Osteogenic potential of periodontal ligament stem cells and immortalized cementoblasts under mechanical vibration forces

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Directed by Professor Euiseong Kim

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ABSTRACT

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After tooth replantation or transplantation, application of occlusal forces is capable to promote periodontal ligament (PDL) healing and prevent ankylosis. However, previous studies regarding the effect of occlusal forces on PDL healing were lack of biological mechanisms due to the insufficient *in vitro* study and uncertain *in vitro* model. Therefore, based on the acknowledge that

mechanical vibration force can imitate occlusal forces, the mechanical vibration machine with four given frequencies as an *in vitro* model to assess the osteogenic differentiation potential of periodontal ligament stem cells (PDLSCs) and immortalized cementoblasts (ihCEMs) at different time points have been established. In this work, cell viability assay showed that both PDLSCs and ihCEMs had the adverse effect in the early time but were resistant to mechanical vibration stimulation (MVS) at different frequencies in the long run. Moreover, the osteogenic gene expression indicated that MVS has a transient and frequency-dependent effect on the osteogenic potential of PDLSCs while it has no influence on ihCEMs at each frequency and each time point. Interestingly, 150 rpm MVS could decline osteogenic differentiation in the early time and have no harmful effect in the long run. For further confirmed, the relative *in vivo* experiment revealed that PDLSCs cell sheet treated with 150 rpm MVS presented ligament-like tissue healing pattern and had decreased new bone formation in rat calvarial bone defect. In conclusion, it is found that MVS at proper frequency (150 rpm) can prove to be a potential strategy for transiently reduced PDLSCs osteogenic potential, which sheds light on the approach that 150 rpm MVS could be utilized for the prevention of ankylosis and promotion of PDL healing after tooth replantation or transplantation in future.

Keywords: mechanical vibration force, periodontal ligament stem cell, cementoblast, osteogenic differentiation, cell sheet, bone formation

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I. INTRODUCTION

Periodontal ligament (PDL) remodeling is an important procedure during orthodontic tooth movement (OTM) or after tooth replantation and transplantation to achieve PDL homeostasis (Andreasen et al. 1981; Lim et al. 2015; Meikle et al. 2006). The break of PDL homeostasis will lead to periodontal pathologies, such as root resorption or tooth ankylosis (Yang et al. 2012).

Previously, accumulating evidence showed that the presence of mechanical forces, such as occlusal forces or orthodontic forces, are able to distinctively reduce the occurrence of periodontal

pathologies. Andersson et al indicated that the monkeys exposed to hard diet underwent less ankylosis than that with soft diet (Andersson et al. 1985). Similarly, Mine et al reported that in rats model, application of occlusal force decreased the risk of ankylosis (Mine et al. 2005). Moreover, Yang et al pointed out that pre-application of orthodontics force might promote PDL healing (Yang et al. 2012). Whereas, some studies suggested excessive forces might lead to pathogenic bone resorption (Andreasen et al. 1981; Mine et al. 2005).

Based on the aforementioned results, mechanical force stimulation seems to be an indispensable factor in PDL healing. However, these reported animal studies were either hard to identify the precious information such as types and intensities of these reported mechanical forces or explain the biological mechanism of periodontium tissue response to mechanical force. Therefore, it is essential to build up an *in vitro* model to go into the depth that which is the proper mechanical force and how this mechanical force work on PDL remodeling.

From a molecular perspective, the osteogenic activity of periodontal ligament stem cells (PDLSCs) and cementoblasts (CMs) plays a key role in PDL remodeling, such as remaining the width of PDL space (Andreasen et al. 1981; Nemoto et al. 2009; Wu et al. 2019). From a cellular point of view, both PDLSCs and CMs are a population of mechanoresponsive cells with osteogenic differentiation capacity (Lindskog et al. 1985; Pavlin et al. 2001). Numerous studies indicated that mechanical forces stimulation might up-regulate osteogenic gene expression of PDLSCs and CMs, such as runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OCN) (Huang et al. 2009; Wescott et al. 2007; Yu et al. 2009; Zhang et al. 2012). On the other hand, some studies indicated that the alteration of osteogenic potential of PDLSCs and CMs by mechanical stimulation are transient and frequency-dependent (Yu et al. 2009; Zhang et al. 2012). Overall, this evidence suggested that the osteogenic differentiation potential of both cells was susceptible to mechanical stimulation. However, these reported results were inconsistent because of the use of varied

mechanical protocols. Nowadays, various *in vitro* mechanical loading models for imitating mastication or orthodontic treatment has been reported (Zhang et al. 2012; Yang et al. 2015; Leethanakul et al. 2018). Among them, mechanical vibration stimulation (MVS) can be a system to mimic the conditions under which mastication occurs (Yang et al. 2015). Moreover, some studies pointed out that MVS have the frequency-dependent effect on osteogenic activity in PDLSCs (Leethanakul et al. 2018; Zhang et al. 2012). Taking all these factors into account, studying the osteogenic differentiation potential of PDLSCs and CMs under an MVS model can help better understand the effect of MVS to specific periodontium cells during PDL remodeling.

Motivated by this, in this study, I attempted to use the MVS with different frequencies (30 rpm (0.5 Hz), 90 rpm (1.5 Hz), 150 rpm (2.5 Hz) and 210 rpm (3.5 Hz)) as an *in vitro* model to assess the alteration of osteogenic differentiation capacity of PDLSCs and immortalized cementoblasts (ihCEMs) at different time points. The proliferation ability and osteogenic differentiation potential of PDLSCs and ihCEMs were evaluated at the cellular levels. More relevantly, *in vivo* bone formation was analyzed by transplantation of PDLSCs cell sheets into rat calvarial defects model according to the *in vitro* study results.

II. MATERIALS AND METHOD

1. Isolation, Culture and Characteristics of PDLSCs and ihCEMs

Primary PDLSCs were obtained from healthy patients who were scheduled for third molar extraction or premolars extraction for orthodontic reasons at Yonsei University Dental Hospital, Seoul, South Korea. The study was approved by the Institutional Review Board of Yonsei University Dental Hospital (Institutional Review Board number: 2-2017-0009). Informed consent was obtained from each patient before extraction. After extraction, freshly extracted teeth were rinsed with Dulbecco's modification of Eagle's medium (DMEM, Gibco, New York, NY, USA) supplemented with 3% Penicillin-Streptomycin (PS, Gibco). The PDL tissue attached to the mid-third of the root surface was gently scraped off, cut into small pieces and washed 2 times with wash medium (DMEM with 3% PS). Minced tissues were seeded in cell culture dishes with complete medium (DMEM with 1% PS and 10% Fetal Bovine Serum (FBS, Gibco)) in a humidified atmosphere of 5% CO_2 at 37°C. Until subconfluence, cells were passaged at regular intervals depending on their growth characteristics using TrypLE^{TM} Expression (Gibco). PDLSCs between passages three and four were used in this study. Immortalized cementoblasts (ihCEMs), which obtained from Department of Histology, Yonsei University Dental College, were isolated and cultured using the method described by Kitagawa et al (Kitagawa et al. 2006). The cells were cultured in Minimum Essential Medium Eagle - Alpha Modification (α-MEM, Gibco), containing 1% PS and 10% FBS as the complete medium at 37°C in a humidified air with 5% CO_2. The cell surface marker expression of the PDLSCs were determined by flow cytometry (FACS verse; BD Biosciences, Franklin Lakes, NJ, USA) using BD Stem flow human MSC analysis kit (BD Biosciences). The expression of MSC surface markers (CD45, CD73, CD90 and CD105) were analyzed by v.10.0 FlowJo software (FlowJo, BD Biosciences).

