomes as they convey the regenerative factors including specific nucleic acids and proteins from parental cells.

Our study evaluated the protective and regenerative potential of hBMSC exosomes in the application of periodontal regeneration in ligature-induced experimental periodontitis in rats. hBMSC exosomes significantly reduced periodontal inflammation and alveolar bone loss in an experimental periodontitis model. When applied as a therapeutic agent, hBMSC exosomes did not show statistically superior effects compared to control. Interestingly, natural inflammation resolution and tissue regeneration was observed in the study, which may be worthwhile for further research to understand the underlying mechanism. Overall, this study suggested that hBMSC exosome may be a key agent as a host modulation therapy for periodontitis.

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SUPPLEMENTARY DATA

Supplementary Table 1. Primers used for RT-qPCR detection in this study

	Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')	Product Length	Tm (C)	
Housekeeping Gene	GAPDH	AGTGCCAGCCTCGTCTCATA	GGTAACCAGGCGTCCGATAC	77	60	
	Actin-b	CGCGAGTACAACCTTCTTGC	CGTCATCCATGGCGAACTGG	70	60	
	Hprt1	TCCTCCTCAGACCGCTTTTC	ATCACTAATCACGACGCTGGG	78	60	
M1: pro-inflammatory	IL1b	TAGCAGCTTTCGACAGTGAGG	TCTGGACAGCCCAAGTCAAG	100	60	
	IL6	CATTCTGTCTCGAGCCCACC	GCTGGAAGTCTCTTGCGGAG	91	60.5	
	TNFa	ATGGGCTCCCTCTCATCAGT	TGGTTTGCTACGACGTGGG	100	60	
	IL23	AAAAGTGACGTGCCCGTAT	TGCAAACAGAACTGGCTGTTG	80	60	
	IL12b	GAGGCCAGCAGCAGAATAA	GGTGGGTCCGGTTTGATGAT	77	59	
	IL12a	GCCTGCTTACCACTGGAAGT	GCAGGCAGCTCCCTCTTATT	85	59	
	IL17a	GGAGAATTCATCCATGTGCC	ATGAGTACCGCTGCCTTAC	76	60	
	IL17b	CACACAGCCTGCTCTTCCTT	GCCCCTTCTTTTGCCTTTG	84	60	
	IL17c	ACTGCCACTGTGATGTGTCT	AATGGGGGATCTTGGTGGGT	71	60	
	IL17d	CCCCACCTCCTAACAAAGTCTG	GAGGGCATCCAGACCAGTGTG	70	60	
	IL17f	TTCTATTGCGTGACCCGGAG	GGAGCCCTTCATGCTGGATT	87	60	
M2: inflammation resolution	IL10	TGCGACGCTGTCATCGATTT	AGACACCTTTGTCTTGGAGCTT	96	60	
	TGFb1	CTGCTGACCCCACTGATAC	AGCCCTGTATTCCGTCTCCT	94	60	
	ARG1	ACAAGACAGGGCTACTTTTCAGG	TGCCGTGTTACAGTACGAG	95	60	
	MRC1	TCAACTCTTGGACTCACGGC	GAACGGAGATGGCGCTTAGA	83	59	
	IL1ra	TCCTTCTCATCCTTCTGTTTTCGT	CCCAGATTCTGAAGGCTTGC	87	59	
	IL4	AACAAGGAACACCACGGAGA	TGTTCTTCAAGCACGGAGGT	98	59	
	IL13	GATCCACATCTCCCCCTGTG	GGGAAGTCTTCTGGTCTTGTGT	78	59	
	TGM2	GTTCTGAAGGACCGTAGCC	CATTGCAGTTGACCATGCCG	89	60	
	Anxa1	GGAAGACAAGGCAATACAAAGATAC	ACATATTCCTGCTCTTGCTTTTCA	100	58	
	Tnfaip3/A20	TGCTACGACACTCGGAACTG	GTAAGTCCACTCCGGG	93	59	
	ALOX15	GGTTGGTTGGACAGCATGGA	AATTCTGCTTCCGAGTCCCG	73	60	
	ALOX15B	GACCGTGAAAGACTTGGACCT	TCAGCTCTGTAACGCAGACT	92	59	
	ALOX5	GGCGGTCGCGACTCCTAT	GCCTGTATTTGCGCTTCTCG	85	60	
	Edil3/Del1	CTGTCAGGATTGGCGGATGA	TCTGATGCAACCTCCACAACA	92	59	
	Ptgs2/Cox2	TCCCATGGGTGTGAAAGGAAA	CTTGGGGATCCGGGATGAAC	95	60	
	Osteoclast Differentiation	Tnfsf11/RANKL	GAGCGCAGATGGATCCTAACAA	GTCGAGTCTGCAAACTGT	97	60
		Tnfrsf11b/OPG	AGAGAAGCACCTAGCACTGG	GTCCAAGAACCAGGAGTG	99	59
BET	BRD2	GTGACTCCCCACAAGCTCC	ATCCTTTTCCCTGGTGCTGC	83	60	
	BRD3	CTAGCGTCTCAGAGTGCCCT	TCTCAATCTTGTCCAGGGC	82	58	
	BRD4	CTAGCGTCTCAGAGTGCCCT	TCTCAATCTTGTCCAGGGC	82	59	

VITA

Jin Ha Kim was born in South Korea. She graduated from Deep Run High School in Glen Allen, VA in 2006, and matriculated from University of Virginia in Charlottesville, VA in 2011 with a B. Sc. in Biology. Jin began the Premedical Graduate Health Sciences Certificate Program at Virginia Commonwealth University in 2018. Through this program, she started research for his Masters in Physiology and Biophysics degree, with an anticipated matriculation date of April 2020.