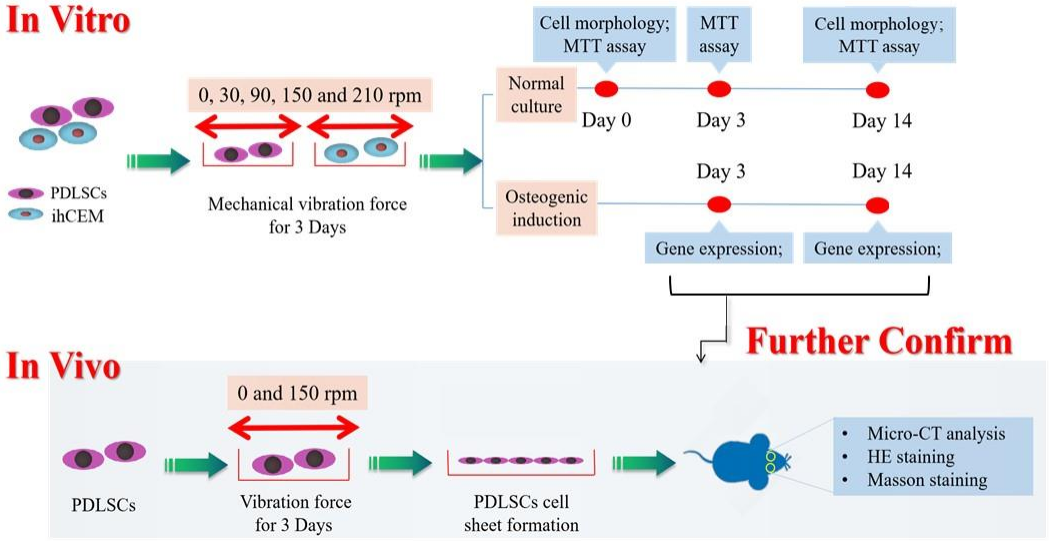


Figure 1. Study design. The whole experiment procedure of this study.

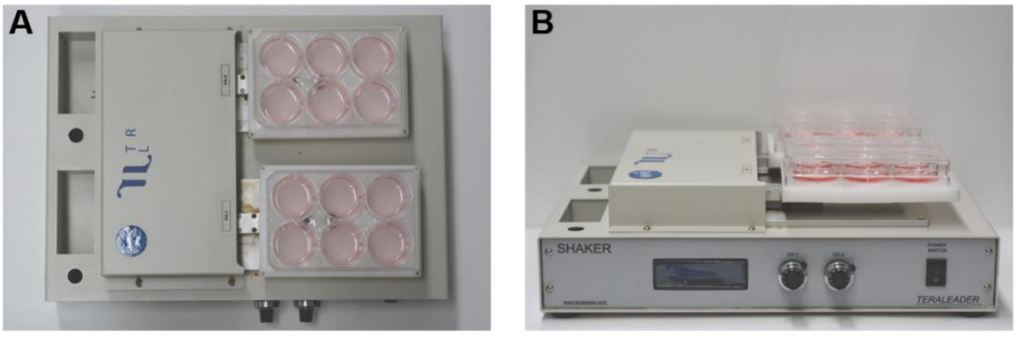
2. Induction of Osteogenic Differentiation

PDLSCs and ihCEMs were seeded in 12-well plates at a density of 5×10^4 cells per well and incubated in complete medium until they reached 80%-90% confluence. Next, cells were incubated in the osteogenic induction medium (complete medium supplemented with 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA)), 1.8 mM KH2PO4 (Sigma-Aldrich), 10 mM B-Glycerol phosphate and 100 nM Dexamethasone (Sigma-Aldrich) for 3, 14, 21 days, respectively. Cells maintained in complete medium were as non-osteogenic induction group and medium were replaced at 2-day intervals. Subsequently, to evaluate the osteogenic potential of PDLSCs and ihCEMs, alkaline phosphatase (ALP) staining was performed by the ALP staining Kit (Sigma-Aldrich) after 3 days and 14 days osteogenic induction. In addition, after 14 days and 21 days induction, mineralization was assessed by Alizarin Red staining with Alizarin Red S solution

(ACROS ORGANICS, Somerset, NJ, USA) according to the manufacturer's instructions. Images were scanned by microscope (Olympus BX43, Tokyo, Japan).

3. Application of Mechanical Vibration Stimulation to PDLSCs and ihCEMs

Prior to mechanical stimulus, cells were incubated overnight in complete medium in order to promote cell attachment. 24 hours later, the medium was changed to fresh complete medium. Subsequently, for stimulating PDLSCs and ihCEMs with mechanical vibration forces, six-well plates cultured with PDLSCs and ihCEMs were placed on the platform of a sample shaker machine (Teraleader, Daejeon, Korea) (Fig. 2). The mechanical vibration stimulations at different frequencies (30 rpm, 90rpm, 150 rpm and 210 rpm) for 3 days were indicated as MVS treated groups. Cells without mechanical vibration stimuli were regarded as no-MVS treated group. All of the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells morphology in each group was identified by light microscope (Nikon, Tokyo, Japan) on day 0 and days 14 after mechanical loading (Fig. 1).



Front View Lateral View

Figure 2. View of mechanical vibration machine. Six-well plate with cells in complete medium was placed on the vibratory transducer. (A) Front view of mechanical vibration machine. (B) Lateral view of mechanical vibration machine.

4. Cell Viability Assay

Cell viability of PDLSCs and ihCEMs on days 0, 3 and 14 after mechanical loading were assessed using MTT assay (Thiazolyl blue tetrazolium bromide; VWR, Radnor, PA, USA), respectively (Fig. 1). Briefly, PDLSCs and ihCEMs were cultured in 6-well plate at a density of 1×10^5 cells per well. On days 0, 3 and 14 after mechanical loading, MTT was dissolved with phosphate buffered saline (PBS), added to each well with a final concentration of 0.5 mg/mL and incubated for 2 hours at 37°C. The formazan product was generated then solubilized with dimethyl sulfoxide (DMSO, VWR). The cell viability was assessed as OD values measured at 570 nm on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

5. Total RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For RT-PCR, PDLSCs and ihCEMs were seeded at 1×10^5 cells per well in 6-well plates. After mechanical loading, cells were replaced with osteogenic induction medium which were supplied every 2 days. Total RNA was isolated from cells using RNeasy mini kit (Qiagen, Hilden, Germany) on days 3 and 14 of osteogenic induction, respectively (Fig. 1). cDNA was generated from 1000 ng extracted RNA using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific)

according to the manufacturer's instructions. RT-PCR was performed with Quantstudio 3 System (Applied Biosystems, Waltham, MA, USA) using the Taqman gene expression assays (Applied Biosystems). Beta-actin (hs01060665 β-actin) was used as endogenous control. Runt-related transcription factor 2 (hs01047973_ma Runx2), Alkaline phosphatase (hs00758162_ma ALP), Osteocalcin (hs01587814_g1 OCN) was used as read-out of osteogenesis. Reactions were performed in triplicate and relative changes to housekeeping gene were calculated by the $2^{-\Delta\Delta Ct}$ method.

6. Formation of PDLSCs Cell Sheet

PDLSCs were seeded onto 35 mm dishes (5×10^5 cells/dish) and cultured in complete medium. After 24 hours, MVS at 150 rpm frequency was applied on PDLSCs for 3 days. Cells without mechanical stimuli were regarded as no MVS treated group. After mechanical loading, the medium was replaced with an osteogenic induction medium that described as before. Medium was replaced every 2 days. After 3 days of incubation, the formed cell sheets were detached as presented at Figure 3 (Fig. 3A-3D).

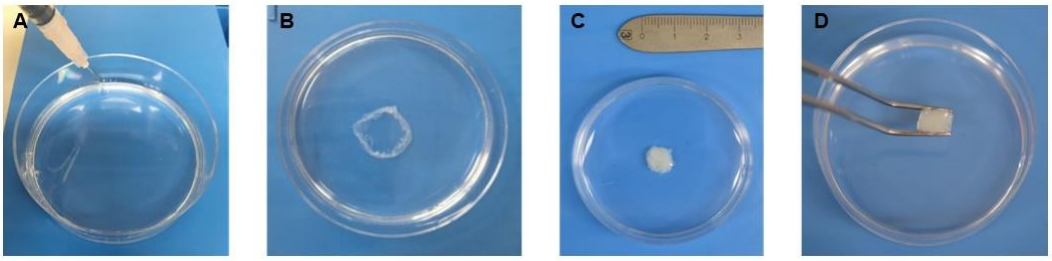


Figure 3. The production of PDLSCs cell sheets. (A-D) The process of PDLSCs cell sheet production. (A) Isolation of the PDLSCs cell sheets with needle. (B) Morphology of monolayer

PDLSCs cell sheet. (C) Morphology of double layers PDLSCs cell sheets (D) Double layers PDLSCs cell sheets can be picked up by forceps.

7. Transplantation of PDLSCs Cell Sheets into Rat Calvarial Defects

All animal experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Yonsei University, and were approved by the committee (protocol number 2019-0185).

In total, 6 female Wistar rat (8-week-old, weighing 200-230 g) were used to establish a rat calvarial bone defects. The rats calvarial defects were divided evenly and randomly into three groups (n = 4 per group): (1) negative control group: defects left untreated; (2) no-MVS treated group: defects treated with PDLSCs cell sheets without MVS; (3) 150 rpm MVS treated group: defects treated with PDLSCs cell sheets with 150 rpm MVS. The calvarial defect model was established as follow (Fig. 4A-4D). The rats were anaesthetized with 3% isoflurane (Hana Pharm, Seoul, Korea) delivered in 100% oxygen and further anaesthetized by intraperitoneal injection with rompun (Bayer Korea, Seoul, Korea). After that, a 1.5-2 cm incision in the midline sagittal direction was made to expose the cranium (Fig. 4A). Then bilateral cranial bone defects (diameter, 5 mm) were created (2 defects/rat) using a low-speed dental engine with trephine bur (Fig. 4B-4C) then were transplanted with PDLSCs cell sheets (Fig. 4D). The periosteum was re-positioned and sutured with 4-0 absorbable sutures; the external incision was closed with 3-0 absorbable sutures. Rats were sacrificed 15 weeks post-surgery. The collected samples were fixed with 4% paraformaldehyde (Sigma-Aldrich) and prepared for Micro computed tomography (Micro-CT) analysis and histological staining.

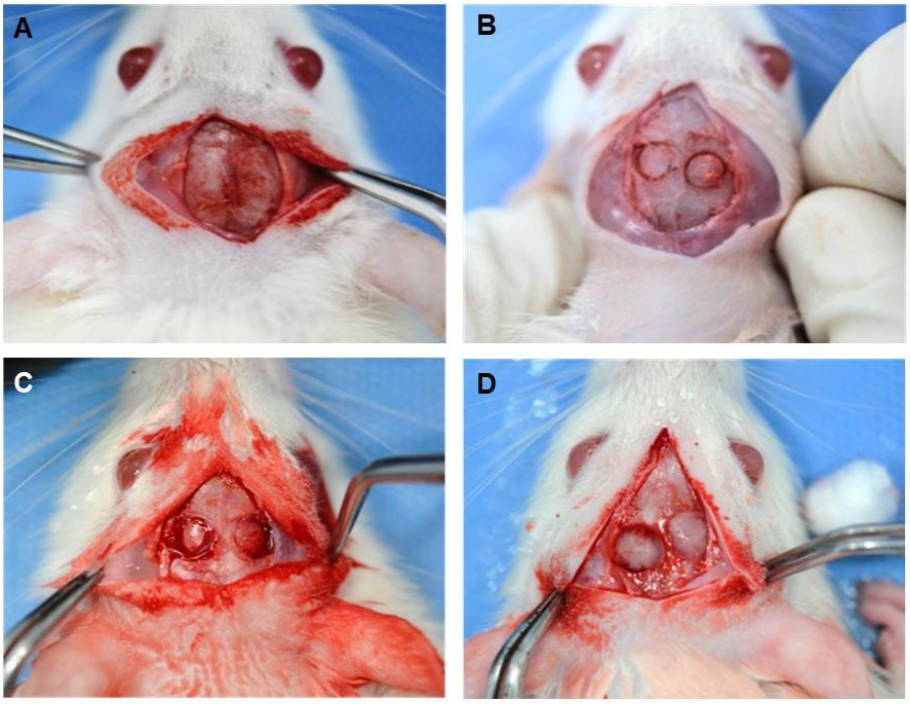


Figure 4. The transplantation of PDLSCs cell sheets into the rat calvarial defects. (A-D) Surgical procedure of transplantation PDLSCs cell sheets into rat calvarial defects. (A) The rat calvarial bone was exposed. (B) The 5 mm defects were created by trephine bur bilaterally. (C) The dura and brain beneath the defects were exposed. (D) PDLSCs cell sheets were placed on the defects; no-MVS treated group (left); 150 rpm MVS treated group (right).

8. Micro Computed Tomography (Micro-CT) Analysis

To evaluate the bone formation at the calvarial defects sites, specimens (n = 4 for each group) were scanned using a high-resolution Micro-CT scanner (SkyScan 1173, Bruker, MA, USA). After Micro-CT scan, the visualization of bone was made with three-dimensional reconstruction. The

measurements of Micro-CT involved the percentage of bone volume relative to total volume (BV/TV) and trabecular thickness (Tb.Th) by Image J software (U.S. National Institutes of Health, Bethesda, MD, USA).

9. Histological Evaluation

After micro-CT scanning, specimens from rats (n = 4 for each group) were then decalcified in phosphate-buffered saline containing 5% EDTA (Duksan, Gyeonggi-do, Korea) and 4% sucrose (JUNSEI, Tokyo, Japan) (pH = 7.4) at room temperature for 10 days, with a solution change every 3 days. Thereafter, the decalcified specimens were embedded in paraffin using standard procedures. All wax blocks were sectioned serially in 5 μm thickness with microtome (RM 2235, Leica, Germany). Serial sections were mounted on slides (MARIENFELD, Lauda-Königshofen, Germany) and dried in incubator at 56℃ for 1 hour. Slides were stained with Mayer's Hematoxylin and Eosin Y (H&E, Cancerdiagnostics, Durham, NC, USA) and Masson-goldner trichrome (Biognost, Zagreb, Croatia) according to the manufacturer's recommended protocol, respectively. Images were obtained on a microscope (Olympus BX43).

10. Statistics

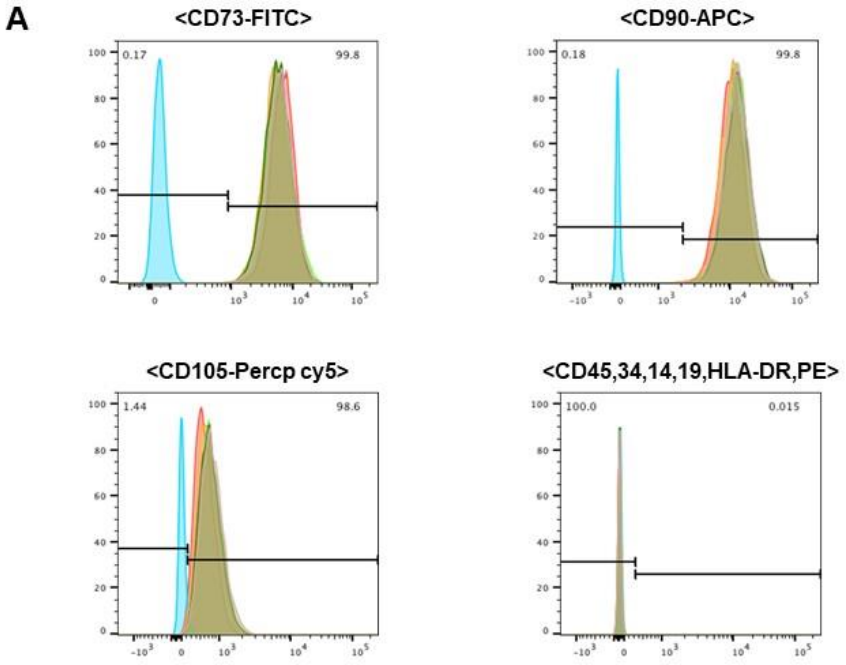
All experiments were performed at least in triplicate, and the data were all presented as means ± standard deviation (SD). One-way analysis of variance followed by Tukey post hoc test was used to determine the differences between two groups. All of the statistical analysis was

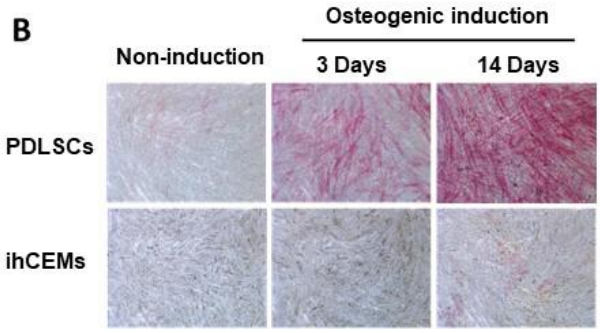
performed with IBM SPSS statistics 24.0 software (IBM, Armonk, NY, USA). The significance level was established at 0.05.

III. RESULTS

1. Identification and Assessment of Osteogenic Differentiation of PDLSCs and ihCEMs

PDLSCs with different frequencies of MVS (30 rpm, 90 rpm, 150 rpm, 210 rpm) for 3 days were identified on the basis of stem cell markers by flow cytometry analysis. It revealed that the cells in each experiment group were positive for CD73 (99.8%), CD90 (99.8%), and CD105 (98.6%) and negative for CD45 (0.015%) averagely (Fig. 5A). Additionally, PDLSCs and ihCEMs were induced in osteogenic induction media for several weeks to evaluate their osteogenic differentiation potential. ALP activity showed that PDLSCs exhibited osteogenic potential after 3 days induction and presented more extensive on days 14. ihCEMs showed osteogenic potential on days 14 (Fig. 5B). ARS staining showed that PDLSCs formed ARS-positive mineralized modules after 14 days of induction and accumulated more calcium deposits on days 21. ihCEMs started to appear mineralized spots on days 14 and became slightly extensive on days 21 (Fig. 5C). The above results demonstrated that MVS with these given frequencies would not weaken the stemness of PDLSCs. Also, both PDLSCs and ihCEMs are capable of osteogenic differentiation at different time points.





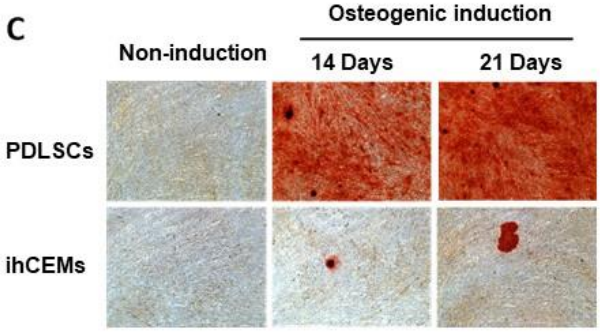


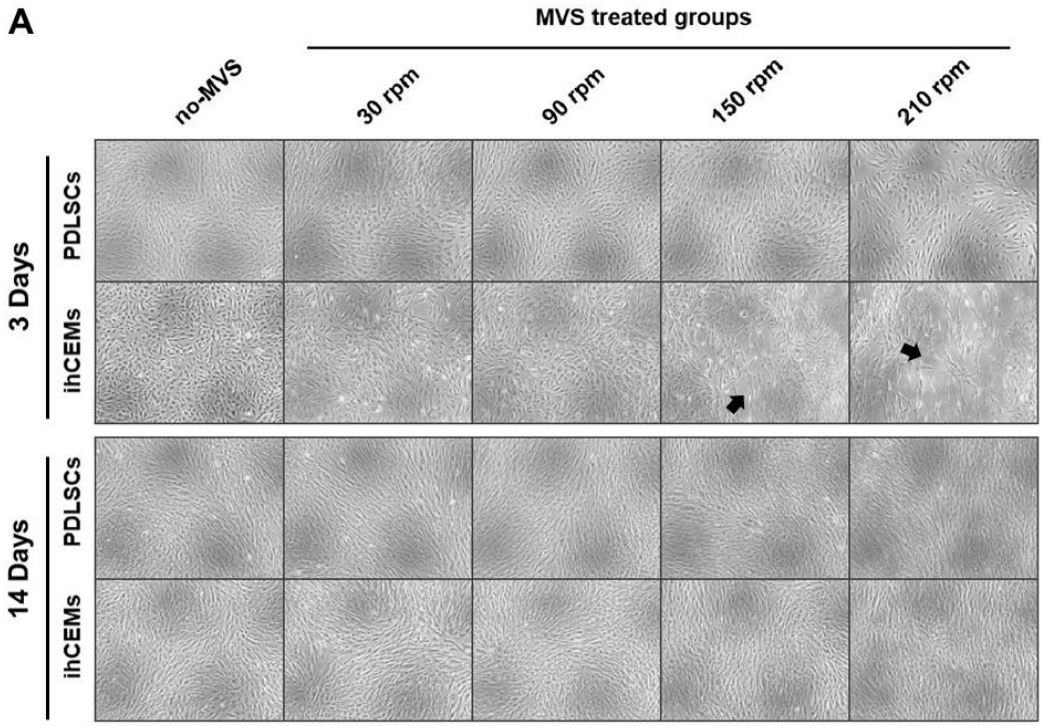
Figure 5. Characterization of periodontal ligament stem cells (PDLSCs) and immortalized cementoblasts (ihCEMs) and assessment of osteogenic differentiation. (A) Flow cytometry analysis of PDLSCs under different frequencies (30 rpm, 90 rpm, 150 rpm, 210 rpm) with averagely positive for CD73, CD90, and CD105 but negative for CD45. (B) ALP staining of PDLSCs and ihCEMs were verified on days 3 and 14 of osteogenic induction (40X microscopy images were shown). (C) ARS staining for evaluating mineralization assay of PDLSCs and ihCEMs were performed on days 14 and 21 of osteogenic induction (40X microscopy images were shown).

2. Morphological Observation and Cell Viability Evaluation of PDLSCs and ihCEMs Stimulated by Mechanical Vibration Forces

PDLSCs and ihCEMs cells morphology were observed under microscopy on day 0 and days 14 after MVS, respectively. Both PDLSCs and ihCEMs showed cell dispersion on 150 rpm and appeared more disperser on 210 rpm. However, on days 14, both PDLSCs and ihCEMs presented well cell-cell attachment and no morphological difference between each group (Fig. 6A).

Cell viability of PDLSCs and ihCEMs were examined by measuring the mean values of OD at days 0, 3 and 14 after MVS using MTT assay. In general, both PDLSCs and ihCEMs presented a downward trend of cell viability with increasing frequency of mechanical stimulation on day 0. Contrarily, both of them appeared opposite trend on days 3 and still remained steady on days 14 (Fig. 6B and 6C). In detail, on day 0, for PDLSCs, compare to the no-MVS treated group, the cell viability of all the MVS treated groups were significant declined as the growth of frequency (Fig. 6B). For ihCEMs, the cell viability significant slightly increased at 30 rpm and 90 rpm but gradually significant decreased at 150 rpm and 210 rpm (Fig. 6C). On days 3 and 14, cell viability was

significant increase in each MVS treated group with the frequency grown up in PDLSCs while only significant rose at 30 rpm or 150 rpm in ihCEMs. However, both of them remained stabilized between these two time points (Fig. 6B-6C). This result indicated that MVS at different frequencies (30 rpm, 90 rpm, 150 rpm, 210 rpm) have no cell viability damage on PDLCs or ihCEMs.



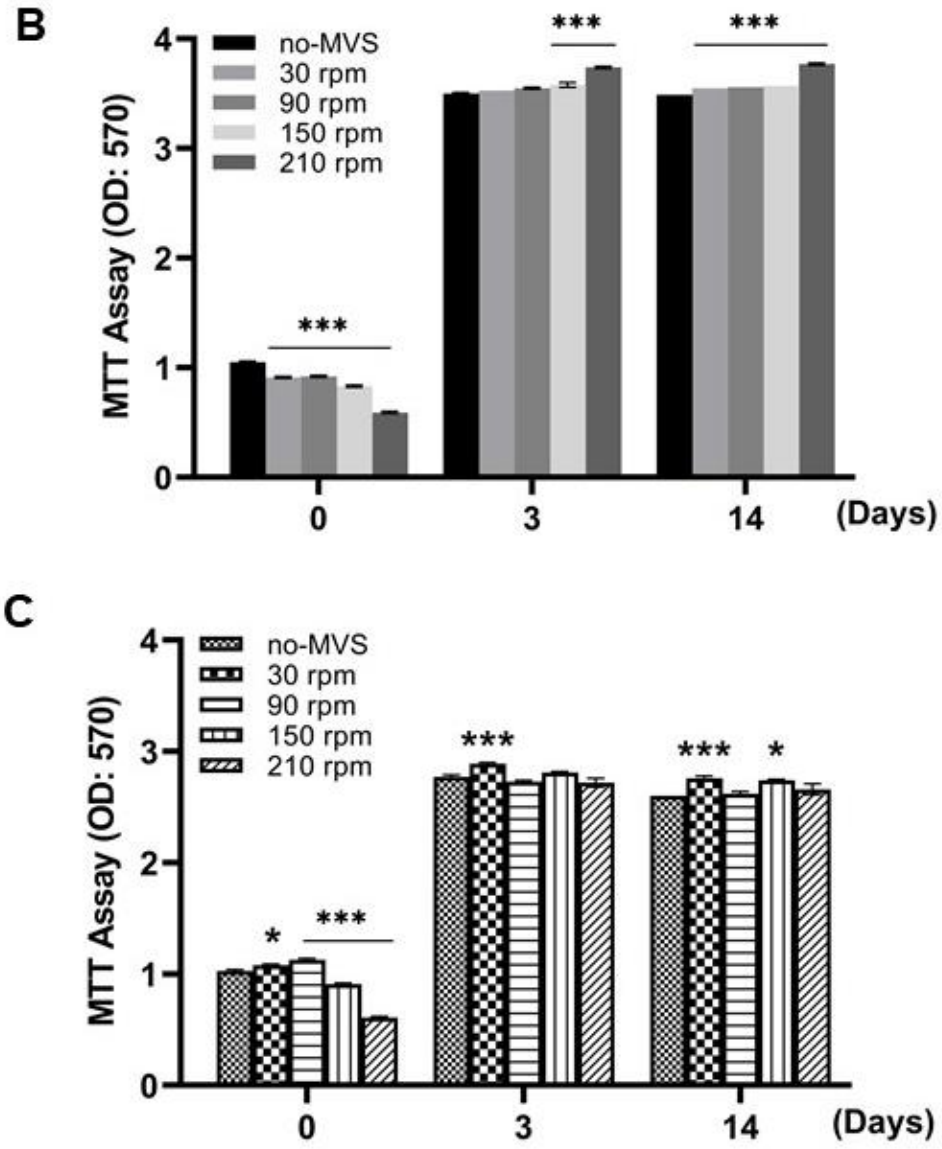


Figure 6. Cell morphology and cell viability of mechanical vibration stimulated PDLSCs and ihCEMs at different time point. (A) Cells morphology of mechanical vibration stimulated PDLSCs and ihCEMs on days 3 and 14 using microscopy (40X microscopy images were shown).

Black arrow: cell dispersion area. The effects of different frequencies (30 rpm, 90 rpm, 150 rpm, 210 rpm) MVS on the cell viability of (B) PDLSCs and (C) ihCEMs on days 0, 3 and 14 after MVS. Absorbance values were measured at 570 nm using MTT assay. (Data are presented as the mean±SD. *P<0.05 and ***P<0.001 indicate significant difference compared with no-MVS treated group on each time point).

3. Osteogenic Gene Expression in Mechanical Vibration Stimulated PDLSCs and ihCEMs

To assess the effect of MVS on osteogenic differentiation, both PDLSCs and ihCEMs were subjected to mechanical vibration forces at different frequencies for 3 days, subsequently osteogenic induction for 3 days and 14 days, respectively, the specific osteogenic gene expression levels were analyzed via RT-PCR (Fig. 7A-7F). Generally, the changing patterns were similar, although the osteogenic expression varied among different factors. For PDLSCs, mechanical vibration frequency at 150 rpm significant reduced expression of Runx2, ALP and OCN on days 3 (Fig. 7A-7C). However, on days 14, the expression of the analyzed genes presented a downward trend comparing with the no-MVS treated group. Of which, Runx2 expression was significantly decreased at 30 rpm, 150 rpm and 210 rpm, respectively (Fig. 7A). Specially, OCN expression at 210 rpm have significant enhanced (Fig. 7C). For ihCEMs, osteogenic gene expressions showed no significant difference between each frequency at each time point (Fig. 7D-7F)

According to above results, there is obvious significant difference of osteogenic potential of PDLSCs between the no-MVS treated group and 150 rpm MVS treated group. Therefore, the *in vivo* bone formation potential of PDLSCs under these two situations subsequently have been further

evaluated. Together, these data confirmed that the effect of MVS was transient and frequency-dependent on PDLSCs while ineffective for ihCEMs.

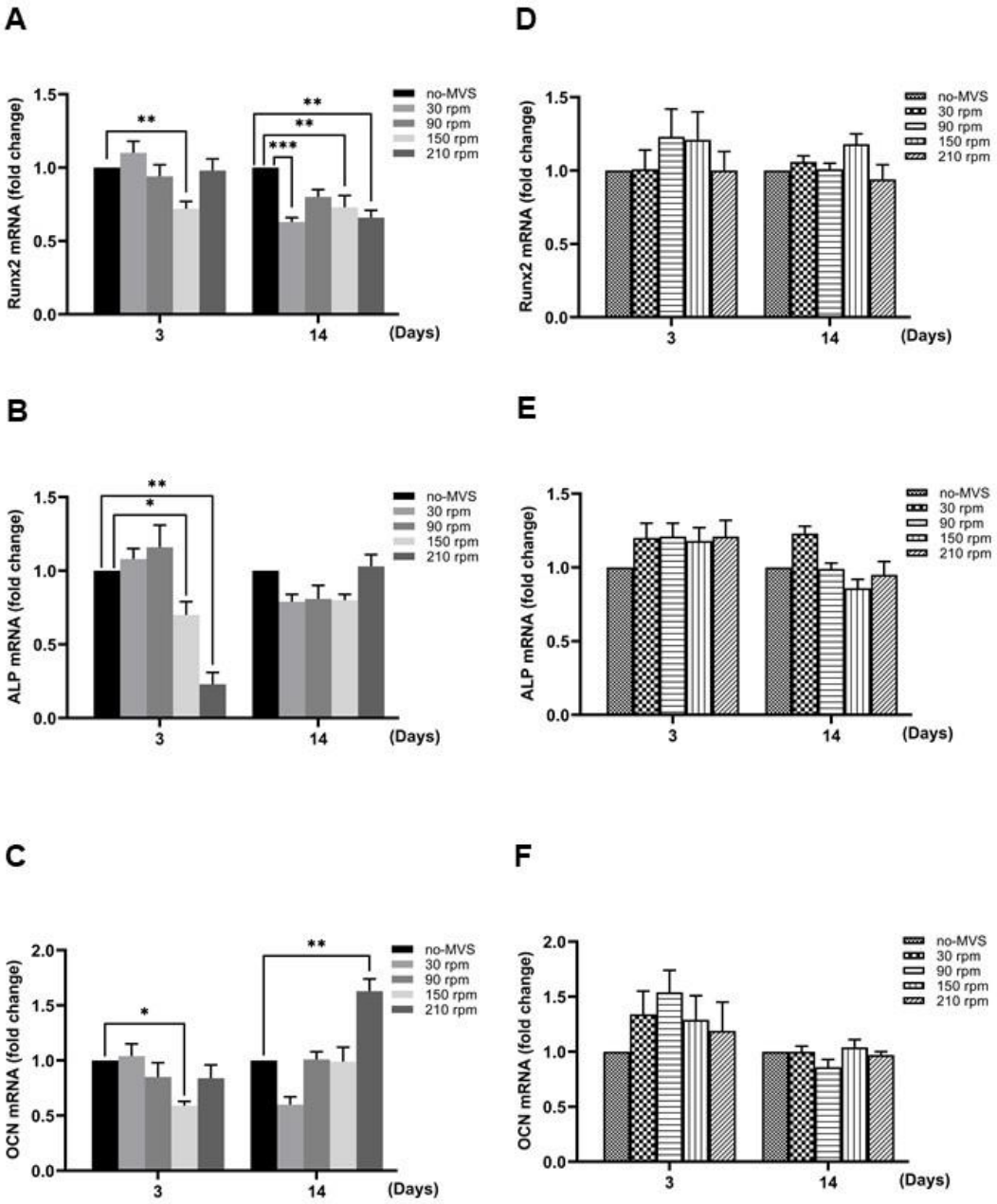
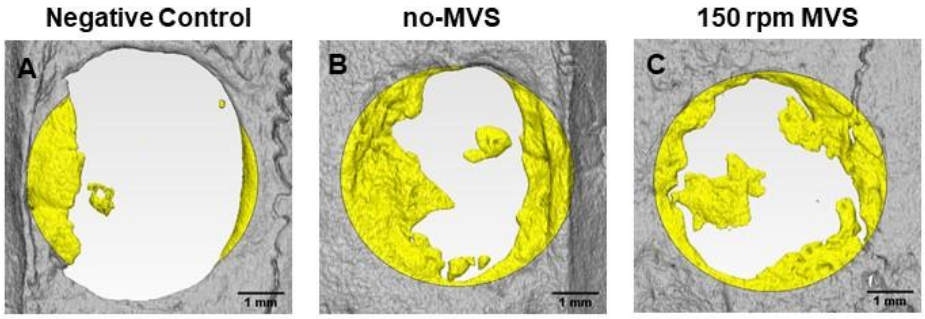


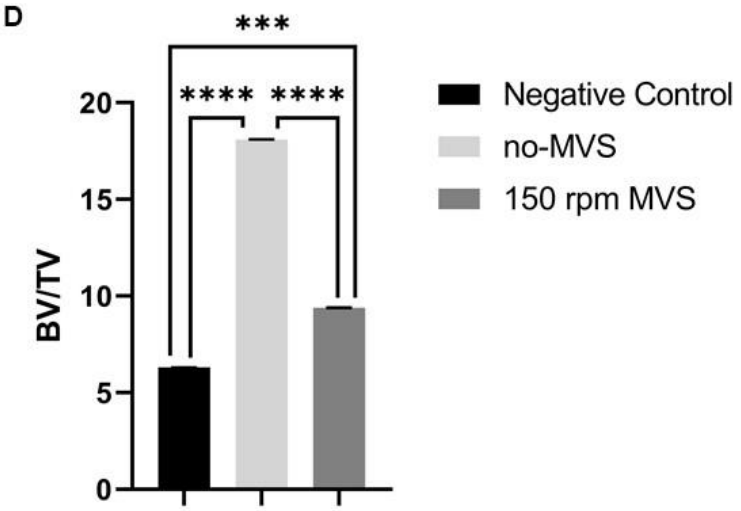
Figure 7. The effects of mechanical vibration stimulation on the osteogenic gene expression of PDLSCs and ihCEMs. Expression levels of osteogenic specific genes are examined in PDLSCs by RT-PCR on days 3 and 14 of osteogenic induction after MVS. (A) Runx2, (B) ALP and (C) OCN. Osteogenic gene expression on days 3 and 14 of osteogenic induction in mechanical vibration stimulated ihCEMs. (D) Runx2, (E) ALP and (F) OCN. (Data are presented as the mean±SD. *P<0.05, **P<0.01 and ***P<0.001 indicate the significant difference compare with the no-MVS treated group on each time point).

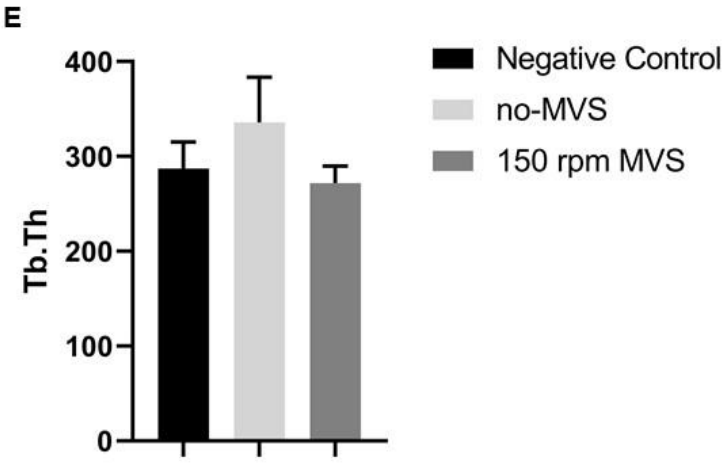
4. Bone Formation *in vivo*

To further evaluate the effect of 150 rpm MVS *in vivo*, PDLSCs cell sheets with or without 150 rpm MVS were transplanted into a rat calvarial bone defects model. The effect was confirmed by radiographic and histological analysis. The results of the three-dimensional (3D) reconstruction by Micro-CT showed that the new bone formation could be observed in both no-MVS treated group and 150 rpm MVS treated group (Fig. 8A-8C). In the results of Micro-CT quantitative analysis, in terms of bone volume per total volume (BV/TV), there was a significant increase in both no-MVS group and 150 rpm MVS group when compared to the negative control group. Most importantly, a significant decrease was performed in 150 rpm MVS group when compared to the no-MVS group (Fig. 8D). Trabecular thickness (Tb.Th), another quantitative result of Micro-CT, also showed the declined tendency on 150 rpm MVS group when compared to no-MVS group (Fig. 8E). Histological analysis including hematoxylin and eosin (H&E) and Masson-Goldner trichrome staining showed that the healing pattern in each group were different. In negative control group, calvarial defects area were filled with well-organized collagen fibrous tissue. In the no-MVS group, disorganized collagen

tissue and newly formed bone tissue could be observed in the defect area. In the 150 rpm MVS group, ligament-like tissue had been observed in the whole defect area. In it, new bone formation could also be found (Fig. 8F-8T). These results indicated that PDLSCs cell sheets treated with 150 rpm MVS could decline the bone formation and promote ligament-like tissue healing in rat calvarium defect.







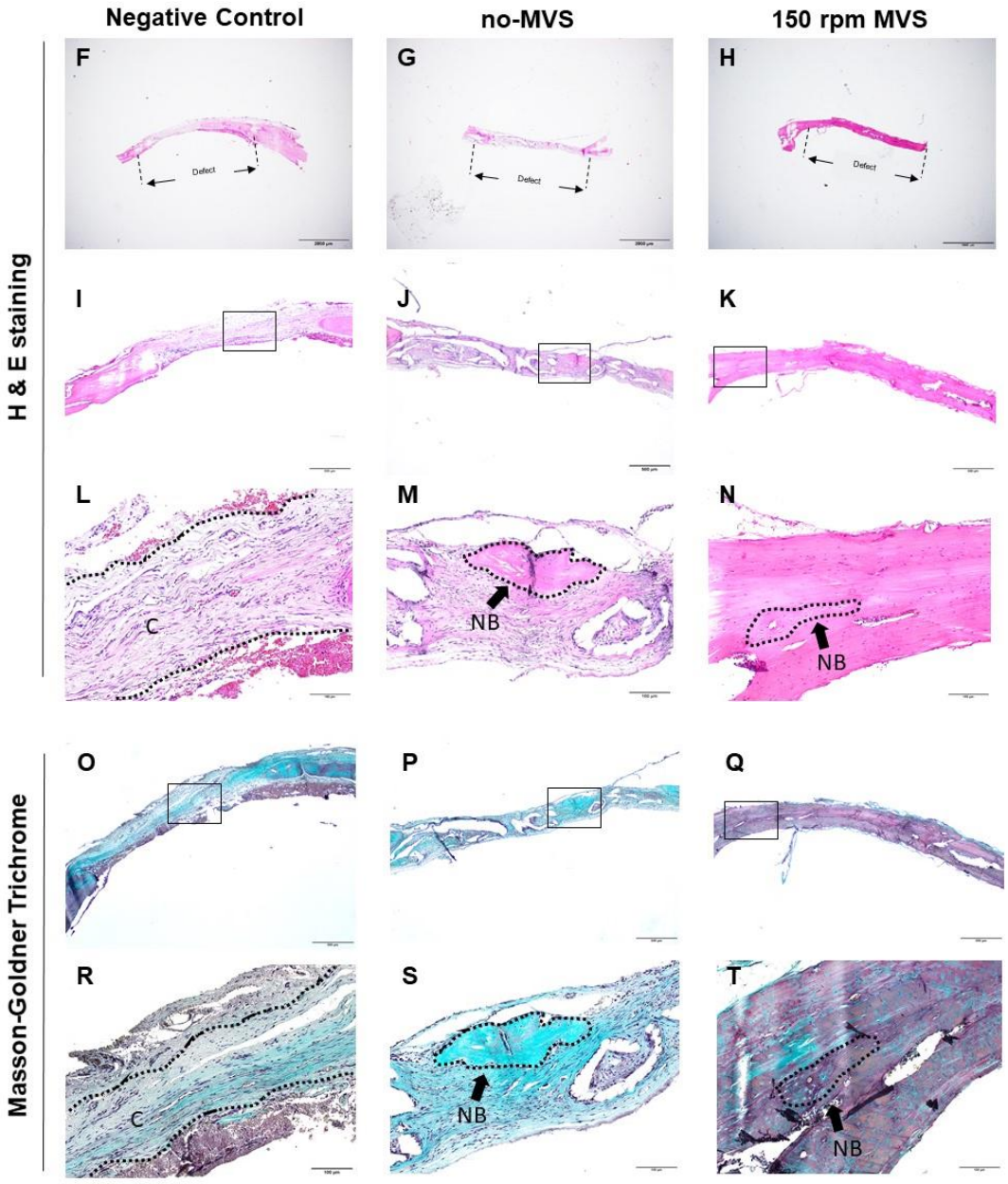


Figure 8. Evaluation of bone formation. 3D reconstruction images of Micro-CT analyses of (A) negative control, (B) no-MVS group and (C) 150 rpm MVS group. Scale bar = 1 mm.

Quantitative analysis of Micro-CT by (D) BV/TV and (E) Tb.Th. (Data are presented as the mean±SD. ****P<0.0001, ***P<0.001). Images of (F-N) H&E staining and (O-T) Masson-Goldner trichrome staining in each group. The black dotted line indicates the newly formed bone area. C: collagen fibrous tissue; NB: new bone; Black arrow: newly formed bone area. Scale bar = 2000 μm (F, G, H), 500 μm (I, J, K, O, P, Q), 100 μm (L, M, N, R, S, T).

IV. DISCUSSION

In this study, it was found that both PDLSCs and ihCEMs showed cell viability reduction just after mechanical vibration stimulation (MVS) but recovered and remained steady after days 3. These results suggested that although the significant reduction effect appeared at the beginning, both PDLSCs and ihCEMs could be resistant to MVS in the long run. Notably, 150 rpm MVS could decline osteogenic differentiation of PDLSCs both *in vitro* and *in vivo*. Additionally, 210 rpm MVS could extensively promote osteogenic differentiation of PDLSCs on days 14. On the contrary, ihCEMs showed no significant difference in response to MVS. These results indicated that the osteogenic potential of PDLSCs was time-dependent and frequency-dependent while ihCEMs were tolerated to MVS. Most importantly, it provides valuable clues that 150 rpm of MVS may be a positive frequency to avoid periodontal pathologies, such as ankylosis.

It is known that both orthodontic forces and occlusal forces have the preference to prevent ankylosis and improve PDL healing after tooth replantation or transplantation (Andersson et al. 1985; Mine et al. 2005; Yang et al. 2001). Orthodontic appliance usually provides the poor experience for patients such as pain, gingivitis, consuming of time and high price (Bradley et al. 2020). On the other hand, previous animal studies indicated that occlusal force could improve PDL healing and reduce ankylosis while excessive occlusal forces could lead to more sever PDL disease (Andersson et al. 1985; Mine et al. 2005; Andersson et al. 1981). Due to the occlusal forces are varied between each person, the proper occlusal force which could promote PDL healing is considered to be out of control. The deficiency of quantification of this “proper occlusal force” lead to the lack of potential therapeutic role of occlusal force in the clinical aspect. Recently, the concept that mechanical vibration force (MVF) can be a system to represent the occlusal force *in vitro* (Leethanakul et al. 2018) has been raised, which means that MVF might have the potential to improve PDL healing and

prevent dentoalveolar ankylosis after tooth replantation or transplantation as occlusal force. A few of previous studies indicated that MVF presented the magnitude-dependent or frequency-dependent effect on PDLSC osteogenic differentiation, which results differed with the change in the mechanical model and protocol (Zhang et al. 2012; Benjakul et al. 2018; Zhang et al. 2015). One of the studies pointed out that mechanical loading model *in vitro* provides a useful, well-controlled method to explore the application of mechanical loading as a factor for PDL reconstruction (Berendsen et al. 2009). Therefore, it is necessary and meaningful to build up a mechanical vibration model, which can imitate the occlusal force *in vitro*, to found out a controllable "proper occlusal force" to promote PDL healing and reduce ankylosis after tooth replantation and transplantation.

Both PDLSCs and cementoblasts play a highly crucial role in maintaining PDL homeostasis during PDL healing and remodeling. PDLSCs always hold the homeostasis in PDL as an unmineralized connective tissue for restoration of normal PDL width. The damage of this homeostasis might lead to undesirable results such as ankylosis or root resorption (Bartold et al. 2006; Nagatomo et al. 2006; Seo et al. 2004; Yang et al. 2012). In addition, the existence of cementum is like a barrier surrounded the tooth to avoid tooth resorption. Once the cementum layer damaged, the periodontium has a limited capacity for regeneration (Beertsen et al. 1997). Therefore, it is necessary to take both above cells into consideration, moreover, the confirmation of cell viability and characteristics of them is an indispensable step for all periodontium regeneration research. In this study, FACS results suggested that the stemness potential of PDLSCs would not be weakened by these MVF. Combined cell viability results with the microscope image of the cells in different time points, it can be hypothesized that the decrease of cell viability might be caused by the dispersion of cells on account of vibration forces. Nevertheless, from days 3, cell viability of both PDLSCs and ihCEMs recovered and were resistant to MVS. Thus, it is suggested that MVF of these

frequencies (30 rpm, 90 rpm, 150 rpm, 210 rpm) had no long-term cytotoxicity effect on PDLSCs and ihCEMs, which might be a safe and promising device for clinical application.

Another essential characteristic of PDLSCs and ihCEMs is their osteogenic differentiation potential. Osteogenic potential of PDLSCs under 150 rpm MVF has significantly decreased at the early osteogenic differentiation stage (days 3), however, it has no further adverse on days 14. Put it another way, this result indicated that PDLSCs under 150 rpm MVF would not have excessive osteogenic differentiation to lead potential ankylosis or have a time-lasting osteogenic reduction to lead potential root resorption, it suggested that 150 rpm MVF might be seen as the "proper mechanical force" to reduce ankylosis after tooth replantation and transplantation. This finding was strengthened by the *in vivo* evaluation of bone formation that the calvarium defect treated with 150 rpm MVS performed the decrease of new bone formation. Another interesting finding in *in vivo* study is that compare with the disorganized collagen healing pattern in no-MVS group, the calvarial defect treated with 150 rpm MVS presented ligament-like tissue healing pattern, which suggested that 150 rpm MVS might have a potential to promote PDL healing. However, further study is needed to confirm the exact PDL healing by PDL specific marker. Moreover, PDLSCs at 210 rpm have a strong mineralization potential on days 14, which could be speculated that 210 rpm MVF might be the excessive and invasive forces that do harmful to PDL healing. The above findings were consistent with the previous reports that physiological levels of mechanical loading can lead to tendon/ligament tissue healing or PDL healing while excessive mechanical force can result in tendon/ligament tissue injuries, root resorption or tooth ankylosis (Gracey et al. 2020; Andersson et al. 1985; Galloway et al. 2013; Andreasen et al. 1981; Mine et al. 2005).

On the other hand, MVS showed no influence on the osteogenic gene expressions of ihCEMs. This result was out of conformity with preliminary researches about the mechanical forces for osteogenic differentiation of cementoblast. The possibility of this inconsistency might be due to the

following reasons. Firstly, the differs in mechanical protocols (Huang et al. 2009; Matsunaga et al. 2016; Yu et al. 2009) could result in various effects. What is more, Wnt signaling, an important pathway in the regeneration of periodontal tissue, might be responsible for inhibition of growth and differentiation of cementoblast (Nemoto et al. 2009) during mechanical vibration loading. Whereas, one study showed another viewpoint that aberrantly elevated Wnt signaling leads to cementum overgrowth (Wu et al. 2019). In this study, MVS almost had no influence on either proliferation ability or osteogenic differentiation potential of ihCEMs. Thus, it is suggested that ihCEMs were resistant with continuous MVS at these given frequencies (30rpm, 90rpm, 150rpm, 210rpm). Nevertheless, the precise mechanism by which Wnt signaling during periodontal development and homeostasis remains undefined. Collectively, based on the above evidence, the results provide a novel clue for the application of MVF at 150 rpm might be a save potential approach for prevention of ankylosis and PDL healing after tooth replantation or transplantation. However, due to different machines have different specifications, the so-called “proper mechanical force” at 150 rpm MVS in this study was only confined to the present specific model, and could not be extrapolated to other *in vitro* models.

Cell sheet technology has been successfully used for tissue regeneration in skin, esophagus mucosa, bone tissue and PDL (Xie et al. 2015). The use of the cell sheet technique could avoid the trypsinization, highly preserve the extracellular matrix (ECM), retain the cell-to-cell junction also differentiated features, and maintain the integrity of the cell sheet during transplantation (Iwata et al. 2009; Kim et al. 2017; Xie et al. 2015). Currently, the technique of PDLSCs cell sheets transplantation has already been widely used in various researches about PDL regeneration (Flores et al. 2008; Iwata et al. 2015; Iwata et al. 2009; Zhou et al. 2012). These studies reported that the application of PDLSCs cell sheets into PDL defects is a sufficient technique that can regenerate complete periodontium, which can achieve new bone, new cementum, and well-oriented collagen

fiber formation. In this study, osteogenic potential of PDLSCs has been focused. Therefore, for integrally understand the effect of 150 rpm MVF on PDL regeneration, further study is needed by transplanting the PDL stem cell sheets with 150 rpm MVF into PDL defects directly.

V. CONCLUSION

In summary, the present study proposes a phenomenon that both PDLSCs and ihCEMs are resistant to MVS at different frequencies (30 rpm, 90 rpm, 150 rpm, 210 rpm). The effect of MVS on osteogenic potential of PDLSCs is time-dependent and frequency-dependent while no effect on ihCEMs.

Moreover, this study described an *in vitro* mechanical vibration model which could imitate occlusal forces and deliver controllable mechanical vibration forces. This presented model is a relatively simple and easily operated machine for research on the effect of cells under mechanical vibration force.

Interestingly, this study proposed that 150 rpm MVS could decline osteogenic differentiation in the early time and have no harmful effect in the long run. Furthermore, PDLSCs cell sheets treated with 150 rpm MVS present ligament-like tissue healing pattern and can reduce new bone formation in rat calvarial defects. These findings bring up the clue that 3 days MVS at 150 rpm has a potential to be the “proper mechanical vibration force” to reduce ankylosis and promote PDL healing.

This study is a primary step on the way to achieve the clinical efficacy. Looking forward to the clinical application in future, the root of replanted or transplanted tooth covered by PDLSCs cell sheets treated with 3 days MVS at 150 rpm might have a potential therapeutic value to reduce ankylosis and improve the prognosis after tooth replantation and transplantation.

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ABSTRACT (KOREAN)

기계적 진동력을 받은 치주인대 줄기세포와 불멸성 시멘트아세포의 골분화능력 연구

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치아 재식혹은 이식 후, 교합력을 가하면 치주 인대(PDL)의 치유를 촉진하고 치주 인대의 유착(ankylosis) 을 예방할 수 있다. 그러나 불확실하고 부족한 생체외(in vitro) 연구로 인해 교합력이 PDL 치유에 미치는 생물학적 매커니즘에 대한 연구는 미비한 수준이다. 따라서 기계적 진동력이 교합력을 모방할 수 있다는 기존의 선행 연구를 바탕으로 생체외 모델에서 기계진동기로 처리한 4개의 주파수와 시간에 따른 치주 인대 줄기세포(PDLSCs)와 불멸성 시멘트아세포(ihCEMs) 의 골분화능을 평가하였다. PDLSCs와 ihCEMs의 세포 생존능 측정 결과, 두 세포 모두 초기에는 생존능력이 감소하였으나 이후에는 주파수별 기계적 진동 자극(MVS)에 모두 내성이 있음을 나타냈다. 골 분화 및 형성능력을 분석한 결과 MVS가 PDLSC의 골 형성에 일시적, 주파수

의존적 영향을 미치는 반면, 각 주파수 및 시점에서 ihCEMs에는 거의 영향을 미치지 않는 것으로 나타냈다. 흥미롭게도, 150 rpm MVS는 초기에 골분화를 낮출 수 있고 장기적으로는 해로운 영향을 끼치지 않는다는 것을 보여주었다. 추가적으로 확인한 생체내 (in vivo) 실험의 결과 150 rpm MVS로 처리된 PDLSCs세포 시트가 랫트 두개골결함 모델에서 골 형성을 감소시키며 인대와 유사한 형태의 조직으로 회복시켰다.결론적으로 적절한 주파수(150rpm)의 MVS는 PDLSCs의 골 분화를 일시적으로 낮추는 잠재적 전략 이 될 것이며 향후 치아 재식이나 이식한 후 발생하는 ankylosis 예방 및 치주인대의 치유촉진에 새로운 길을 열어줄 것이다.

핵심이 되는 말: 기계적 진동력, 치주인대 줄기세포, 시멘트아세포, 골 분화, 세포 시트, 골 형성